UNIVERSITAT DE BARCELONA

Utilización de aceites ácidos y ácidos grasos destilados en alimentación animal: caracterización de estos subproductos y repercusiones de su uso en la estabilidad oxidativa de piensos y carne de pollo

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Utilización de los aceites ácidos y ácidos grasos destilados en alimentación animal: caracterización de estos subproductos y repercusiones de su uso en la estabilidad oxidativa de piensos y carne de pollo

Use of acid oils and fatty acid distillates in animal feeding: characterization of these by-products and repercussions of their use on the oxidative stability of feeds and chicken meat

Memoria presentada por **Elisa Varona Sánchez** para optar al título de Doctor por la Universitat de Barcelona

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RESUMEN

La correcta formulación de piensos es esencial para conseguir dietas que aporten al animal niveles equilibrados de nutrientes, a la vez que se consiga una eficiencia en la producción animal mediante piensos con palatabilidad adecuada, y todo ello, a un precio competitivo. La grasa se considera uno de los ingredientes más relevantes en los piensos, cuyo valor esencial viene dado por el aporte de energía en la dieta a la vez que es una fuente importante de vitaminas liposolubles y ácidos grasos esenciales. En el mercado pueden encontrarse distintos ingredientes grasos de precios diferentes que varían en composición y calidad, lo que acaba repercutiendo en su valor energético. De forma convencional, las grasas y aceites crudos son los que han sido utilizados en la formulación de piensos. Pero en la actualidad, el interés por la sostenibilidad ha promovido la utilización de subproductos de la industria alimentaria en alimentación animal, de forma que se valoricen y se consiga un beneficio ambiental y económico. Es aquí donde se centra el primer objetivo de esta Tesis Doctoral, en la necesidad de caracterizar algunos de los subproductos obtenidos en la refinación de aceites y grasas comestibles de origen vegetal, los aceites ácidos (AA) y ácidos grasos destilados (AGD), estableciendo los parámetros de control críticos para su uso en alimentación animal. Se ha observado tanto una alta variabilidad en su composición, como una relación entre la composición y calidad de los AA y AGD con el proceso de obtención (refinación química o física) de estos, así como, con el origen botánico del aceite crudo del que proceden.

Además de su caracterización, para avanzar en el estudio de estos subproductos y su potencial como ingredientes de las dietas animales, es de gran relevancia conocer cómo afecta la inclusión de estos subproductos en los piensos en cuanto a composición y estabilidad hidrolítica y oxidativa de los mismos. Estos aspectos se han abordado mediante un estudio que compara piensos formulados con AA y AGD (de diferente origen botánico) y sus respectivos aceites crudos y refinados, fabricados según tres procesos tecnológicos distintos y almacenados a diferentes tiempos y temperaturas. Los principales resultados mostraron que la formulación de piensos con subproductos ricos en ácidos grasos libres (AA y AGD) no supone un problema relevante en la hidrólisis lipídica ni en la estabilidad oxidativa, sin embargo, la formulación con aceites crudos y refinados de piensos en forma de harina podría resultar en un contenido de ácidos grasos libres (AGL) mucho mayor de lo esperado.

Finalmente, se ha estudiado cómo se ve afectada la composición y estabilidad oxidativa de la fracción grasa de la carne de pollo mediante un estudio *in vivo* donde se alimentaron animales con subproductos ricos en AGL de diferente origen botánico (soja y palma) y con sus

correspondientes grasas crudas o refinadas. La carne obtenida no presentó diferencias significativas entre un subproducto y sus correspondientes grasas crudas y refinadas, pero si en función del origen botánico de estos ingredientes grasos.

ABSTRACT

In general, to achieve a greater efficiency in animal production, the correct formulation is essential to get diets that provide the animal with balanced levels of nutrients, as well as palatable diets to ensure efficient growth performance, and all at a competitive price. Fat is considered one of the most relevant ingredients in animal feeds, whose essential value is given by its energetic value, at the same time that it is an important source of fat-soluble vitamins and essential fatty acids. Different fatty ingredients at different prices can be found on the market, varying in composition and quality, and thus, in their energy value. Traditionally, crude fats and oils have been used in feed formulation. However, at present, interest in sustainability has promoted the use of by-products from the food industry, to valorize them and to achieve environmental and economic benefits. It is here where the first objective of this thesis focuses, on the need to characterize some by-products obtained from refining of edible vegetable oils and fats, the acid oils (AO) and fatty acid distillates (FAD). A high variability in their composition and a relationship between the refining process (chemical or physical refining) as well as the botanical origin and the final composition and quality of the AO and FAD has been observed.

In addition to their characterization, to progress in the study of these by-products and their potential as feed ingredients, it is of great relevance to know how the inclusion of these by-products in feed affects their composition and hydrolytic and oxidative stability. All these aspects have been addressed in another study which compared feeds formulated with AO and FAD (of different botanical origin) and their corresponding crude and refined oils, produced by three different technological manufacturing processes and stored at different times and temperatures. The main results have shown that the formulation of feeds with by-products rich in free fatty acids (AO and FAD) did not pose a relevant problem of lipid hydrolysis or oxidative stability, whereas the formulation with crude and refined oils could result in a higher than expected free fatty acid (FFA) content in mash feeds.

Finally, broilers were fed with feeds formulated with by-products rich in FFA of different botanical origin (soybean or palm) or their corresponding crude and refined oils, to study how the composition and oxidative stability of the lipid fraction of chicken meat was affected. The meat obtained did not show significant differences between a by-product and its corresponding raw and refined oils, but rather depending on the botanical origin of these fat ingredients.

ABREVIACIONES

AA	Aceites ácidos
AC	Índice de acidez (*acidity*)
ADG	Ganancia diaria de peso (*average daily gain*)
AG	Ácidos grasos
AGD	Destilados ricos en ácidos grasos (ácidos grasos destilados)
I/S	Relación ácidos grasos insaturados/ácidos grasos saturados (U/S ratio) (en este cálculo los ácidos grasos con 12 carbonos o menos, independientemente de que sean o no saturados, se consideran como ácidos grasos insaturados, incluyéndose en el numerador de la ratio, y los ácidos grasos *trans* se consideran como ácidos grasos saturados y, por tanto, se incluyen en el denominador). Esta ratio se utiliza para predecir el valor energético de las grasas.
AGL	Ácidos grasos libres
AGMI	Ácidos grasos monoinsaturados
AGPI	Ácidos grasos poliinsaturados
AGI	Ácidos grasos insaturados
AGS	Ácidos grasos saturados
AS	Aceite ácido de soja (*acid soybean oil*)
BS	Mezclas de aceites ácidos de aceites de semillas (*blends of acid oils from seed oils*)
CP	Aceite crudo de palma (*crude palm oil*)
CS	Aceite crudo de soja (*crude soybean oil*)
Contenido LHP	Contenido de hidroperóxidos lipídicos (*lipid hydroperoxides*) medido mediante el método del naranja de xilenol tras 0,5 h de incubación, mide el contenido de LHP de la muestra.
DAG	Diacilgliceroles
EMA	Energía metabolizable aparente
ED	Energía digestible
FCR	Tasa de conversión de alimento (*feed conversion ratio*)
HAP	Hidrocarburos aromáticos policíclicos
I	Impurezas insolubles
AGI/AGS	Relación ácidos grasos insaturados/ácidos grasos saturados (*UFA/SFA ratio*). Este cálculo se hace en base a la presencia de dobles enlaces de los ácidos grasos, incluyendo todos los ácidos grasos insaturados en el numerador y todos los ácidos grasos saturados en el denominador. Esta relación se utiliza para predecir la oxidabilidad de la fracción lipídica.

IT	Periodo de inducción medido mediante instrumento Rancimat a 120 °C (*induction time by Rancimat at 120 °C*)
LFAD	Ácidos grasos destilados de aceite de coco y mezclas de ácidos grasos destilados de aceites de coco y palmiste (aceites laúricos) (*lauric fatty acid distillates*)
M	Humedad y materia volátil (*moisture and volatile matter*)
MAG	Monoacilgliceroles
MDA	Malondialdehido
MIU	Humedad y materia volátil + impurezas insolubles + materia insaponificable (*moisture and volatile matter + insoluble impurities + unsaponifiable matter*)
NEM	Material no eluible (non-elutable material)
O	Aceites ácidos de orujo de oliva y mezclas de aceites de orujo de oliva y de oliva (*olive acid oils*)
OFAD	Ácidos grasos destilados de aceite de orujo de oliva y de aceite de oliva (*olive fatty acid distillates*)
***p*-AnV**	Índice de *p*-anisidina (*p-anisidine value*)
PCA	Análisis de componentes principales (*principal component analysis*)
PFAD	Ácidos grasos destilados de aceite de palma (*palm fatty acid distillates*)
POL	Compuestos políméricos
PV	Índice de peróxidos (*peroxide value*)
RP	Aceite refinado de palma (*refined palm oil*)
RS	Aceite refinado de soja (*refined soybean oil*)
SCP	Mezclas de aceites ácidos de aceites de semillas, manteca de cacao y aceite de palma (*blends of acid oils from seed oils, cocoa butter and palm oil*)
SO	Aceites ácidos de aceites de soja (*acid oils from soybean oils*)
SP	Mezclas de aceites ácidos de aceites de palma y semillas (*blends of acid oils from seed and palm oils*)
SU	Aceites ácidos de aceites de girasol (*acid oils from sunflower oil*)
SU-SO	Mezclas de aceites ácidos de aceites de girasol y soja (*blends of acid oils from sunflower and soybean oils*)
TAG	Triacilgliceroles
TBA	Ácido tiobarbitúrico
T	Suma de α-, β-, γ- and δ-tocoferoles
T3	Suma de α-, β-, γ- and δ-tocotrienoles
T+T3	Suma de tocoferoles y tocotrienoles
U	Materia insaponificable (*unsaponifiable matter*)

Valor Final LHP	Contenido de hidroperóxidos lipídicos (*lipid hydroperoxides*) medido mediante el método del naranja de xilenol tras 96 h de incubación, mide el contenido de LHP formados tras 96 h de incubación y estima la estabilidad oxidativa de la muestra
VC	Compuestos volátiles (*volatile compounds*)

ABREVIACIONES EN ARTÍCULOS EN INGLÉS

AA	Arachidonic acid
ALC	Sum of alcohols
ALD	Sum of aldehydes
AO	Acid oils
AS	Acid soybean oil
BS	Blends of acid oils from seed oils
C	Cooked meat
CHP	Cumene hydroperoxide
CP	Crude palm oil
CR	Cooked and refrigerated storage meat
CS	Crude soybean oil
DAG	Diacylglycerols
DE	Dietary energy
DHA	Docosahexaenoic acid
EIC	Extracted ion chromatogram
EPA	Eicosapentaenoic acid
F	Fresh meat
FA	Fatty acids
FAD	Fatty acid distillates
FFA	Free fatty acids
FFA-AC	Free fatty acids- acidity (determined by titration)
FFA-SE	Free fatty acids fraction (determined by size molecular exclusion chromatography)
FOX	Ferrous-oxidation xylenol-orange method
HC	Sum of hydrocarbons
I	Insoluble impurities
IT	Induction time measured by the Rancimat at 120 °C
KET	Sum of ketones
LFAD	Fatty acid distillates from coconut oil and blends of fatty acid distillates from coconut and palm kernel oils

LHP Content	Content of lipid hydroperoxides measured by the xylenol orange method after 0.5 h of incubation, measures the LHP content of the sample
LHP Final Value	Amount of lipid hydroperoxides measured by the xylenol orange method after 96 h of incubation, measures the content of LHP formed after 96 h of incubation and estimates the oxidative stability of the sample
M	Moisture and volatile matter
MAG	Monoacylglycerols
MDA	Malondialdehyde
AME	Apparent metabolizable energy
MIU	Sum of moisture and volatile matter + insoluble impurities + unsaponifiable matter
MUFA	Monounsaturated fatty acids
NEM	Non-elutable material value
n-6/n-3 ratio	n-6 polyunsaturated/ n-3 polyunsaturated ratio
O	Acid oils from olive pomace oil and blends of acid oils from olive pomace and olive oils
OFAD	Fatty acid distillate from olive pomace and olive oils
***p*-AnV**	*p*-anisidine value
PCA	Principal component analysis
PFAD	Fatty acid distillates from palm oil
PG	Propyl gallate
POL	Polymeric compounds
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
RP	Refined palm oil
RS	Refined soybean oil
SCP	Blends of acid oils from seeds oils, cocoa butter and palm oil
SFA	Saturated fatty acids
SIM	Selected ion monitoring
SO	Acid oils from soybean oils
SP	Blends of acid oils from seed and palm oils
SU	Acid oils from sunflower oil
SU-SO	Blends of acid oils from sunflower and soybean oils
TAG	Triacylglycerols
T	Sum of α-, β-, γ- and δ-tocopherols
T3	Sum of α-, β-, γ- and δ-tocotrienols
T+T3	Sum of tocopherols and tocotrienols
TBA	2-thiobarbituric acid

U	Unsaponifiable matter
U/S ratio	Unsaturated fatty acids/saturated fatty acids ratio (the calculation includes the saturated fatty acids with 12 carbons or below as unsaturated fatty acids (in the numerator -U of the ratio), and trans-fatty acids as saturated fatty acids (in the denominator -S), it is used to predict the energy value of fats
UFA	Unsaturated fatty acids
UFA/SFA ratio	Unsaturated fatty acids/saturated fatty acids ratio (the calculation includes all the unsaturated fatty acids, regardless of the configuration of the double bonds, in the unsaturated fatty acids (numerator -UFA) and all the saturated fatty acids in the saturated fatty acids (denominator -SFA). This ratio is used to predict oxidizability of the lipid fraction.
VC	Volatile compounds

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1. INTRODUCCIÓN

1. Introducción

La producción de aceites a gran escala parte de los aceites crudos obtenidos mediante extracción, los cuales a menudo presentan altos niveles de acidez y/o defectos organolépticos. Por tanto, en muchas ocasiones los aceites crudos no son aptos para su consumo directo, sino que previamente es preciso eliminar todas las sustancias indeseables que contienen. Actualmente, en nuestra sociedad la refinación de los aceites vegetales crudos es un proceso habitual cuyo objetivo consiste en la producción de los aceites refinados aptos para el consumo humano [1], con olores y colores tenues y más resistentes a la rancidez [2]. Algunos de los componentes indeseados en un aceite son los que pueden dificultar el proceso de refinación (este sería el caso de los fosfolípidos ya que pueden saturar las tierras de blanqueamiento o inducir reacciones de oscurecimiento durante la desodorización), mientras que otros podrían dañar el aceite durante su almacenamiento, conduciendo a la reducción de su vida útil por una rancidez prematura, causante de un olor y gusto desagradables [2]. Entre estos constituyentes se encuentran, los ácidos grasos libres (AGL), fosfolípidos, pigmentos, gomas, metales y compuestos volátiles, entre otros [3]. A su vez, durante la refinación también se eliminan contaminantes y pesticidas además de disolventes utilizados en el proceso de extracción [2]. Por ello, un tratamiento adecuado del aceite crudo es imprescindible para conseguir un producto final estable y agradable a los sentidos.

Simplificando, la finalidad de los procesos de refinación se basa en maximizar la eliminación de componentes no deseados minimizando la pérdida de aceite en sí mismo, así como, de otros componentes que sí son deseados desde un punto de vista nutricional [4]. Algunos de estos constituyentes con valor nutricional son los tocoferoles (T) y tocotrienoles (T3), los esteroles o los polifenoles, todos ellos considerados antioxidantes naturales que impiden el desarrollo de procesos de oxidación de las grasas y que se acumulan en algunos subproductos obtenidos en la refinación de aceites comestibles [4]. Dichos subproductos muestran un alto potencial en alimentación animal porque suponen un aporte energético a la dieta animal, y suelen contener ácidos grasos (AG) esenciales y vitaminas liposolubles [5]. El aprovechamiento de dichos subproductos como ingredientes para alimentación animal es una alternativa más sostenible y efectiva para su tratamiento. Su utilización en formulación animal permite tanto que se valoricen los residuos de las refinerías como que se maximice la sostenibilidad de la cadena alimentaria. No obstante, aunque su composición los convierte en potenciales ingredientes para alimentación animal, es preciso su control, asegurando su seguridad y propiedades nutricionales. De aquí la importancia de una correcta caracterización de estos

subproductos para que su aplicación sea segura, ventajosa tanto nutricionalmente para el animal como económicamente para las refinerías y productores de piensos [6,7].

Los subproductos objeto de estudio en esta Tesis, los aceites ácidos (AA) y los ácidos grasos destilados (AGD), son subproductos ricos en AGL obtenidos en los procesos de refinación de los aceites y grasas vegetales. Existen dos tipos de refinación, el proceso químico y el físico, a partir de los cuales se generan respectivamente los AA y los AGD una vez aplicada la etapa de eliminación de los AGL en cada uno de los procesos [8]. En el caso de la refinación química, durante la etapa de neutralización con un álcali se obtienen por centrifugación las pastas jabonosas que posteriormente se acidifican con ácido sulfúrico para obtener los AA, mientras que en la refinación física los AGD proceden de una destilación al vacío a altas temperaturas aplicada durante la etapa de desodorización [8]. La principal característica de ambos subproductos es su alto contenido en AGL lo que les confiere valor energético, a la vez que, dependiendo del origen botánico del aceite crudo, pueden ser una buena fuente de AG esenciales (linoleico y linolénico) y de vitaminas liposolubles (como los T y T3), entre otros componentes nutricionalmente interesantes que ya se han mencionado anteriormente [5].

En general, en alimentación animal las grasas son incluidas como ingredientes en la formulación de piensos ya que participan en la homeostasis energética, y cumplen un papel fundamental en membranas celulares y tejidos, además de en el propio desarrollo del animal [7,9]. También, las grasas influyen en la palatabilidad del pienso e incluso favorecen los procesos de granulación reduciendo la separación de partículas en el pienso [10]. Por todo ello, el uso de estos subproductos en pienso presenta un alto interés, particularmente en dietas de cerdos y pollos [11]. El problema actual se encuentra en que a pesar de estar incluidos en el Catálogo Europeo de ingredientes para piensos establecido en el Reglamento de la Comisión N°68/2013 [12], debido a su alta variabilidad de composición y a su falta de estandarización [13], su utilización por parte de productores de piensos y ganaderos no está totalmente instaurada, ya que hay una desconfianza en la eficacia de su uso.

Por todo ello, los objetivos principales de esta Tesis Doctoral son, en primer lugar, estudiar la composición y calidad de los AA y AGD, caracterizarlos y establecer las posibles fuentes de su alta variabilidad. De esta forma, se contribuye a que se valoricen subproductos de la industria de la refinación de aceites vegetales de consumo humano, logrando tanto un beneficio medioambiental como económico. El siguiente objetivo es evaluar si la diferente composición y calidad de estos subproductos una vez incluidos en el pienso genera cambios en la composición lipídica y estabilidad (hidrolítica y oxidativa) del mismo, y posteriormente evaluar la composición lipídica y estabilidad oxidativa de la carne de pollo alimentado con estos piensos.

En ambos casos, tanto en el estudio de piensos como en el de carnes, el uso de los subproductos AA y AGD se ha comparado con el uso de aceites del mismo origen botánico crudos y refinados, los dos usados como controles, y en el caso de las grasas crudas se trata además de las habitualmente utilizadas en la formulación de dietas animales.

2. ANTECEDENTES

2. Antecedentes

2.1. Importancia de las grasas y aceites en alimentación animal

Generalmente las grasas y aceites contienen un 95-98% de triacilgliceroles (TAG), una pequeña parte de monoacilgliceroles (MAG), diacilgliceroles (DAG) y AGL, y un 1-2% de materia insaponificable donde se incluyen esteroles, T y T3, entre otros compuestos [14]. Las grasas constituyen una fuente rentable y concentrada de energía, siendo metabolizadas mucho más eficientemente que carbohidratos o proteínas. De hecho, con la grasa añadida la densidad calórica del pienso se incrementa 2,25 veces en comparación con similar peso seco de proteínas o carbohidratos [15,16]. Es por ello que habitualmente se incorporan a las dietas animales cuya formulación busca incrementar la eficiencia energética mediante la reducción del consumo, para reducir los costes y mejorar la producción incrementando las tasas de crecimiento animal. En las formulaciones, las grasas aportan otras ventajas como por ejemplo una mejora de la palatabilidad de la dieta lo que modifica el consumo de pienso por parte del animal, favorecen la digestión y absorción de otros nutrientes presentes en la dieta, y proporcionan vitaminas liposolubles, fosfolípidos y ácidos grasos esenciales [10,15–17]. El aporte de grasa es necesario pues se trata de la principal forma de almacenamiento, protegiendo al animal frente a la pérdida de calor y el daño mecánico gracias a los depósitos subcutáneos en la piel. Además, los lípidos forman parte de las membranas celulares, tienen función vitamínica y hormonal y a su vez participan en el metabolismo celular y en el reconocimiento e inmunidad celular [9]. De hecho, estudios anteriores han observado como la función reproductiva y el rendimiento productivo en animales de granja (bovinos, porcino y aves) mejora tras la inclusión de grasas en las formulaciones de piensos, siendo relevante la optimización de la cantidad y tipo de grasa elegida [17,18].

Aunque en general las grasas son muy digeribles (aprox. un 80%), altos niveles de grasas en la formulación de los piensos pueden presentar efectos nutricionales negativos en la digestibilidad de la fibra o en la absorción del calcio y de los propios AG mediante la formación de sales de calcio de ácidos grasos [19]. De acuerdo con Atteh y Leeson [20] y Wohl et al. [21] la grasa, especialmente de alto contenido en ácidos grasos saturados (AGS), puede perjudicar la absorción de diferentes minerales (calcio, magnesio y zinc) debido a la formación de jabones insolubles, de forma que tanto el AG como el mineral queden no biodisponibles. En el caso del calcio esto podría llegar a afectar de forma negativa a la mineralización del hueso de los animales en crecimiento [20,21]. Esta formación de jabones es más pronunciada en presencia de altos

niveles tanto de grasa como de minerales en la dieta; también, en el caso de AGS frente a los ácidos grasos poliinsaturados (AGPI), dado que, la absorción de los primeros ya es menos eficiente por lo que aún se verá más perjudicada y, por último, en animales jóvenes ya que su capacidad para digerir y absorber es más limitada que en el caso de animales adultos [5]. De esta forma, añadir mayor cantidad de grasa o de minerales en una formulación animal puede finalmente afectar de forma negativa a la absorción de ambos, de aquí la importancia de seleccionar contenidos apropiados de estos dos ingredientes, valorando a su vez el tipo de fuente grasa, ya que dependiendo del grado de saturación o de si los AG se encuentran en forma libre o no, la formación de jabones se podrá ver modificada.

Por último, el papel de los lípidos es de gran importancia en la fabricación del pienso ya que reducen el contenido de polvo, beneficiando los procesos de producción al proporcionar un efecto cohesivo y lubricante [10,22] Además, se utilizan para proveer al pienso de una estructura y textura determinadas por ejemplo en la extrusión, actuando como plastificantes y emulsionantes disminuyendo la viscosidad del producto y reduciendo así la energía aplicada durante el proceso [23]. Sin embargo, cuando dichos contenidos de grasa exceden un punto crítico pueden afectar negativamente los procesos de granulado o extrusionado si en vez de una correcta mezcla entre ingredientes se produce la separación de los lípidos [23,24]. Además, niveles de grasa por encima del 12%, a pesar de reducir la generación de polvo como efecto positivo gracias a su efecto lubricante, pueden perjudicar la dureza del pienso granulado, característica esencial en la calidad física del producto obtenido [25]. Y ya no sólo la cantidad de grasa añadida, sino también el tipo de fuente grasa puede afectar la tasa de expansión durante la extrusión [26]. De forma general, en las dietas de ganado basadas en forrajes la cantidad de grasa añadida no supera el 6% de la ración total en base a materia seca [19]. Según Heugten et al. [7] en el caso de los cerdos, el contenido de lípidos en las formulaciones dietéticas es desde un 0,5 a un 7%, ya que cantidades superiores podrían causar problemas en la fabricación del pienso como los que se han mencionado anteriormente [23,25,26], resultando difícil mantener un nivel adecuado de dureza en el producto final (en granulados y extrusionados), así como problemas en la manipulación del pienso.

2.2. Tipos de grasas y aceites usados en alimentación animal: caracterización y valor energético

De acuerdo con el *Economic Research Service* de Estados Unidos, la producción mundial de aceites vegetales en el período 2019/20 es de 207,28 millones de toneladas métricas, siendo el orden de producción de los principales aceites el siguiente: aceite de palma (35,33%), aceite

de soja (27,95%), aceite de colza (13,50%) aceite de girasol (10,36%), aceite de oliva (1,51%), aceite de coco (1,74%), aceite de algodón (2,48%), aceite de cacahuete (3,06%) y aceite de palmiste (4,13%) (Figura 1) [27].

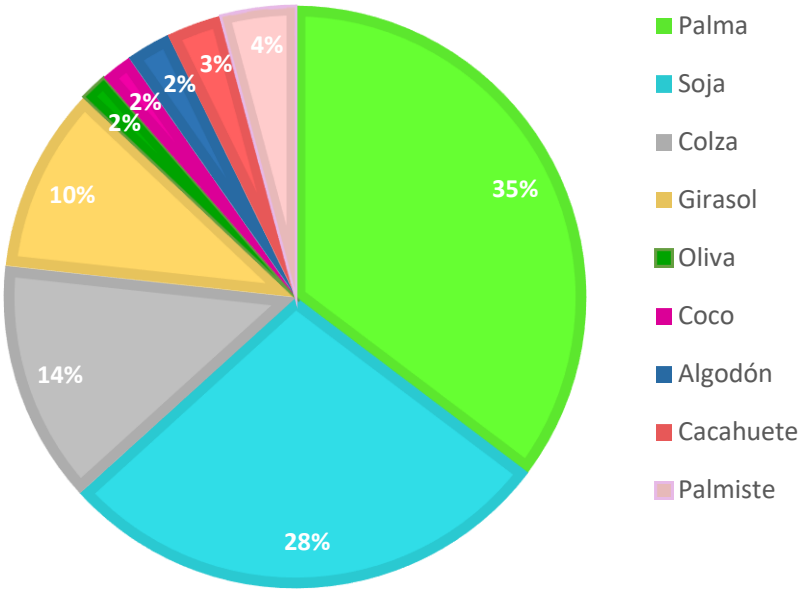


Figura 1. Producción mundial de los principales aceites vegetales. Período 2019/2020. Fuente: *Economic Research Service* de Estados Unidos, https://www.ers.usda.gov/data-products/oil-crops-yearbook/ [27].

Estos aceites y grasas vegetales, así como otras grasas de origen animal (manteca de cerdo o grasas de aves), constituyen las fuentes primarias de grasas y aceites crudos que son los que normalmente se usan en alimentación animal. Además, es posible encontrar subproductos, que permiten sustituir las grasas crudas debido a su potencial energético, usándose directamente o tras su procesado o mezcla con otras materias lipídicas [10]. El Catálogo Europeo de ingredientes para piensos recogido en el Reglamento de la Comisión N°68/2013 lista los ingredientes aceptados a nivel europeo para alimentación animal donde se incluyen, entre otros, varios subproductos grasos (como las pastas jabonosas, los AGD o las lecitinas, entre otros), así como, los parámetros que deben declararse de forma obligatoria en cuanto a su composición [12]. Además, los lípidos de una dieta animal también proceden, aunque en cantidades más bajas, de otros ingredientes del pienso que no son las propias materias grasas como, por ejemplo, de los cereales (maíz, cebada, avena y trigo) o en menor cantidad, de las harinas de semillas oleaginosas (algodón, soja) conteniendo principalmente ácidos grasos insaturados (AGI) de 18 átomos de carbono y de 1-3 dobles enlaces [9].

Por tanto, en el mercado de la alimentación animal pueden encontrarse diversos ingredientes grasos, los cuales varían en diversos aspectos como disponibilidad, precio, composición y calidad. En la formulación de piensos el valor de las diferentes grasas y aceites

viene definido básicamente por la energía que aportan, según la especie animal que se considere, energía metabolizable aparente en el caso de pollos (EMA) o energía digestible (ED) en cerdos, la cual dependerá de su digestibilidad y absorción. En el caso de animales monogástricos, la digestión y absorción de los lípidos viene principalmente definida por factores relacionados con la composición de la grasa, así como por factores relacionados con el animal (edad, sexo o especie) [7,10,28]. Con relación a la composición de la fuente lipídica, influyen tanto el tipo de AG y TAG que la constituyen (el grado de saturación y la longitud de la cadena carbonada de los AG, el contenido de AGL o la posición de estos dentro del TAG), como la calidad de la grasa. De forma práctica en el campo de la nutrición animal, la calidad de grasa viene determinada por la cantidad de compuestos que diluyen su valor energético. Entre ellos encontramos tres parámetros, la humedad y materia volátil (M), las impurezas insolubles (I) y la materia insaponificable (U), que sumados dan lugar a un valor global conocido generalmente como MIU (*moisture and volatile matter + insoluble impurities + unsaponifiable matter*), el cual es un parámetro de gran relevancia en el sector a la hora de seleccionar las fuentes grasas usadas en las dietas animales teniendo en cuenta los parámetros productivos finales. A su vez, se conoce como NEM (material no eluíble; *non-elutable material*) a una mezcla heterogénea de compuestos donde, entre otros, pueden encontrarse compuestos no nutricionales (incluyéndose el MIU además de otros productos oxidados y polimerizados [29]). Es común en el caso de las formulaciones para no rumiantes, como cerdos o pollos, el uso de mezclas de diferentes fuentes grasas para mejorar su valor nutricional [30].

El proceso general de digestión de los lípidos consiste básicamente en su solubilización en el medio acuoso intestinal mediante la formación de micelas mixtas y su posterior absorción en el intestino delgado. Según la revisión de Ravindran et al. [28] este sería el caso de AG de cadena media y larga, DAG y vitaminas liposolubles, mientras que los AG de cadena corta y los MAG no necesitan emulsionarse y son absorbidos directamente de forma pasiva una vez en contacto con las microvellosidades intestinales. Sin embargo, en la revisión de Wealleans et al. [31] son los AG de cadena corta y media los que se solubilizan como componentes individuales mientras que los MAG y los AG de cadena larga son los que requieren de la formación de micelas para ser transportadas hacia los enterocitos en el intestino delgado. Por tanto, de forma general, debido a la insolubilidad de los lípidos en el medio acuoso intestinal, la función de las sales biliares es reducir la tensión interfacial actuando como emulgentes formando las micelas mixtas. A continuación, intervienen la colipasa y la lipasa pancreática rompiendo el enlace éster en las posiciones *sn*-1 y *sn*-3 de los TAG, liberando 2 AGL y un MAG con el ácido graso en posición *sn*-2 (Figura 2). En cuanto a la secreción de sales biliares, esta se ve influenciada por la cantidad y

tipo de grasa en la dieta, y se ve limitada en animales jóvenes. Respecto al tipo de grasa añadida en la formulación, tanto la longitud de cadena como el número de insaturaciones influyen en la absorción de la grasa, siendo los AGI los que se absorben de forma más eficiente en comparación con los AGS, así como los AG de cadena media se absorben mejor que los de cadena larga [32]. Esto se debe a que los AGI presentan mayor capacidad de formar micelas respecto a los AG de cadena larga, sobre todo si estos últimos son saturados, y a su vez, favorecen la incorporación de los AGS en ellas, mejorando la absorción de AG totales [7,28,33]. También, es relevante considerar la presencia de MAG en la absorción, ya que son necesarios para la eficiente solubilización y absorción de los AGL [34]. Respecto a las micelas permiten el paso mediante difusión pasiva del material lipídico al interior del enterocito, donde a continuación se produce en el retículo endoplasmático la re-esterificación de los AGL y MAG para formar de nuevo los TAG [10]. Finalmente, los TAG son empaquetados en los quilomicrones, los cuales pasan al sistema sanguíneo que los transporta a los adipocitos donde se almacenan [10].

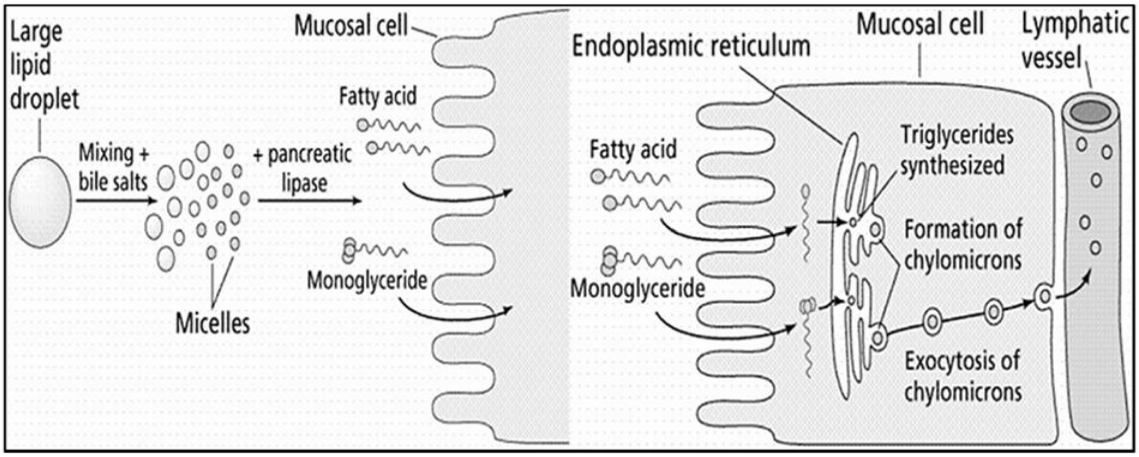


Figura 2. Proceso de digestión y absorción de los lípidos. Fuente: Kerr et al. [10].

Considerando lo anterior, Wiseman et al. [35] evaluó los factores con mayor influencia en la EMA en pollos: grado de saturación de los AG, contenido en AGL y edad de los animales. Observó un incremento exponencial de la EMA al aumentar el grado de insaturación, definido por la ratio entre AG insaturados y saturados, denominada relación I/S (*U/S ratio,* donde los AG con 12 carbonos o menos, independientemente de que sean o no saturados, se consideran como AGI, incluyéndose en el numerador de la ratio, y los AG *trans* se consideran como AGS y, por tanto, se incluyen en el denominador, este cálculo de la ratio se utiliza para predecir el valor energético de las grasas. Dicho aumento de EMA al aumentar la relación AGI/ AGS se observó sobre todo en pollos adultos respecto a los jóvenes, así como un decrecimiento lineal de la EMA a medida que se incrementaba el contenido de AGL, y finalmente una posible interacción entre AGL y la relación I/S. De aquí que posteriormente Wiseman desarrollase una ecuación para

calcular la EMA en pollos o la ED en cerdos en la que asumía la influencia de ambos parámetros, es decir, tanto el contenido en AGL como la relación I/S [30]. En un estudio posterior observó el efecto en la reducción de la EMA por el NEM [36], como ya se ha mencionado, parámetro que algunos autores consideran una buena estimación de la suma de M, I, U y material oxidado, pudiendo actuar todos ellos como diluyentes de energía, perjudicando el crecimiento del animal [10]. Pero a pesar de la clara influencia de la composición y calidad de las fuentes grasas sobre la energía que el animal podrá obtener de ellas, así como sobre otros aspectos productivos, de salud y de calidad de producto final, no siempre existen límites o recomendaciones establecidos para su control de cara a su uso en alimentación animal.

En cuanto a la composición en AG y otros componentes minoritarios (como los que forman parte de la U) de las grasas y aceites de origen vegetal, no sólo dependen de la especie o variedad vegetal, sino que también intervienen otros factores como las condiciones climáticas, el tipo de suelo, la madurez y salud de la planta o la temporada de cultivo [3,37]. Dentro de estos componentes minoritarios de U se encuentran principalmente los T y T3 (vitamina E), y también, compuestos fenólicos, esteroles, hidrocarburos, pigmentos (carotenoides y clorofilas), trazas de contaminantes o pesticidas, entre otros. Los componentes minoritarios con acción antioxidante, como por ejemplo los T y T3 (vitaminas liposolubles), carotenoides o compuestos fenólicos, están presentes de forma natural en la mayoría de los aceites y grasas de origen vegetal, y proveen a éstas de una mayor estabilidad a la oxidación evitando su deterioro (rancidez) y prolongando así su vida útil [38]. Sin embargo, la reducción de los contenidos de T y T3, así como, de los AGPI puede darse a raíz de los procesos de oxidación, a la vez que se incrementan los productos de oxidación afectando todo ello a la composición y calidad de las materias grasas [10].

Los AGPI libres o ligados a los TAG son susceptibles a la oxidación mediante procesos complejos de autooxidación, fotooxidación y oxidación enzimática [38]. Además de los procesos de oxidación, la rancidez hidrolítica de grasas y aceites puede deberse a procesos de hidrólisis mediante la ruptura de los TAG por acción enzimática en presencia de agua liberando AGL. El proceso de autooxidación se trata del más común de todos los procesos de oxidación, siendo una reacción de los lípidos con oxígeno atmosférico (oxígeno triplete, $^3O_2$) produciendo una reacción en cadena de radicales libres [38,39]. La autooxidación consta de tres etapas: iniciación, propagación y terminación [40,41]. En la iniciación, un hidrógeno es sustraído de un lípido insaturado (RH), dando lugar a un radical alquilo (R•) en presencia de una fuente de energía (la luz o las altas temperaturas) o mediante un catalizador (metales) (Ecuación 1) [42]. Dicha sustracción del hidrógeno será más o menos fácil según el grado de insaturación de los AG o su

esterificación, siendo los AGL más susceptibles a ser oxidados que los AG esterificados con glicerol [41].

- **Iniciación:**

  RH → R• + H • (1)

A continuación, durante la etapa de propagación, el radical alquilo reacciona con el oxígeno triplete ($^3O_2$) formándose un radical peroxilo (ROO•) (Ecuación 2), el cual sustrae un nuevo hidrógeno de otro lípido insaturado generándose los hidroperóxidos (ROOH) y otro radical alquilo que continúa la reacción en cadena de radicales libres (Ecuación 3) [41,42].

- **Propagación:**

  R• + $^3O_2$ → ROO• (2)

  ROO• + RH → ROOH + R• (3)

Finalmente, el proceso termina cuando se combinan dos radicales formando un compuesto dimérico no radicalario (Ecuaciones 4-9) [43]. En estados avanzados de la oxidación se incrementa significativamente las reacciones entre radicales formando compuestos poliméricos de mayor peso molecular [39].

- **Terminación:**

  R• + R• → RR (4)

  R• + ROO• → ROOR (5)

  RO• + RO• → ROOR (6)

  RO• + R• → ROR (7)

  ROO• + ROO• → ROOR + O_2 (8)

  2RO• + 2ROO• → 2ROOR + O_2 (9)

Por otra parte, los hidroperóxidos como productos de oxidación primaria son inestables y terminan fragmentándose en compuestos de oxidación secundaria, de menor peso molecular (y por lo tanto muchos de ellos volátiles), y que suelen ser hidrocarburos alifáticos o suelen contener grupos carbonílicos en su estructura, como los aldehídos, cetonas. El tipo de compuestos de oxidación secundaria formados dependerá del AG que ha dado lugar al hidroperóxido [44,45].

Por tanto, la tasa de oxidación de una grasa o aceite está influenciada principalmente además de por el perfil de AG (a mayor número de dobles enlaces mayor susceptibilidad a la oxidación), por la forma en la que se presentan esos AG (libre o esterificado con el glicerol formando los TAG), por la presencia de componentes minoritarios antioxidantes (T y T3), así

como por la presencia de agentes prooxidantes, entre otros. Pero también, además de la naturaleza de la grasa o aceite, otros factores ambientales a los que está expuesto durante el procesado y almacenamiento pueden afectar a las reacciones de oxidación, por ejemplo, las altas temperaturas pueden acelerar los procesos de oxidación [38]. Para predecir la oxidabilidad de la fracción lipídica, se utiliza la relación AGI/AGS (*UFA/SFA ratio*), la cual se calcula en base únicamente a la presencia de dobles enlaces en los AG, incluyendo todos los insaturados en el numerador y todos los saturados en el denominador.

De esta forma, la oxidación lipídica ocurre en los ingredientes grasos de forma inevitable y va acompañada de una reducción del valor nutricional, relacionado con la pérdida de AGPI y antioxidantes, y con un incremento en los productos de oxidación, lo que implica una pérdida del valor energético, es decir, una reducción de la eficiencia alimentaria. Por ello, en la caracterización de grasas y aceites se deberían incluir parámetros que indiquen el estado de oxidación de las fuentes lipídicas para mejorar su control de calidad y obtener mejores resultados productivos [46].

2.2.1. Procedencia de los AA y AGD

Los aceites y grasas de origen vegetal podrían clasificarse como aquellos procedentes de frutos (por ejemplo, coco, palma u oliva) o de semillas (soja, girasol y palmiste, entre otras). En la mayoría de los casos, salvo alguna excepción como por ejemplo el aceite de oliva virgen, se requiere del proceso de refinación previamente a su consumo humano. Los procesos de refinación se basan en la eliminación de compuestos no deseados (fosfolípidos, AGL, pigmentos, productos de oxidación, trazas de metales o contaminantes) minimizando la pérdida de aceite, así como de compuestos beneficiosos (antioxidantes) [47]. Los aceites vegetales de consumo humano pueden refinarse mediante dos procesos que principalmente difieren en la forma de eliminar los AGL: refinación química (basada en una neutralización de los AGL) o física (por destilación de estos AGL). La refinación química es el proceso más ampliamente utilizado para aceites insaturados (soja, girasol, colza…) mientras que la refinación física se suele utilizar preferentemente para aceites o grasas más saturados (palma, coco, palmiste) [11,48]. Si comparamos ambos procesos, la refinación química requiere más etapas, genera más diversidad de subproductos e implica un rendimiento de aceite refinado ligeramente menor [49]; en cambio la refinación física al presentar menos etapas implica la generación de menos subproductos y se considera más sostenible desde el punto medioambiental , así como supone un alto rendimiento del aceite refinado obtenido. No obstante, el uso de altas temperaturas y presiones en la refinación física puede dar lugar a alteraciones perjudiciales en el aceite

afectando a la estabilidad oxidativa del aceite refinado y a su contenido en isómeros *trans* y compuestos de oxidación, sobre todo en el caso de grasas más fácilmente oxidables como serían las más insaturadas. Esto explica que la aplicación de uno u otro tipo de refinación vendrá muy definida por el tipo de aceite crudo a refinar [11,50].

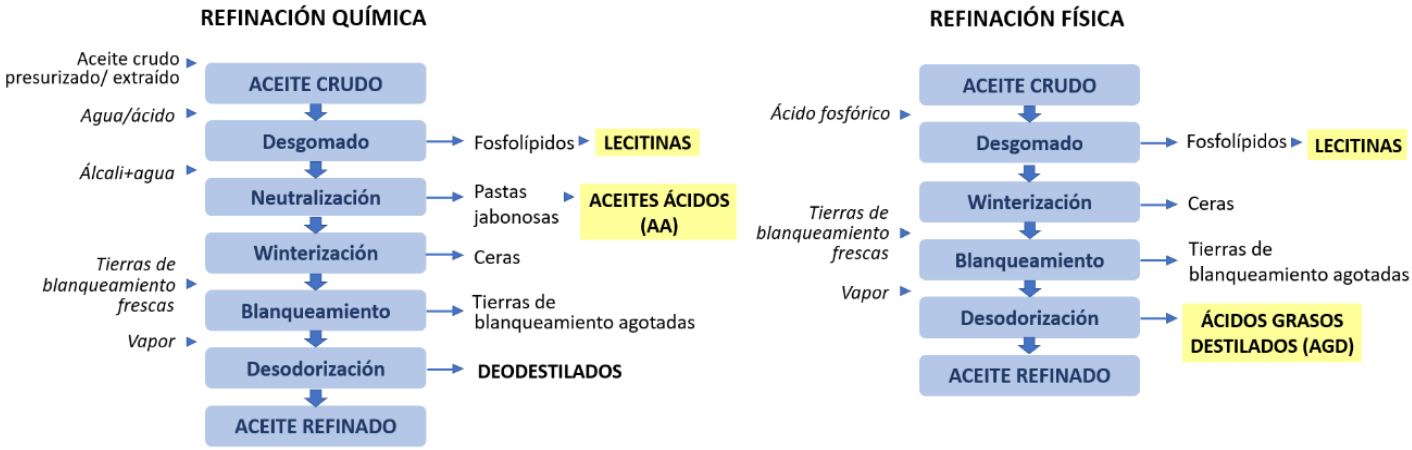


Figura 3. Procesos de refinación de grasas y aceites vegetales. Refinación química y refinación física de grasas y aceites para consumo humano, con la correspondiente obtención de los subproductos destinados a alimentación animal. Fuente: FEDIOL, 2021 [8].

Normalmente, la refinación química consiste en las siguientes etapas: desgomado, neutralización, winterización (opcional), blanqueamiento y desodorización, mientras que en la refinación física las principales etapas son desgomado, winterización (opcional), blanqueamiento y desodorización (Figura 3) [8]. En sus revisiones, Ghazani et al. [4] y Vaisali et al. [50] analizaron las distintas etapas de los dos procesos de refinación de aceites vegetales. En primer lugar, el desgomado es la etapa inicial de ambos tipos de refinación de aceites y grasas comestibles y su objetivo es eliminar los fosfolípidos, trazas de metales y sustancias mucilaginosas, y, además, según FEDIOL [8] entre las sustancias a eliminar en esta etapa también se incluyen partículas, impurezas, carbohidratos y proteínas. Este proceso puede llevarse a cabo únicamente con la adicción de agua, actuando sobre las gomas fácilmente hidratables (por ejemplo fosfatidilcolina, fosfatidilinositol y fosfatidiletanolamina) las cuales finalmente pueden separarse por centrifugación (según FEDIOL alcanzando temperaturas de 100 °C), o a ese tratamiento con agua se le puede añadir otro con ácido fosfórico o cítrico, seguido también de una centrifugación con lo que se consigue eliminar los fosfolípidos no hidratables como sales de calcio o magnesio de ácido fosfatídico [4,50,51]. A pesar de que algunos de los fosfolípidos podrían ser beneficiosos para la salud, éstos deben ser eliminados ya que son mucho más propensos a la oxidación que los acilgliceroles, además, pueden suponer altas pérdidas de aceite al formar emulsiones durante el proceso y por último podrían ser causa de oscurecimiento del aceite una vez almacenado [50,52]. A continuación, en el caso de la refinación química la

segunda etapa consiste en una desacidificación, la cual se denomina neutralización y consiste en tratar el aceite desgomado con un álcali como el hidróxido sódico en exceso teniendo en cuenta la cantidad de AGL del aceite a refinar. Así, los AGL precipitan en forma de jabones (denominados pastas jabonosas o en inglés, *soapstocks*) para ser eliminados mediante una centrifugación o lavado con agua. Así la gran mayoría de AGL se elimina en esta etapa (aquellos que permanezcan en el aceite se eliminarán más adelante en la desodorización). Junto a los AGL también se eliminan algunos productos de oxidación de los AG, así como restos de proteínas, fosfolípidos, carbohidratos, trazas de metales y una parte de pigmentos [8]. En cuanto a la refinación física, la etapa de eliminación de AGL se corresponde directamente con la desodorización, donde el aceite previamente desgomado se trata con altas temperaturas (generalmente 230-240 °C, aunque según FEDIOL [8] y Naz et al. [6] el rango de temperaturas posible es más amplio, entre 180-270 °C). Además de las altas temperaturas, se establecen unas condiciones de vacío (0.5-8 mbar [8]) y se hace pasar una corriente de vapor que arrastra los AGL principalmente, pero también otros compuestos volátiles como pesticidas, hidrocarburos aromáticos policíclicos o productos de oxidación causantes de aromas indeseados [8]. En ambos casos, tanto en la refinación química como física, esta etapa de desacidificación es esencial ya que es preciso eliminar los AGL para garantizar la calidad del aceite refinado, impidiendo las reacciones de oxidación y retrasando así su rancidez. La siguiente etapa, denominada winterización, es opcional en ambos procesos de refinación, y se trata de un proceso de fraccionamiento para eliminar las ceras y los TAG con elevado punto de fusión [8]. Tras esto, se continúa con la etapa de blanqueamiento también aplicada en ambos procesos de refinación, la cual consiste en la eliminación de diversos materiales mediante mecanismos de adsorción en agentes blanqueantes, para conseguir mejorar aún más la estabilidad oxidativa y cualidades sensoriales del aceite [4,51]. Entre los componentes eliminados se encuentran muchos minoritarios como pigmentos (carotenoides, clorofilas) o restos de jabones, fosfolípidos, iones metálicos (hierro y cobre), contaminantes y productos de oxidación (peróxidos) que aún permanecen en el aceite neutralizado [11,53]. Finalmente, la desodorización se trata de la última etapa en ambos procesos. Su objetivo es prolongar al máximo la vida útil del aceite, siendo esencial en el caso de la refinación física ya que como previamente se ha mencionado consiste en la etapa donde se eliminan los AGL, además de otros contaminantes volátiles odoríferos, productos de oxidación (aldehídos, cetonas, alcoholes) y no intencionadamente pero de forma inevitable, también una parte de MAG, DAG y compuestos minoritarios de U (T, T3, esteroles, polifenoles, escualeno), todos ellos componentes que pueden volatilizarse durante la destilación a altas temperaturas y bajas presiones [6]. Algunos factores que pueden afectar la eficiencia de

esta última etapa son la temperatura, la presión, el tiempo o la cantidad de vapor utilizado afectando a la calidad final del aceite, así como a la pérdida de dichos componentes minoritarios de U los cuales poseen cualidades positivas, que interesaría mantener en el aceite refinado. Por ello, las condiciones de la desodorización se basan en la calidad inicial del aceite. Además, durante la desodorización tanto en la refinación química como en la física, debido a las condiciones de destilación a altas temperaturas pueden producirse reacciones secundarias no deseadas que afectan negativamente al aceite, como polimerización de los TAG, ciclación de los AGI, formación de AG *trans* con un drástico incremento sobre todo en el caso de la refinación física, siendo la temperatura entre 220-230 °C el punto crítico por encima del cual parece aumentar sustancialmente la isomerización, así como la formación de compuestos tóxicos como los ésteres de 3-monocloropropano-1,2-diol (3-MCPD) y los ésteres glicídicos (GE) [48,54,55].

Por todo ello, durante la refinación se produce una reducción de componentes bioactivos de U ya sea por su eliminación en la destilación y acumulación en los subproductos obtenidos, o por su destrucción debido a su inestabilidad. Es el caso por ejemplo de los T y T3 con actividad Vitamina E, los cuales son antioxidantes naturales que impiden las reacciones de oxidación evitando el daño oxidativo, o de los fitoesteroles que impiden la absorción del colesterol a nivel intestinal a la vez que poseen, entre otras, propiedades antiinflamatorias [6]. Algunos estudios han analizado la pérdida progresiva de T o fitoesteroles tras los procesos de refinación, y básicamente destacan un decrecimiento en el contenido individual y total de los T tras ambos procesos de refinación, aunque este efecto es más notable en la refinación física [3,55–58]. Concretamente, la etapa de desodorización de este tipo de refinación supuso la mayor reducción del contenido total de T, debido a las condiciones del proceso (altas temperaturas aplicadas) y a su alta volatilidad. Por otro lado, en la refinación química se observó una mayor reducción del contenido total de T en el desgomado y la neutralización, a causa de la inestabilidad de los T ante un largo contacto con el aire y el álcali. Y aunque ligeramente, los T también fueron afectados durante las primeras etapas de la refinación, también observándose diferencias significativas en los T totales tanto en la refinación física como química tras el desgomado o después del blanqueamiento, debido a la adsorción y oxidación de los T en las tierras de blanqueamiento, pero no fueron observados cambios tras la winterización [3,56–59].

Por tanto, durante los procesos de refinación se generan varios subproductos (Figura 3). En el caso de la refinación química, durante la etapa del desgomado se obtiene una pasta rica en fosfolípidos, que contiene básicamente fosfolípidos hidratables, AGL y aceite neutro (acilgliceroles). A partir de esta pasta desecada se obtienen las distintas lecitinas. A continuación, tras la neutralización se producen las pastas jabonosas que se someten a un tratamiento con

ácido sulfúrico en exceso, dando lugar al AA constituido principalmente por AGL, aceite neutro y componentes minoritarios. Las pastas jabonosas se generan en una tasa de alrededor del 6% y están constituidas por un porcentaje de grasa y otro de agua [59]. Si únicamente se considera la pérdida de aceite y no la suma de aceite y agua que constituiría las pastas jabonosas, la pérdida sería de un 1,6-2% [60]. A partir de las pastas jabonosas secas, es decir, considerando sólo la parte grasa sin humedad, se puede obtener un rendimiento del 85% de AA. Por lo tanto, considerando la pérdida de aceite que suponen las pastas jabonosas y el rendimiento en la obtención del AA a partir de estas, los AA supondrían un 1,36-1,7% del aceite de partida. De todas formas, hay que tener en cuenta que la cantidad de subproducto obtenido finalmente dependerá de la acidez del aceite crudo inicial (que puede ser muy variable en función de la calidad del aceite crudo), ya que cuanto más ácido sea, se obtendrá una mayor cantidad de pastas jabonosas y por consiguiente más AA. En el caso de los AGD, como se obtienen directamente tras la etapa de desodorización en la refinación física, equivalen propiamente a la pérdida de aceite que se produce. Según estudios previos, la refinación del aceite de palma supone la obtención de un 4% de AGD [61]. Después, durante la etapa de blanqueamiento se obtienen las tierras de blanqueamiento agotadas, que contienen entre un 30-50% en peso de aceite. Y, por último, durante la desodorización de la refinación química se generan los deodestilados, constituidos principalmente por compuestos muy volátiles (e.g. compuestos de oxidación secundaria), compuestos bioactivos que forman parte de U (e.g. vitamina E y esteroles) que incrementan el valor añadido de estos subproductos y AGL [56,62–64]. La principal diferencia respecto a los AGD obtenidos en la refinación física, es que el contenido en AGL es mucho más bajo en los deodestilados de refinación química, con valores desde 10 a 24,5% frente a un 76,2-83,6% en los AGD de refinación física, mientras que el U es mayor (en torno a 9,2-15% de T y 9-17,6% de esteroles frente a un 1,4-4,3% y 1,8-6,9%, respectivamente en los AGD) [53,65]. Es por esto que la caracterización de los deodestilados se basa en la identificación y cuantificación del contenido en T, T3 y esteroles y su extracción y recuperación, ya que son compuestos minoritarios de alto valor comercial como fuente de antioxidantes naturales. Además, el hecho de extraerlos del deodestilado es muy importante puesto que también se acumulan en este los contaminantes orgánicos como los hidrocarburos aromáticos policíclicos (HAP) que reducen la seguridad de su uso en la industria alimentaria [11,65,66].

En el caso de la refinación física se obtienen los mismos subproductos con la excepción de los AA, ya que esta etapa no se lleva a cabo en este tipo de refinación. Además, como se ha comentado anteriormente, los AGD obtenidos en la etapa de desodorización en la refinación física se caracterizan por su mayor contenido en AGL y menor contenido en compuestos

bioactivos (componentes de la U) [65]. La utilización de estos subproductos en distintas áreas, además de cumplir con los requerimientos de la industria a nivel medio ambiental, da un valor añadido al sector de la refinación de aceites y grasas de consumo humano. Las pastas jabonosas pueden utilizarse con fines industriales para el crecimiento de microorganismos, como fertilizantes en agricultura, como fuente novedosa para la preparación de biodiesel y por último para alimentación animal una vez convertidos en AA [67]. Los AA en alimentación animal (especialmente en cerdo y pollo) resultan muy interesantes por su bajo precio, sus buenos resultados a nivel nutricional observados en los parámetros productivos, debido a los altos contenidos en AGL que aumentan la energía de la dieta y mejoran la palatabilidad, además contienen componentes bioactivos muy valorados (e.g. vitamina E como antioxidante natural) [11,62]. Aproximadamente se componen de AGL (65-70%), aceite neutro (TAG en un 20-30%), componentes minoritarios e I (sobre un 5%) [56]. De forma similar, los AGD resultan interesantes para la elaboración de piensos debido a su precio altamente competitivo, su alto contenido en AGL y componentes de la U con valor nutricional (T y T3). También pueden tener otras aplicaciones como en la industria cosmética (por sus altos contenidos en compuestos bioactivos) o para la fabricación de biodiésel (por el alto porcentaje en AGL) [6].

2.2.2. Caracterización de los AA y AGD

El uso de los AA y AGD está autorizado a nivel europeo, ya que se encuentran incluidos en el Catálogo Europeo de ingredientes para piensos [12]. Al igual que con otros ingredientes, para garantizar la seguridad, eficacia y calidad de estos subproductos en alimentación animal, es necesario conocer su composición. En el Catálogo Europeo de ingredientes para piensos, se encuentran algunas declaraciones obligatorias para ambos subproductos como es el caso del contenido de grasa bruta o el porcentaje de humedad cuando sea superior al 1%. También se indica la necesidad de especificar en dicho Catálogo los contenidos de I generadas como consecuencia del proceso de obtención cuando sean superiores al 0,1% [12]; sin embargo, en el caso de los AA y los AGD no se especifica un contenido máximo de I concreto. Además, al igual que ocurre con otros ingredientes, estos subproductos deben cumplir con la normativa que fija los niveles máximos de contaminantes entre los cuales figura el caso de las dibenzo-*p*-dioxinas o los dibenzofuranos [68,69], los cuales precisan un control de sus niveles en las grasas utilizadas para la fabricación de piensos, sobre todo en el caso de los AGD donde se presentan en mayores cantidades respecto a AA [70]. Por otro lado, existen normas de calidad y control elaboradas por asociaciones de fabricantes de piensos y ganaderos, como por ejemplo las establecidas por la FEDNA, pero son de carácter voluntario y para los AA y AGD solo recomiendan contenidos

máximos para ciertos parámetros de composición u oxidación [71]. Por ejemplo, recomiendan un contenido de I máximo de 0,15%, valores de MIU menores de 5% o de NEM menores de 7%, así como un contenido de peróxidos menor de 10 meqO_2/kg [28,71]. Sin embargo, la composición de los AA y AGD puede variar considerablemente dependiendo tanto de la procedencia o tipo de aceite crudo, también según el tipo de refinación, así como de las condiciones de refinación empleadas, las cuales a su vez son dependientes de la calidad inicial del aceite crudo. De esta forma, todos estos factores afectarán finalmente a la composición tanto del aceite refinado como de los subproductos generados. Además, en las refinerías en algunas ocasiones mientras se van refinando diferentes tipos de aceites, los subproductos se recogen en un único tanque (esto puede suceder tanto para AA como para AGD), obteniéndose mezclas de subproductos cuya composición es aproximada o desconocida por los propios productores. A esto se le suma el hecho que estos subproductos pueden presentar diferente viscosidad, formándose distintas capas en el propio tanque en función de los lotes refinados, complicando la homogeneidad de las mezclas. Por otro lado, también es cierto que se dan casos en los que los productores mezclan de forma intencionada distintos subproductos de forma que obtienen una composición determinada en la mezcla final, para adaptarla a los requerimientos nutricionales de los animales para los que se destinen.

Todo esto conduce a que estos subproductos presenten una gran variabilidad en su composición, incluso entre lotes de un mismo productor, lo que lleva a una clara necesidad de caracterizarlos. Dicha caracterización debe estudiar parámetros de composición, valor nutricional y oxidación, de tal forma que se puedan establecer cuáles son las fuentes de variabilidad en todos ellos y estableciendo recomendaciones de su uso basándose en los contenidos de diferentes parámetros que puedan afectar al valor nutricional, a la energía que aportan, o incluso que puedan afectar negativamente a la salud de los animales cuando algunos parámetros superan un límite determinado. Sin embargo, pocos son los estudios que hayan evaluado exhaustivamente la composición de estos subproductos o hagan recomendaciones de su uso en alimentación animal. En este sentido, Gunawan y Yi-Hsu [72] muestran en su revisión las características de diferentes subproductos procedentes de la etapa de desodorización en la refinación de aceites vegetales (soja, maíz, girasol, colza, oliva y palma), concretamente AGD procedentes de refinación física y deodestilados obtenidos en la refinación química. La diferencia principal entre ambos es el contenido en AGL, superior en los AGD (>70%) frente a los deodestilados (30-50%); sin embargo, el contenido en U se mantuvo inferior en los primeros (5-10%) frente a los deodestilados con un rango entre 25-33%. A su vez, indicaron la presencia de AG *trans* en ambos tipos de subproductos, cuya formación se veía favorecida según la

composición del aceite inicial (mono o poliinsaturado) y por las altas temperaturas alcanzadas durante la etapa de desodorización. También, revisaron el contenido en acilgliceroles (TAG, DAG y MAG) que fue superior en los deodestilados (10-20%) frente a los AGD (<8%). Diferenciaron los subproductos procedentes de soja, maíz, girasol, colza y oliva como fuentes ricas en T y, por otro lado, los T3 fueron encontrados en la palma. A su vez, tanto los AGD como los deodestilados constituían fuentes importantes de fitosteroles, tanto libres como conjugados, así como de hidrocarburos como el escualeno (este último, concentrado especialmente en la refinación de la palma como AGD). Concluyeron que según las fuentes iniciales estos subproductos tendrán características significativamente diferentes, que a su vez modifican sus usos y valor. Además, en otro estudio, Nuchi et al. [13] evaluaron diferentes parámetros de degradación en diferentes subproductos, entre ellos los AA (n = 25) y los AGD (n = 16), junto con otros subproductos de la industria (subproductos hidrogenados (n = 6), aceites extraídos de tierras de blanqueo (n = 2), aceites de fritura (n = 8), lecitinas (n = 8), grasas animales (n = 36) y aceites de pescado (n = 9)). Realizaron una caracterización de estos subproductos y observaron diferencias en los compuestos de oxidación entre AA y AGD, sugiriendo que tanto el perfil de AG como las condiciones respectivas de refinación y almacenamiento afectaban a estos parámetros de forma relevante. Por ejemplo, los AGD mostraron un alto valor del *p*-AnV (índice de *p*-anisidina), que es un parámetro para determinar los compuestos aldehídicos de oxidación secundaria (valor medio de 35,0), mientras que el contenido de compuestos poliméricos (POL) (parámetro complementario que también evalúa la oxidación avanzada) fue superior en los AA (media de 0,7% en AA frente a un 0,0% para los AGD) lo cual perjudica la digestibilidad del subproducto. En este último caso, debido al elevado peso molecular de los POL se ve dificultada su destilación y por ello, era de esperar que no se encontraran en los AGD. En cuanto al PV (índice de peróxidos, *peroxide value*) en ambos casos se registraron valores muy bajos, lo cual lo relacionaron con su descomposición por el avance de los procesos de oxidación o por los tratamientos térmicos que implica el proceso de refinación. Respecto a parámetros de composición, observaron un mayor contenido de T en AGD (media 2595,5 mg/kg) respecto a AA (media 415,2 mg/kg) y también alto contenido de T3 en el caso de los AGD (media 507 mg/kg), concluyendo que, a pesar de las drásticas condiciones de los procesos de refinación, dichos componentes minoritarios se verán acumulados en los subproductos, dando un valor añadido a su uso en alimentación animal, sobre todo en el caso de los AGD. Además, observaron mayores contenidos de M en el caso de AA que en AGD, debido a la cantidad de agua utilizada en el proceso que favorece su acumulación en el subproducto. Estos contenidos de M correlacionaron con los MAG debido a la relación entre agua y reacciones de hidrólisis. Por otro lado, en esta caracterización, el valor máximo del índice

de acidez se correspondía a los AGD (155,2 mg de KOH/g), mientras que los AA poseían mayores contenidos de TAG y otros componentes del aceite inicial. Sin embargo, concluyen que la calidad de estos subproductos no está estandarizada, lo que implica que no hay establecidos niveles aceptables de degradación que aseguren dicha calidad. De aquí la necesidad de conocer más a fondo los aspectos de composición y degradación de los AA y AGD, y cómo el tipo de refinación o el origen botánico de los aceites crudos influye en su calidad. Además, es preciso evaluar el valor de parámetros como el MIU por su repercusión sobre el valor energético de estos subproductos. Esto es necesario para que su utilización asegure la mejora de la calidad nutricional y de los parámetros productivos y, finalmente, de la calidad de la carne.

Como previamente se ha mencionado, lo que principalmente caracteriza a ambos subproductos (AA y AGD) es su alto contenido en AGL que les confiere un alto valor energético, esencial en alimentación animal [11]. Sin embargo, varios son los factores que afectan la digestión y absorción de las grasas y de esta forma a la energía que proporcionan. Tal y como se ha comentado en anteriores secciones, algunos dependen de la propia composición de la grasa, otros de su calidad, y otros dependen directamente del animal [28]. En este sentido, el estudio del valor energético de los AA y AGD es crítico puesto que son fuentes grasas económicas y disponibles en el mercado de la alimentación animal. Aunque la composición en AG de los AA y AGD es, en grandes rasgos, similar a la de los aceites crudos de los que proceden [13,72], la diferencia principal está en su estructura molecular, ya que, en estos subproductos, los AG en gran parte se encuentran en forma libre en vez de esterificados con el glicerol como lo están en las grasas crudas [73]. Varios estudios, entre ellos Wiseman y Salvador [74] observaron que el incremento en AGL viene acompañado de una reducción en los valores de EMA en dietas para pollos, la cual parecía influenciada por el grado de saturación de la grasa, sobre todo en aves jóvenes, que de acuerdo con lo observado por Sklan en 1979 [34] estaría explicado por una menor absorción debida a una menor secreción biliar y formación de micelas. Este hecho, fue de nuevo estudiado por Wiseman et al. [35] y por Vilà y Esteve-García [75], donde ya concluyeron que el efecto de los AGL viene determinado por la relación I/S. Observaron que al aumentar los AGL incrementando también la proporción de AGS, la EMA se vio reducida, sin embargo, ocurría lo contrario si se aumentaban los AGI. Más recientemente, Rodríguez-Sánchez et al. [76] también obtuvieron resultados similares, donde la grasa saturada tenía un mayor impacto en la disminución de la absorción de los AG que el hecho de que éstos estuviesen presentes en la forma libre. De hecho, estudios en pollos en etapas de crecimiento-terminación alimentados con dietas elaboradas con subproductos, no implicaron un efecto negativo en la absorción de los AG con un contenido de AGL de hasta el 35% [77], o en el caso de incluir en la

dieta AA de soja como fuente rica en AGL e insaturada, la utilización de los AG no se vio afectada hasta un 56,3% de AGL con una relación I/S de 3,88 [78], al igual que ocurrió si se utilizaba AGD de palma, como fuente rica en AGL y saturada, siempre que la dieta no superase el 30% de AGL manteniendo una relación I/S de 2,61 [79]. Sin embargo, Doppenberg et al. [24] indicaron previamente que el valor energético para todo tipo de grasas, con cualquier relación I/S y para toda especie animal, se vería reducido cuando el contenido en AGL alcanza el 50% o por encima. De hecho, debido a la importancia de los AGL y la relación I/S, ambos parámetros fueron incluidos en la ecuación que propuso Wiseman et al. [30] para calcular al EMA para pollos y la ED para cerdos de diferentes edades, y que aún hoy en día es una de las más utilizadas para este fin.

2.3. Estabilidad de los piensos en función de sus ingredientes

2.3.1. La grasa en la formulación de dietas animales

La formulación de piensos consiste en la combinación de diferentes ingredientes con el objetivo de aportar al animal cantidades de nutrientes que cubran sus necesidades nutricionales. Estas necesidades varían en función de la especie y de la etapa de crecimiento en la que se encuentra el animal. Así, los requerimientos nutricionales variarán tanto en el contenido de energía, como de proteínas, agua, minerales y otros componentes que el animal no puede sintetizar y que deben ser ingeridos a través de la dieta (incluyendo vitaminas, algunos aminoácidos y AG esenciales). Por tanto, los requerimientos nutricionales establecidos pretenden mantener las funciones básicas como homeostasis, reproducción, crecimiento y desarrollo, entre otras. De hecho, se pueden encontrar diferentes especificaciones nutricionales para aves y porcino como, por ejemplo, las tablas brasileñas de composición de alimentos y requerimientos nutricionales en aves y cerdos [80], también las tablas de piensos francesas [81], las holandesas [82] o las del Reino Unido [83], así como referencias más antiguas, por ejemplo, las tablas de valores energéticos de ingredientes para piensos en aves de la World´s Poultry Science Association (WPSA) [84] o del National Research Council (NRC) [85]. Pero es la Fundación Española para el Desarrollo de la Nutrición Animal (FEDNA), la que establece las recomendaciones nutricionales de referencia para la formulación de piensos por las empresas del estado español [71]. En el caso de las formulaciones para aves, son mayores los requerimientos nutricionales en comparación con otras especies, siendo necesario elaborar dietas más precisas considerando a fondo la composición química y el valor energético de los ingredientes utilizados con el fin de obtener piensos nutricionalmente equilibrados [86]. Además

de considerar el valor nutricional de cada ingrediente que se verá reflejado en el desarrollo animal, se formula también desde un punto de vista económico, considerando los costes finales para los granjeros y la industria cárnica, ya que dietas mal formuladas podrían limitar el crecimiento animal aumentando los costes o dar lugar a carnes de composición inadecuada para su comercialización o uso posterior en la elaboración de productos cárnicos.

La inclusión de las grasas en las formulaciones de dietas animales cumple diferentes funciones (como ya se ha indicado en la sección 2.1). Resumiendo, la grasa es una fuente concentrada de energía y pequeños cambios en la cantidad de grasa añadida pueden producir efectos significativos en el aporte calórico de la dieta, siendo la homeostasis energética fundamental en la alimentación y bienestar animal. Por ello, la grasa añadida debe considerarse un componente de la nutrición animal tan importante como lo son proteínas o hidratos de carbono [17]. De hecho, como se mencionó previamente, los TAG proveen más del doble de energía por gramo que la que pueden aportar carbohidratos o proteínas [7,24,87]. De forma general, para alcanzar los requerimientos energéticos de los animales es imprescindible conocer el valor energético de los distintos ingredientes, de esta forma se podrá conseguir una correcta formulación. En este sentido, las tablas anteriormente mencionadas de composición y energía de diferentes ingredientes para piensos pueden guiar a la hora de formular las dietas. Además, tal y como se ha comentado en la sección 2.2., también se pueden encontrar las ecuaciones de predicción de energía que, si se conocen las variables de composición de las fuentes grasas, permiten calcular de forma aproximada el potencial energético de estas, siendo la ecuación de Wiseman una de las más utilizadas en la actualidad [30] basada en el grado de saturación de los AG y la cantidad de AGL.

El contenido en AGL es uno de los factores más relevantes que afectan el valor energético final de la dieta, así como la longitud de cadena de los AG, la posición del AG en la molécula del TAG, y también, el grado de saturación de los AG, representado mediante la relación de I/S [24,31]. Considerando este último factor, es un hecho bien reconocido la mayor capacidad de los AGI de ser incorporados a las micelas, serán más digeribles que los AGS; por ello, una mayor relación I/S conlleva mayor digestibilidad de la grasa añadida [31], así como un aporte beneficioso de AG esenciales. Pero el efecto de esta ratio no es lineal, sino que es dependiente de la edad y de la especie, encontrándose diferencias entre animales jóvenes o adultos y también diferencias entre aves y cerdos [30]. Por ejemplo, en animales jóvenes está bien descrito que las grasas insaturadas se utilizan mejor que las saturadas [31,79,88]. Sin embargo, tanto en el NRC [85] como en las Normas FEDNA para la formulación de piensos para pollos [89] y cerdos de engorde [90] únicamente se encuentran recomendaciones para el ácido

linoleico como único AG esencial para el que su requerimiento en la dieta ha sido demostrado, y el cual varía en función de la fase fisiológica en la que se encuentre el animal. Según el NRC, los requerimientos de linoleico para aves en crecimiento y adultas son del 1%, y en el caso de FEDNA, para avicultura establece un mínimo entre 0,8-0,5% en función de la edad, y un máximo sólo en cebo (2,6%) y en acabado (2,3%). Además, también según FEDNA, para porcino el mínimo es de 0,1% (recomendables niveles superiores al 0,7%) mientras que el máximo en piensos de finalización es del 1,5%. El hecho de recomendar un nivel máximo en las fases finales de crecimiento se hace para evitar canales con grasas excesivamente insaturadas, ya que al tener un bajo punto de fusión y por tanto líquidas a temperatura ambiente, podrían dar problemas de comercialización debido a carnes más blandas.

Por lo que respecta a los contenidos en AGL, su efecto negativo sobre la digestibilidad de las grasas se verá también influenciado por esta relación I/S y por la edad del animal, produciendo una mayor reducción de la digestibilidad y absorción cuando los animales son más jóvenes y cuanto más baja es la relación I/S [24,74]. El contenido en AGL de la grasa incorporada en un pienso puede verse incrementado debido a reacciones de hidrólisis en las que tiene lugar la ruptura de los enlaces éster de los TAG dando lugar a un aumento de los AGL. Estas reacciones de hidrólisis pueden deberse a la acción de las enzimas lipasas (enzimas endógenas procedentes de otros ingredientes comentadas en la siguiente sección 2.3.2), al contenido de agua (se trata de uno de los sustratos requeridos para la reacción hidrolítica), al nivel inicial de AGL (la tasa de hidrólisis de los TAG es proporcional al contenido inicial de AGL) y la temperatura [91,92]. De este modo, adquieren relevancia tanto la humedad residual en un pienso, así como la temperatura alcanzada en el proceso de fabricación. A su vez, se ha descrito que estos AGL podrían estar sujetos a una mayor tasa de reacciones de oxidación ya que son más susceptibles a la misma que los TAG [91,93]. Sin embargo, el desarrollo de reacciones de hidrólisis durante la vida útil de un pienso, el incremento en AGL y la posibilidad de un incremento en la oxidación de este, son factores poco estudiados.

En cuanto a la longitud de cadena de los AG, también supone una diferencia en la digestión debido a que esta aumenta cuando se reduce dicha longitud, siendo los AG de cadena corta digeridos y absorbidos de forma más rápida y completa que los de cadena media y larga, e igualmente ocurre al comparar los de cadena media frente a los AG de cadena larga [31]. Además, las posiciones *sn*-1 y *sn*-3 de los AG en el TAG son las de mayor especificidad para la lipasa pancreática, liberándose para su absorción dos AGL y un MAG con un AG en posición *sn*-2 [31]. Sin embargo, en función de qué AG específico se encuentre en dichas posiciones del glicerol, puede haber diferencias en la digestibilidad y absorción de diferentes grasas; por

ejemplo, cuando AG de cadena larga se encuentran en las posiciones *sn*-1 y *sn*-3 podrá verse reducida la digestión y absorción respecto a dicho tipo de AG situado en la posición *sn*-2 [10,28]. A su vez, el grado de saturación de los AG situados en las diferentes posiciones del TAG pueden modificar el grado de oxidación de este, ya que AGPI en las posiciones *sn*-1 y *sn*-3 serán más fácilmente oxidables que si estas posiciones son ocupadas por AGS [10].

Sin embargo, en el cálculo energético, la ecuación de Wiseman no tiene en cuenta la calidad de la grasa, entendida como el contenido de compuestos no energéticos, como los incluidos en el MIU y en el NEM.

- **Ecuación sugerida por Wiseman et al.** [30]**:**

  EMA (pollos) o ED (cerdos) (MJ/kg)= A + B (AGL) + C $e^{D\,(I/S)}$

  (Donde A, B, C y D son valores de constantes que difieren según especie y edad de los animales).

De hecho, en algunas grasas, como por ejemplo los subproductos grasos, el contenido de diluyentes de energía y compuestos oxidados varía ampliamente [13], dificultando dicha relación entre composición de la materia grasa y cálculo de la energía de la dieta, donde además interfieren factores inherentes al animal (edad, especie…). Una reciente adaptación de la ecuación de Wiseman refleja el potencial del MIU como diluyente de la energía, tras obtener los valores de energía para diferentes grasas y aceites disponibles como ingredientes en alimentación de no rumiantes (n = 724), mediante la aplicación de una nueva ecuación basada en la ecuación de Wiseman corregida con el valor del MIU [94].

- **Ecuación sugerida por Wealleans et al.** [94]**:**

  Energía= (A + B (AGL) + C $e^{D\,(I/S)}$) K

  (Donde K es una constante que representa el factor de corrección de los compuestos diluyentes de energía, calculada como: 1-(MIU/100)).

Como ya se ha comentado, entre los compuestos diluyentes de energía se incluyen los productos de oxidación. Cabe tener en cuenta que la calidad de la fracción lipídica del pienso puede verse disminuida con el tiempo debido a la alta susceptibilidad de las grasas y aceites a sufrir reacciones de degradación como las de hidrólisis u oxidación, lo que afectará tanto al valor energético del pienso, como a la estabilidad y vida útil de éste. A su vez, la pérdida de calidad de la grasa será determinante en el valor nutricional del pienso. La posible oxidación de la fracción lipídica del pienso conlleva tanto un incremento de compuestos oxidados diluyentes de energía como una pérdida de compuestos con propiedades nutricionalmente interesantes como los AGI

y las vitaminas liposolubles y otros antioxidantes lo que, en último lugar, puede afectar a los parámetros productivos de los animales y a la calidad final de la carne [95]. Por ello, los procesos de oxidación e hidrólisis de las grasas en los piensos también requieren especial atención. De hecho, Riaz y Alam [16] recogieron un resumen de las consideraciones sobre la inclusión de grasas en alimentación de distintas especies animales hecha por la NRC o también según la *American Feed Industry Association* (AFIA), donde especifican que para aves es preciso estabilizar la grasa del pienso mediante antioxidantes añadidos que protejan los AGI frente a la oxidación.

La estabilidad oxidativa del pienso dependerá inicialmente de la composición de la grasa añadida, pero también influyen las condiciones de fabricación del mismo ya que temperaturas muy elevadas como ocurre en granulado y extrusionado, pueden tener un efecto negativo al promover la oxidación lipídica y al degradar antioxidantes naturales, pero a la vez se destruyen enzimas como, por ejemplo, lipasas [96–98]. Si estas lipasas permanecen activas en el pienso, durante el almacenamiento según las condiciones de temperatura y humedad podrían promover reacciones de hidrólisis incrementando los AGL que son más susceptibles a oxidarse [92]. Además, durante el almacenamiento puede tener lugar la oxidación de la grasa en los piensos, estando favorecida por la presencia de oxígeno, metales (Fe, Cu…), calor y luz. Por lo que refiere a la composición de la grasa, tal y como se ha comentado anteriormente, la susceptibilidad de las grasas a la oxidación dependerá de su perfil de AG y de su contenido en prooxidantes y antioxidantes [99]. Además, como se ha apuntado más arriba, el contenido de agua en la grasa y en el pienso puede favorecer las reacciones de hidrólisis lipídica que pueden dar lugar a más AGL más susceptibles a oxidarse [91].

Por último, diferentes criterios de calidad se tendrán en cuenta para la fabricación de piensos, en los cuales la grasa cumple un papel fundamental. Una ventaja de la grasa añadida a los piensos es que reduce la energía mecánica necesaria durante el proceso de producción, gracias a su efecto plastificante y lubricante, creando una capa que recubre las partículas de los piensos que reduce las pérdidas en forma de finas partículas [23]. En el caso de los piensos granulados, la adición de grasa variará en función de la consistencia deseada en el pienso según las especies a las que se destinen. Por ejemplo, en pollos de carne para aumentar el consumo y así la energía ingerida, se requieren piensos granulados blandos, por lo que será necesario incrementar el contenido de grasa en la formulación. Este también sería el caso de los lechones destetados o de conejos, pero en estos últimos, la dureza también debe ser optimizada en el granulado ya que cuando aumenta demasiado, se reduce el consumo de pienso, más en animales jóvenes. En cambio, en el ganado lechero se busca un pienso de mayor dureza, por lo

que la grasa añadida será menor [24,100]. Es por esto, que es de especial importancia el control de los niveles de grasa que se añaden ya que puede verse afectada la calidad del pienso, produciéndose por ejemplo un granulado demasiado blando y fácilmente rompible en el caso de un exceso de grasa. A su vez, hay que considerar también la cantidad de grasa añadida respecto a la edad del animal, ya que los animales jóvenes poseen menor capacidad para ingerir el pienso [24], por lo que requerirán dietas con mayor energía, aunque, por otro lado, su capacidad para formar micelas con los AG es más baja, ya que la concentración de sales biliares está reducida en las primeras semanas de crecimiento, por lo que el aporte de energía no será tan necesario como el de proteína. De acuerdo con Leeson y Summers [5], independientemente de otras consideraciones nutricionales o económicas, en todas las formulaciones animales es necesario un mínimo de un 1% de grasa añadida, para así conseguir todas las ventajas anteriormente mencionadas que aporta este nutriente. Ayed et al. [101] indican que la grasa añadida normalmente en los piensos comerciales es de un 2-7% en materia seca y que piensos con cantidades superiores a un 8% generan problemas digestivos en los animales, diarrea y reducción del consumo de pienso.

Se puede concluir que la digestión, absorción y, por tanto, la energía variará principalmente según la fuente grasa y en base a las distintas especies animales y su edad, pero también las distintas reacciones de degradación que afectan a la estabilidad del pienso, incluyéndose aquí reacciones de oxidación e hidrólisis de la fracción lipídica del mismo, que son de gran relevancia pudiendo afectar a la calidad de la grasa añadida y finalmente a su valor nutricional. De esta forma, la estabilidad del pienso será clave para conseguir una correcta nutrición animal, ya que se mantienen en mayor o menor grado el aporte de AG y antioxidantes naturales, evitándose la formación de sustancias no deseada.

2.3.2. Otros ingredientes

Como se ha mencionado en el apartado anterior, a la hora de formular los piensos se busca conseguir piensos estables, y puesto que los aceites y grasas son probablemente el ingrediente más problemático en este sentido, requieren especial atención tanto en la fabricación como en el almacenamiento de los piensos. Con esto se hace referencia a la estabilidad oxidativa e hidrolítica de la grasa utilizada en las formulaciones, tratando de evitar o disminuir estas reacciones de degradación de forma que se prolongue la vida útil de los piensos. Para entender la repercusión de estas reacciones de deterioro de la grasa hay que partir del hecho de la reducción de digestibilidad y valor nutricional cuando hay un exceso en el contenido de AGL altamente susceptibles a la oxidación.

Previamente se ha explicado que los AGL son los productos primarios de la lipolisis a partir de los TAG. Este proceso causante de la rancidez de las grasas de los piensos puede ser catalizado por unas enzimas denominadas lipasas, en presencia de un alto contenido de humedad o elevadas temperaturas [91]. En cuanto a las lipasas, éstas son unas enzimas endógenas las cuales se encuentran en todos los cereales (siendo los más habituales como ingredientes en alimentación animal el trigo, la cebada o el maíz) [102]. Son enzimas encargadas del metabolismo de las grasas, mediante la ruptura de los enlaces éster de los TAG liberando los AGL, lo cual conlleva una disminución de la absorción debido a la menor eficiencia en la formación de micelas de los AGL, también por la falta de MAG, lo que se traduce en una reducción del valor energético del pienso [34,103,104]. Este proceso de lipolisis endógena resulta un proceso rápido, que transcurre de forma inevitable en los piensos durante su almacenamiento, sobre todo según cuales hayan sido las condiciones de fabricación y del mismo almacenamiento (Figura 4). Cuando en los procesos de fabricación de los piensos son aplicadas temperaturas elevadas, como sería el caso de los procesos de granulado y extrusionado, se produce la inactivación de estas enzimas a la vez que se reduce el contenido de humedad, de forma que la actividad enzimática, y por consiguiente la lipolisis endógena, se mantendrá reducida durante el posterior almacenamiento del pienso [91,104]. Por el contrario, los piensos fabricados como harinas, donde no se necesitan temperaturas tan altas, mantendrán activas las lipasas y por tanto serán mayores sus contenidos en AGL durante su almacenamiento. Además, el aumento de AGL generados tras la acción hidrolítica de las lipasas supone una pérdida de calidad de la grasa, ya que los AGL promueven las reacciones de oxidación a una tasa más rápida que aquellos AG esterificados con una molécula de glicerol formando parte de los TAG [41,105].

Además de las lipasas procedentes de las plantas, muchos son los microorganismos reconocidos por su alto potencial como productores de estas enzimas hidrolíticas, incluyéndose aquí bacterias, hongos y levaduras [106]. La presencia de micoflora en piensos o ingredientes para piensos puede deberse a la previa contaminación fúngica durante el crecimiento, cultivo, procesado o almacenamiento de diferentes ingredientes utilizados en las formulaciones (como granos de cereales) [107]. Por tanto, una vez contaminados los ingredientes, dichos hongos pueden ser encontrados en los piensos, donde suponen una reducción de la estabilidad hidrolítica debido a este aumento de las lipasas.

De esta forma, debido al incremento en el contenido de AGL y su potencial rancidez, se ve perjudicada la energía de la dieta en primer lugar, así como según estudios anteriores, la salud, crecimiento y finalmente la producción animal debido al consumo de grasas oxidadas [95,108,109].

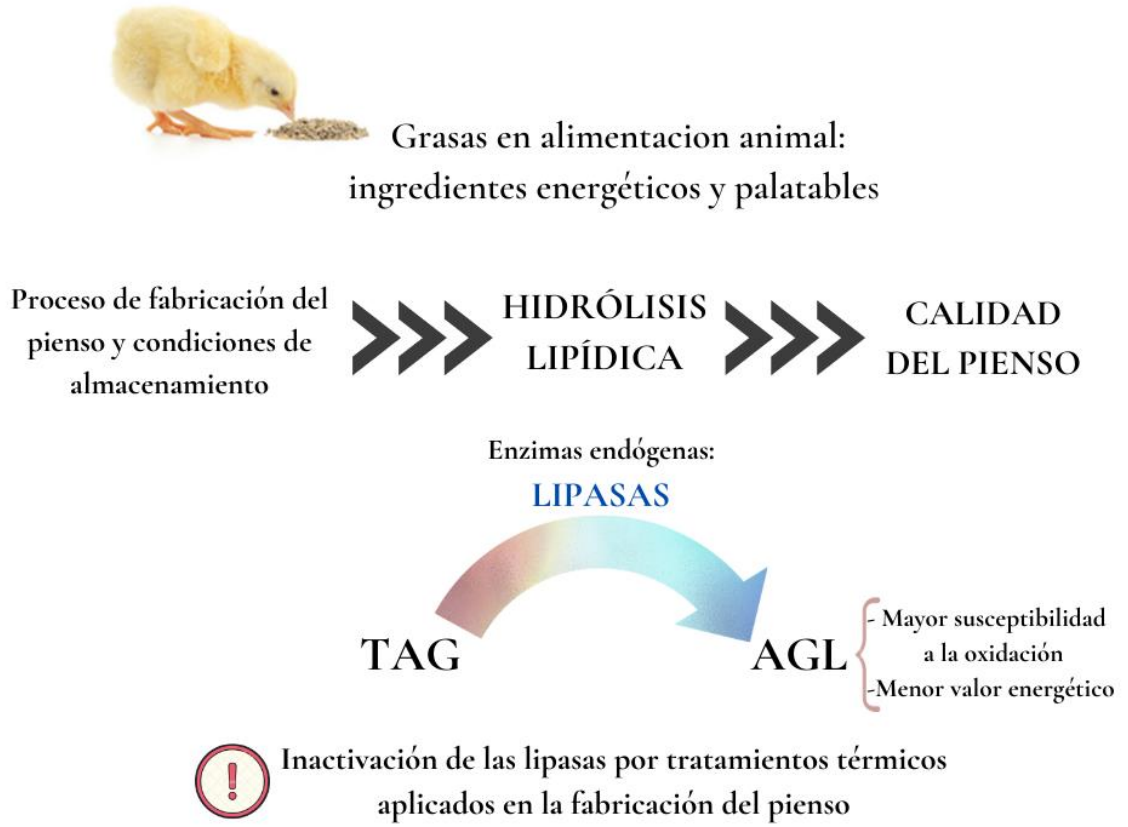


Figura 4. Esquema hidrólisis enzimática en piensos. Efecto de los procesos de fabricación en la calidad de los piensos debido a la posible inactivación de la actividad de las enzimas lipasas.

A su vez, otro ingrediente de gran utilidad para mantener la estabilidad oxidativa de los piensos durante el almacenamiento son los antioxidantes. En la formulación de piensos se utilizan para impedir la rancidez de la grasa debido a la oxidación, principalmente cuando el origen de la fuente grasa es muy insaturado. Las grasas y aceites comestibles contienen antioxidantes de forma natural, como los T y T3 [110], carotenoides, polifenoles o esteroles, los cuales protegen hasta cierto grado frente a la oxidación, aunque finalmente el proceso de oxidación es un proceso inevitable. Por tanto, dependiendo de la grasa utilizada en la dieta, se partirá de un contenido de antioxidantes naturales determinado, y también de forma intencionada se pueden añadir estos compuestos en la formulación (generalmente como α-T acetato). Incluso, algunos cereales como el trigo y la cebada, utilizados de forma habitual en las formulaciones, pueden afectar a los contenidos de estos antioxidantes en los piensos, ya que son una fuente rica en T y T3 (el trigo especialmente en β-T3 y la cebada en α-T3) [111,112]. Sin embargo, el proceso de fabricación puede a su vez influir en el contenido total de estos componentes, causando pérdidas sustanciales de éstos debido al tratamiento térmico aplicado, generalmente en los procesos de granulación y extrusión [96,113].

2.4. Influencia de las grasas de la dieta sobre la composición lipídica y la estabilidad oxidativa de las carnes

2.4.1. Las grasas de la dieta y su efecto sobre la composición lipídica y estabilidad oxidativa de las carnes

La composición de la carne puede estar influenciada por diferentes factores, donde se incluyen la especie animal, la alimentación del animal, el aturdimiento antes del sacrificio, el propio método de sacrificio, así como las condiciones de almacenamiento de la carne [114]. En la alimentación del animal, la formulación de dietas se trata de una herramienta comúnmente utilizada para mejorar el valor nutricional del pienso a la vez que se mejoran los parámetros productivos, manteniendo un estado saludable en el animal vivo. También, con la alimentación se consigue un control de la calidad de la carne producida, pudiéndose prolongar su vida útil. Se trata de un hecho bien reconocido que la composición lipídica de las dietas animales se ve reflejada en la composición lipídica de la carne, siendo el tejido adiposo el que presenta el mayor contenido en AG, los cuales también se encuentran en el músculo, con un perfil de AG muy similar en ambos tejidos [115,116]. Es decir, el perfil de AG observado en un tejido adiposo animal, abdominal o subcutáneo, o en el músculo dependerán del perfil de AG de la grasa utilizada en la fabricación del pienso, ya que los AG que se depositan en estos tejidos dependen de los AG ingeridos [116–118]. En el caso de los pollos, el tejido adiposo abdominal es un buen indicador de la grasa corporal, estrechamente determinado por la composición en AG de la dieta, así como por el balance general entre esta y los procesos de lipogénesis y lipolisis [101,119]. También, en la revisión de Kouba et al. [120] se indica este hecho, así como que la relación entre composición en AG de la dieta y composición lipídica del tejido adiposo es mucho más fuerte en el caso de los animales monogástricos, como pollos y cerdos, que en el caso de los rumiantes. Por tanto, además del fuerte impacto de la dieta sobre los tejidos grasos abdominal, subcutáneo e intermuscular (grasa de la canal) donde la clase lipídica mayoritaria son los TAG, también la composición en AG del músculo y del tejido adiposo intramuscular (grasa veteada del músculo) constituido principalmente por fosfolípidos con un alto contenido en PUFA, se ve a su vez afectado aunque en menor medida por la composición de la dieta siendo ésta, como ya se ha mencionado, una estrategia para producir carne con un deseado valor nutricional y con unas características tecnológicas determinadas en cuanto a la calidad de la carne [118,121–123]. Algunas características que definen la calidad de la carne dependerán de la composición en AG en el tejido adiposo, como es el caso de la dureza, la cual variará en función del punto de fusión de los AG, también la vida útil de la carne la cual dependerá del grado de

saturación de los AG o la jugosidad y textura pueden verse modificadas según el contenido total de grasa en el músculo [116,122].

De esta forma, la grasa añadida a la formulación afectará primero a la composición del pienso y, a su vez, se reflejará en la composición del tejido adiposo y muscular de la carne, de aquí el interés en la modificación de las dietas, para conseguir finalmente la composición de AG deseada en la carne, contemplándose parámetros como la relación I/S o la relación n-6/n-3. Por ejemplo, Alagawany et al. [124], muestran en su revisión la importancia de una ratio n-6/n-3 adecuada, la cual puede conseguirse suplementando los piensos con grasas o aceites ricos en AGPI, puesto que a continuación la carne se verá enriquecida con este perfil de AG. En otra revisión, Wood et al. [116] indicaron que el mayor impacto de la dieta en el tejido adiposo y músculo en el caso de los cerdos se observó en las proporciones de los AG C12:0, C14:0 y el C18:2 n-6, ya que los dos primeros proceden principalmente de la dieta y el tercero totalmente, sin embargo, los AG de 16 y 18 átomos de carbono tanto saturados como monoinsaturados son sintetizados por el animal y por tanto no dependieron tanto de la propia dieta.

A su vez, debido a que los AGPI son también más susceptibles al daño oxidativo, especialmente aquellos con más de dos dobles enlaces esto, como ya se ha indicado, puede perjudicar la calidad final de la carne, y así su vida útil, al verse afectada la estabilidad oxidativa (rancidez) que depende tanto de la cantidad de estos sustratos (AGPI) en el músculo como del contenido en antioxidantes (T y T3) y especies prooxidantes [118,122]. De hecho, la influencia de la dieta en la composición de la carne no sólo ocurre sobre los AG, sino también sobre otros compuestos lipídicos como son los T y T3, compuestos con actividad vitamínica E y antioxidantes naturales que también se verán reflejados en la composición de la carne según el perfil de estos en la fuente grasa usada para producir los piensos. Además, estos antioxidantes naturales incorporados en los tejidos actúan de forma efectiva reduciendo la oxidación lipídica durante el procesado o almacenamiento de la carne, ya que favorecen el balance oxidativo entre agentes antioxidantes y sustratos considerándose nutrientes esenciales en la mejora de la calidad de la carne y así en su valor nutricional [116,118]. Por ejemplo, tal y como se detallará en la siguiente sección, cambios del color u olores desagradables de la carne han sido relacionados con la oxidación lipídica (estrechamente relacionada con el perfil de AG) y la concentración de antioxidantes naturales (vitamina E) [116,118]. De esta forma, la calidad de la carne y por tanto su vida útil tanto tras el procesado (cocinado) como durante el almacenamiento, vendrá influenciada por la composición en AG y antioxidantes en la carne (Figura 5), ambos reflejados directamente de la dieta (de aquí la importancia en su control por medio de esta), los cuales afectarán la estabilidad oxidativa (rancidez con la consiguiente pérdida de color y producción de

aromas desagradables, muchos relacionados con un incremento de los compuestos secundarios de oxidación) así como la dureza de la carne.

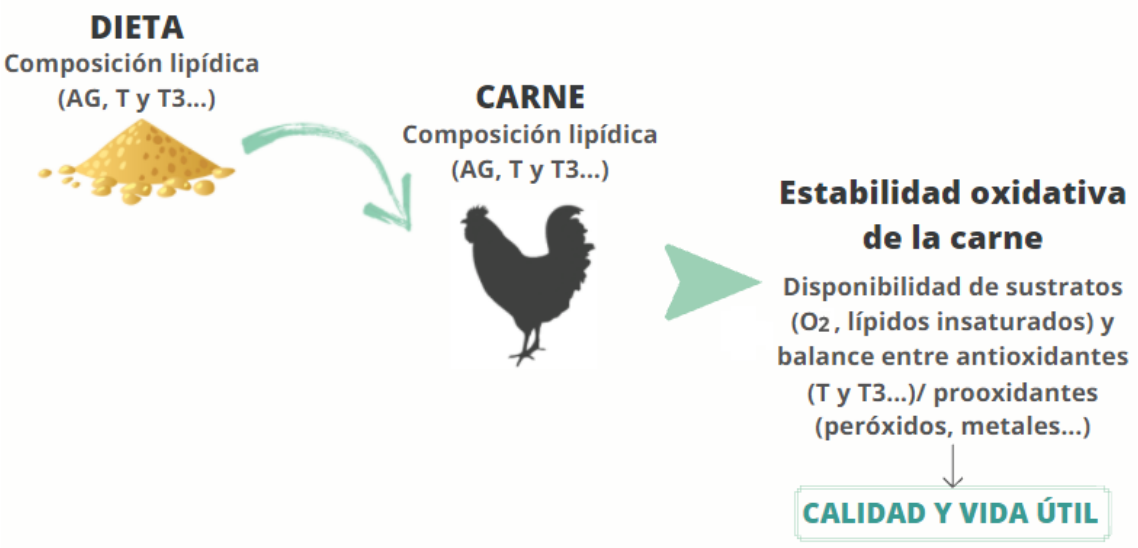


Figura 5. Influencia de la dieta en la composición y estabilidad oxidativa de la carne. Abreviaciones: AG, ácidos grasos; T, tocoferoles; T3, tocotrienoles.

Por lo tanto, estos antioxidantes tienen un papel relevante cuando se proporcionan dietas ricas en AGPI y su suplementación (T y T3) debe ser ajustada de acuerdo con el contenido de estos sustratos (AGPI) [125]. Dado que los AGPI, y en especial aquellos con más de dos dobles enlaces, son más susceptibles al daño oxidativo que los AGS, un incremento en AGPI en carne sin aportar un contenido adecuado en antioxidantes (T y T3) podría afectar a la estabilidad oxidativa de la carne (rancidez), perjudicando su calidad final, y su vida útil [118,122]. Además del contenido en AGPI de la grasa, cabe tener en cuenta también la calidad oxidativa de la grasa utilizada como ingrediente en el pienso ya que, si las grasas utilizadas presentan un elevado grado de oxidación, su contenido en T y T3 puede ser menor del esperado y esto puede también comprometer la estabilidad oxidativa de la carne, ya que supone bajos niveles de vitamina E en los tejidos [126]. A su vez, las grasas oxidadas supondrían un incremento del aporte de compuestos de oxidación, primaria o secundaria, en función de su estado oxidativo. A pesar de que son escasos los estudios que traten hasta qué punto los productos de oxidación son absorbidos, Kanazawa y Ashida [127] observaron en ratas la descomposición de los productos de oxidación primaria (hidroperóxidos) en productos de oxidación secundaria (aldehídos) a nivel estomacal, los cuales pasaban al intestino donde eran absorbidos. Este hecho implicaría una reducción de la estabilidad oxidativa de la carne ya que también se verán reducidos los contenidos de antioxidantes. En otro estudio en peces también confirmaron que estos animales, al igual que los vertebrados parecen estar protegidos frente a la absorción de los hidroperóxidos

por su previa descomposición en el estómago, mientras que los productos de oxidación secundaria sí se absorben a nivel intestinal [128].

El proceso de oxidación es considerado la principal causa no microbiana de deterioro de la calidad de la carne y productos cárnicos, la cual se inicia con el sacrificio del animal o durante algunos procesos que ocurren tras el sacrificio, continuando de forma progresiva durante el almacenamiento de la carne [40,43]., De hecho, hay un interés desde el punto de vista económico por evitar que se produzcan estas reacciones de oxidación, ya que conducen a una reducción de la vida útil de la carne, con las consecuentes pérdidas [43]. De forma general, la oxidación de la grasa en la carne supone una disminución de la aceptación del producto por el consumidor al verse alterada la calidad sensorial tras la formación de compuestos que producen malos olores o tras la pérdida de color, así como también se puede ver afectado el valor nutricional de la carne como consecuencia de la oxidación de AG esenciales o debido a la reducción de la vitamina E, y por último, con la oxidación lipídica se generan y acumulan productos de oxidación que pueden absorberse y ser potencialmente tóxicos para el ser humano [116,129,130]. De aquí que sea preciso llevar un control en la manipulación, durante los procesos de sacrificio y más tarde en el almacenamiento de la carne, para así prevenir en cierta manera estas reacciones de oxidación no deseadas, prolongando la vida útil de la carne.

La oxidación lipídica de la carne es un proceso de múltiples mecanismos y reacciones complejas, la cual consta de tres etapas principales: iniciación, propagación y terminación. En las revisiones de Min y Ahn [40] y Domínguez et al. [43] se indica cómo durante este proceso tiene lugar una reacción en cadena de radicales libres, que dependerá de distintos factores, tanto intrínsecos como extrínsecos. Respecto a los primeros, se encuentran tanto el contenido de grasa y la composición lipídica de la carne, como la presencia de prooxidantes y antioxidantes en la misma. Estos factores vienen principalmente definidos por la composición de la dieta, como ya se ha explicado previamente, la cual se verá reflejada en la composición de la carne. Por otro lado, las reacciones de oxidación en la carne se verán influenciadas por parámetros extrínsecos, donde se incluyen los tratamientos de procesado de la carne, como el deshuesado, trituración o cocinado, entre otros. También influirán la presencia de aditivos como nitritos u otros antioxidantes y las condiciones durante el almacenamiento, como pueden ser el contacto con el oxígeno, la luz o distintas temperaturas de almacenamiento, así como el período durante el cual la carne es almacenada, ya que con el paso del tiempo el deterioro de la calidad lipídica aumenta. A su vez, la estabilidad lipídica de la carne después del sacrificio puede cambiar según factores biológicos del animal como la especie, el sexo y la edad, el tipo de músculo seleccionado o por el estado nutricional del animal en el momento del sacrificio [131,132].

Como ya se ha mencionado, el tipo de dieta consumida por los animales tendrá una gran influencia en la posterior susceptibilidad de la carne a la oxidación, incluso *post-mortem*. La presencia de AGI constituye el mayor determinante para la oxidación lipídica de la carne, sobre todo los AGPI ya que son muy susceptibles a esta, los cuales forman parte de los fosfolípidos de las membranas celulares [129]. Al producirse la ruptura de dichas membranas celulares durante el sacrificio del animal o la manipulación de la carne, los AGPI entran en contacto con agentes prooxidantes desencadenándose las reacciones de oxidación que cursan mediante la formación de radicales libres y que producen los hidroperóxidos lipídicos [40,129]. Entre los agentes prooxidantes se encuentran aquellos que promueven las reacciones de oxidación, por ejemplo, las especies reactivas de oxígeno formadas por reacciones metabólicas, las cuales interactúan con los AG, proteínas o ácidos nucleicos. Otros prooxidantes son los metales, principalmente el hierro en sus distintas formas, pero especialmente cuando se encuentra libre [129]. Los hidroperóxidos son productos de oxidación primaria caracterizados por su baja volatilidad, su carencia de olor y su inestabilidad ya que pueden descomponerse formando los productos de oxidación secundaria altamente volátiles causantes de los malos olores, tales como aldehídos, cetonas, alcoholes, entre otros. Los aldehídos son de hecho, los componentes volátiles que más contribuyen a la alteración sensorial de la carne, con límites muy bajos para generar los malos olores en la carne, siendo algunos grupos predominantes los n-alcanales, *trans*-2-alquenales y el malondialdehído (MDA), entre otros [43]. En esta reacción de oxidación pueden intervenir los antioxidantes protegiendo frente a la oxidación de la grasa en la carne. Entre ellos se encuentran los antioxidantes antirradicalarios como, por ejemplo, los T y T3 que se incorporan también a las membranas lipídicas celulares. Estos actúan cediendo un hidrógeno a los radicales inestables (muy reactivos) y ellos mismos se transforman en un radical estable (poco reactivo) y como consecuencia se ralentiza la cadena radicalaria y la oxidación [43]. Hay que tener en cuenta también que el efecto protector de los T y T3 dependerá de factores como el contenido de los mismos suplementado en la dieta, el grado de saturación y estado oxidativo de las grasas añadidas al pienso, el estado fisiológico del animal y finalmente el balance neto entre agentes prooxidantes, agentes antioxidantes y sustratos en la carne [118]. De hecho, una de las herramientas de la industria cárnica para prolongar la vida útil de la carne mediante la prevención o ralentización de la oxidación, es la adición de antioxidantes como aditivos. Dicha estrategia está siendo sustituida por la manipulación de la dieta con la inclusión de grasas con altos contenidos de antioxidantes naturales, así como la modificación del empaquetado de forma que permita controlar la evolución de la oxidación [43,130].

2.4.2. Efecto del uso de subproductos grasos (AA y AGD) sobre la composición lipídica y estabilidad oxidativa de las carnes

A pesar de que el principal objetivo de la refinación de los aceites vegetales para consumo humano es la eliminación de compuestos no deseados para aumentar la vida útil del aceite, a la vez es inevitable que ocurran pérdidas en el contenido de compuestos con actividad antioxidante como es el caso de la vitamina E o los compuestos fenólicos. Los AA y AGD obtenidos en los procesos de refinación, tanto química como física respectivamente, se caracterizan por su alto contenido en AGL ya que proceden de la etapa donde estos son eliminados durante la refinación, pero a su vez, otra de sus características que les confiere un alto valor comercial es su contenido en componentes minoritarios que conforman la denominada U. Dentro de U en los aceites de semillas se encuentran compuestos bioactivos como por ejemplo la vitamina E, esteroles, alcoholes triterpénicos (compuestos fenólicos), carotenoides e hidrocarburos saturados e insaturados (dentro de esos últimos se encuentra el escualeno) [133,134]. En cuanto a los ocho homólogos que constituyen la vitamina E se encuentran los T (α-T, β-T, γ-T y δ-T) y T3 (α-T3, β-T3, γ-T3 y δ-T3), todos ellos antioxidantes naturales muy potentes que previenen de la oxidación de grasas y aceites durante el almacenamiento. Concretamente, los AGD obtenidos en la refinación física del aceite de palma (PFAD) son considerados una fuente rica en estos compuestos, siendo los T3 dentro de la vitamina E aquellos que predominan (principalmente γ-T3, seguido de δ-T3 y α-T3) y en el caso de los fitoesteroles el β-sitoesterol es el mayoritario, seguido del estigmasterol y campesterol [133,135]. En cuanto al escualeno, se trata del mayor constituyente de la U en el aceite de oliva (llegando a constituir más del 50% de la U de este tipo de aceite), siendo junto a la vitamina E un compuesto de interés por su actividad antioxidante [136]. El hecho de que estos subproductos sean ricos en estos compuestos se debe a las condiciones de refinación aplicadas, que favorecen su acumulación en el subproducto (sección 2.2.1). En el caso de los AGD, es la etapa de desodorización con la aplicación de altas temperaturas y vacío, la que promueve la mayor la eliminación y acumulación de estos componentes en el subproducto, pero también en las etapas de neutralización y blanqueamiento para la obtención de los AA se produce esta pérdida de compuestos bioactivos y la acumulación en los correspondientes subproductos [11]. Por ejemplo, tanto la vitamina E como el escualeno ven drásticamente reducido su contenido en el aceite refinado debido a la etapa de desodorización principalmente siendo acumulados en los subproductos, dotando a estos de un alto valor comercial por su capacidad de prolongar la estabilidad oxidativa. Así, el hecho de incorporar estos subproductos en los piensos para la alimentación animal, además de la fuente energética que suponen al ser subproductos grasos,

contienen estos componentes bioactivos, que se verán reflejados en la composición del pienso y más tarde pasarán a formar parte de la carne del animal, favoreciendo la estabilidad oxidativa *post-mortem*.

De hecho, algunos estudios han demostrado como la suplementación de las dietas animales con algunos de estos compuestos no sólo supone un beneficio *post-mortem,* previniendo la rancidez en la carne, sino también *in vivo* reduciendo el estrés oxidativo en el animal. Por ejemplo, en la revisión de Salami et al. [137] se muestra cómo dietas de pollos suplementadas con vitamina E fueron relacionadas con el aumento de la actividad de enzimas antioxidantes como la glutatión peroxidasa o la superóxido dismutasa en los tejidos, mostrando así un alto potencial a la hora de reducir el estrés oxidativo en los animales. A su vez, la suplementación con vitamina E también mejoró el estado oxidativo de la carne, con la reducción de la oxidación lipídica y el consiguiente beneficio para la nutrición humana [137,138]. También, se han descrito efectos fisiológicos positivos de los fitoesteroles como captadores de radicales libres o por el aumento de la actividad antioxidante de enzimas endógenas, efectos que suponen en general una mejora del estatus antioxidante de la carne mejorando finalmente la calidad de esta [139,140]. En cuanto a la suplementación del escualeno en dietas para pollos, en un estudio donde fue extraído de los deodestilados, se concluyó una reducción en la acumulación de MDA, así como un aumento de la actividad antioxidante enzimática en el músculo, todo ello de nuevo con el beneficio de una mayor estabilidad de la carne [141]. A su vez, Yang et al. [142] o Englmaierovà et al. [143] observaron que la adición de diferentes tipos de carotenoides (astaxantina o licopeno) en las formulaciones animales suponía una forma efectiva de mantener la estabilidad oxidativa de la carne *post-mortem* debido a su acción antioxidante. También, en otro estudio se mostró cómo los compuestos fenólicos, concretamente los taninos, tras ser suplementados en dietas para corderos eran transferidos al músculo donde contribuían a mejorar el estado antioxidante del mismo [144]. Sin embargo, la suplementación de la dieta con acetato de α-tocoferol es considerada la más efectiva en la prevención de la oxidación lipídica de la carne, ya que el α-tocoferol ha demostrado la mayor eficiencia a nivel biológico previniendo la oxidación lipídica *in vivo*, y también mejora la estabilidad oxidativa de la carne *post-mortem*, suponiendo finalmente una fuente de vitamina E para el consumo humano [125]. En la revisión de Bou et al. [118] se indica la mayor eficiencia de la suplementación con acetato de α-tocoferol frente a la suplementación con plantas ricas en compuestos fenólicos o aceites esenciales extraídas de las mismas. Por tanto, la suplementación de las dietas con estos compuestos es una forma efectiva de reducir el estrés oxidativo *in vivo*, así como

posteriormente, mejorar la estabilidad oxidativa de las carnes gracias a estos compuestos con actividad antioxidante, entre otras.

Además, sobre los AA y AGD es preciso comentar la alta variabilidad en su composición, incluyendo los T y T3, así como la posible acumulación de productos de oxidación en estos subproductos (sección 2.2.1). Sin embargo, en la actualidad son escasos los estudios que evalúen con detalle la composición de estos subproductos en cuanto a productos de oxidación y cómo afectarán a la oxidación posterior de la carne y tejidos, es decir además de la falta de estandarización de los AA y AGD es preciso avanzar en el conocimiento de su utilización en piensos y su efecto en la calidad final de la carne. A pesar de ello, Nuchi et al. [13] y Sarojini y Dutta [145] explican la necesidad de controlar el contenido de los productos de oxidación en los AA y AGD ya que su contenido en dietas animales puede modificar la composición lipídica de la carne debido al efecto en el grado de oxidación, especialmente con grasas altamente insaturadas. A su vez, como se ha comentado anteriormente, la presencia de estos productos de oxidación puede afectar al contenido de antioxidantes en el pienso, que más tarde puede modificar el contenido de antioxidantes en la carne, viéndose comprometida la estabilidad oxidativa de la misma.

2.5. Hipótesis del estudio

Haciendo una visión general a lo anteriormente comentado se evidencia la falta de una completa caracterización de los AA y AGD para su uso en alimentación animal, ya que como se ha indicado, pocos son los estudios que incluyan información sobre la composición, estabilidad y calidad nutricional de estos subproductos disponibles en el mercado español. Los estudios disponibles han realizado una primera caracterización sobre un grupo reducido de muestras, y/o no han proporcionado datos sobre algunos parámetros relevantes para el valor energético final del subproducto como es el caso de I y U en el estudio de Nuchi et al. [13]. Por otro lado, también ha quedado evidenciado que las normas de comercialización o las guias de calidad para estos productos no contemplan una caracterización exhaustiva de los mismos, en especial, por lo que refiere a los parámetros de oxidación.

Por ello, la hipótesis de partida es que la composición y estado oxidativo de los AA y AGD del mercado español es muy variable y que los valores de muchos parámetros relevantes para el valor energético o la estabilidad de estas grasas difieren de los característicos para las grasas crudas que son las habitualmente utilizadas en piensos. Su caracterización permitiría desarrollar unas recomendaciones para su control de calidad y estandarización garantizando la calidad de los AA y AGD como ingredientes en las formulaciones animales.

Otro aspecto crítico para incrementar el uso de estos subproductos es conocer cómo la utilización de los AA y AGD puede afectar a la composición y estabilidad oxidativa del pienso, y como éstas pueden verse modificadas según el proceso tecnológico utilizado para elaborar dicho pienso; sin embargo, hay una falta de estudios que aborden estos aspectos.

Dado que los antecedentes muestran que los AA y los AGD son más ricos en AGL que los aceites crudos, y que su contenido en compuestos de oxidación puede también ser diferente, la hipótesis de partida se basa en que la utilización de estos subproductos puede modificar negativamente la composición y estabilidad oxidativa del pienso.

También hay una falta de conocimiento sobre la posible influencia que la incorporación de los AA y AGD al pienso puede tener sobre la composición y estabilidad oxidativa de la carne, lo que en último lugar afectaría a la aceptación y nutrición humana. A pesar de que existen numerosos estudios sobre la utilización de varias fuentes grasas como ingredientes en pienso, incluidos algunos subproductos de refinación, y su repercusión sobre la composición lipídica de la carne, no existen estudios comparativos entre estos subproductos y sus aceites crudos, lo cual

sería necesario para poder determinar si las diferencias en la composición de estos tipos de grasas modifican la composición lipídica de la carne.

Dado que los AA y los AGD difieren de los aceites crudos principalmente en su contenido en AGL U y compuestos de oxidación, más que en su perfil de AG la hipótesis es que tendrán una mayor repercusión en la estabilidad oxidativa de la carne que sobre su composición en AG.

Esta tesis doctoral persigue poder cubrir las carencias detectadas sobre estos aspectos en los antecedentes bibliográficos y poder así verificar las hipótesis planteadas.

3. OBJETIVOS

3. Objetivos

El **objetivo general** de esta Tesis Doctoral es caracterizar los AA y AGD de origen vegetal utilizados como ingredientes para piensos en España con el fin de controlar su calidad y propiedades nutricionales para poderlos utilizar de manera eficaz y eficiente en alimentación animal. Por tanto, con su caracterización y el estudio de su incorporación en piensos para pollos, se persigue generar información que contribuya a aumentar el uso de estos subproductos en alimentación animal, puesto que constituyen una alternativa más económica que las grasas crudas usadas convencionalmente y a la vez su uso contribuye a la sostenibilidad de la cadena alimentaria.

Partiendo de este objetivo general, la presente Tesis Doctoral se divide en varios **objetivos específicos**:

1) Poner a punto métodos analíticos para caracterizar de manera fiable y exhaustiva la composición y calidad de los AA y AGD disponibles en el mercado español, estableciendo sus posibles fuentes de variabilidad, identificando aquellos compuestos de los AA y AGD que pueden interferir de forma negativa en su valor energético y nutricional, y definiendo los parámetros de composición, degradación y estabilidad que resultarán más críticos a la hora de establecer recomendaciones para su control y uso en piensos.

2) Determinar si la diferente composición y grado de alteración de los AA y AGD añadidos al pienso, provocan cambios en la composición y estabilidad de la fracción lipídica del mismo, estudiando para ello, dichos cambios en función del proceso de fabricación del pienso (harina, granulado y extrusionado), así como del tiempo y la temperatura de almacenamiento.

3) Estudiar cómo la diferente composición de los AA y AGD añadidos al pienso afectan a la composición lipídica y estabilidad oxidativa de la carne de pollo.

4. DISEÑO EXPERIMENTAL Y METODOLOGÍA

4. Diseño experimental y metodología

En cuanto al diseño experimental, para abordar el ***primer objetivo***, se recopilaron un total de 92 muestras de AA y AGD de origen vegetal destinadas a alimentación animal y representativas del mercado español (Tabla 1). De estas muestras, 79 fueron AA procedentes de la refinación química y 13 fueron AGD de la refinación física de aceites y grasas vegetales para consumo humano.

Tabla 1. Clasificación de las muestras según el proceso de refinación y el origen botánico.

Proceso de Refinación	***Grupo[1]***	***Origen Botánico***	***Subgrupo: Diferentes mezclas***	***N***	***Total***
Refinación química (Aceites ácidos, AA)	*SCP*	*Mezclas de AA de aceites de semillas, manteca de cacao y aceite de palma*	*Manteca de cacao y aceites de colza, soja y palma (40/30/20/10)*	*2*	*12*
			Manteca de cacao y aceites de palma y semillas[2]	*10*	
	SP	*Mezclas de AA de aceites de palma y semillas*	*Aceites de soja, colza y palma (40/40/20)*	*2*	*5*
			Aceites de girasol, soja, palma, maíz y colza[2]	*3*	
	O	*AA de orujo de oliva y mezclas de AA de aceites de orujo de oliva y de oliva*	*Aceite de orujo de oliva*	*13*	*18*
			Aceites de orujo de oliva y aceite de oliva (90/10)	*5*	
	BS	*Mezclas de AA de aceites de semillas*	*Aceites de girasol (80-90), colza (20-10), trazas de aceites de palma y palmiste y estearina de palma[3]*	*1*	*9*
			Aceites de girasol, maíz y pepita de uva (40/30/30)	*3*	
			Aceites de girasol, soja y maíz[2]	*3*	
			Aceites de girasol, girasol alto oleico, soja, maíz y orujo de oliva[2]	*2*	
	SU	*AA de aceites de girasol*	*Aceite de girasol*	*18*	*18*
	SU-SO	*Mezclas de AA de aceites de girasol y soja*	*Aceites de girasol y soja[2]*	*4*	*15*
			Aceites de girasol y soja (10/90)	*7*	
			Aceites de girasol y soja (80/20)	*2*	
			Aceites de girasol y soja (90/10)	*2*	
	SO	*AA de aceites de soja*	*Aceite de soja*	*2*	*2*
Refinación física (Ácidos grasos destilados, AGD)	*LFAD*	*AGD de aceite de coco y mezclas de AGD de aceites de coco y palmiste (aceites laúricos)*	*Aceite de coco*	*2*	*5*
			Aceites de coco y palmiste[2]	*3*	
	PFAD	*AGD de aceite de palma*	*Aceite de palma*	*6*	*6*
	OFAD	*AGD de aceites de orujo de oliva y de aceite de oliva*	*Aceite de orujo de oliva*	*1*	*2*
			Aceite de oliva	*1*	

[1] Ver columnas "Grupo" y "Origen Botánico" para la definición de las abreviaciones. [2] En algunas mezclas las proporciones fueron desconocidas.[3] Algunas mezclas presentaron trazas de aceites de frutos.

Las muestras fueron obtenidas de diferentes empresas del sector, entre ellas, de refinerías de grasas y aceites comestibles, otras procedían de empresas que elaboran y/o comercializan este tipo de subproductos como ingredientes para piensos tras comprar las pastas jabonosas, los AA o los AGD a las refinerías, y finalmente, otras se obtuvieron de fabricantes de piensos y empresas de producción animal. De todas las muestras recogidas, únicamente **43 procedían de la refinación de una única grasa o aceite**, mientras que **49 fueron mezclas de AA o AGD procedentes de la refinación de diferentes grasas y aceites**, reflejando así la forma habitual en la que se encuentran disponibles estos subproductos en el mercado. Cuando las muestras llegaron al laboratorio, fueron fundidas, homogeneizadas y trasvasadas a viales de menor tamaño, cuyo espacio en cabeza se llenó con N_2 para después ser almacenadas bajo congelación a -20 °C hasta que fueron analizadas. Debido a la diferente naturaleza en composición de las muestras recogidas, se aplicaron diferentes tiempos y temperaturas de calentamiento para su correcta homogeneización, siempre buscando el mínimo tratamiento térmico para minimizar su alteración.

Respecto a la metodología empleada en la caracterización de los AA y AGD, se analizaron parámetros para evaluar la calidad de estos subproductos grasos, incluyéndose parámetros de composición, valor nutricional, oxidación y estabilidad oxidativa. Todas las determinaciones fueron llevadas a cabo por duplicado. Además, debido a que los métodos analíticos oficiales están destinados a grasas y aceites crudos o refinados, mucho más homogéneos en composición que los AA y AGD, estos métodos tuvieron que ser adaptados para el análisis de estos subproductos de forma que permitiesen obtener resultados fiables. Los protocolos analíticos adaptados a los AA y AGD están descritos en una de las publicaciones incorporadas en esta Tesis (sección 5.1.1 y Anexo 8.1, Varona et al. [146]) en la que se detallan las siguientes determinaciones llevadas a cabo:

- Composición en ácidos grasos (AG)
- Contenido en tocoferoles (T) y tocotrienoles (T3)
- Contenido en humedad y materia volátil (M)
- Contenido en impurezas insolubles (I)
- Contenido de materia insaponificable (U)
- Grado de acidez (contenido en ácidos grasos libres, AC)
- Composición en clases lipídicas: compuestos poliméricos (POL), acilgliceroles (TAG, DAG y MAG) y ácidos grasos libres (AGL)
- Índice de peróxidos (PV)

- ❖ Índice de *p*-anisidina (*p*-AnV)
- ❖ Estabilidad oxidativa mediante el instrumento Rancimat (IT)

Para el análisis estadístico global de los datos de la caracterización se llevó a cabo un Análisis de Componentes Principales (PCA) como técnica de análisis multivariante, para explorar la distribución natural y agrupamiento de muestras a la vez que se investigan las correlaciones entre variables. La matriz de datos consistió en 92 filas (muestras) x 29 columnas (correspondientes a las 29 variables): M, I, U, AC (grado de acidez, *acidity*), C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C16:1 n-9, C16:1 n-7, C17:0, C18:0, C18: 1 *trans,* C18:1 n-9, C18:1 n-7, C18:2 n-6, C20:0, C18:3 n-3, C20:1 n-9, C22:0, C23:0, C24:0, relación I/S, *p*-AnV, PV, T y T3 (la media de todas las variables fue escalada y centrada a la unidad de varianza). El sofware utilizado para realizar el PCA fue el SIMCA v13.0 (Umetrics AB, Umea, Sweeden).

Considerando el ***segundo objetivo***, se llevó a cabo un estudio en el que se formularon 6 piensos para pollos de carne, que se diferenciaban en el tipo de grasa añadida que fue añadida (en todos los casos al 6% p/p) sobre un mismo pienso base. Los 6 tipos de grasas añadidas consistieron en:

- ➢ Dos subproductos: el **AA de soja (AS)** como fuente insaturada y el **AGD de palma (PFAD)** como fuente saturada
- ➢ Sus correspondientes grasas crudas: aceite crudo de soja (CS) y de palma (CP)
- ➢ Sus correspondientes grasas refinadas: aceite refinado de soja (RS) y de palma (RP)

La selección del AA de soja fue motivada porque el aceite crudo de soja es la fuente de grasa más utilizada en alimentación de pollos de carne, con lo que el AS podría plantearse como un sustituto más económico y sostenible. La selección del PFAD se hizo para poder estudiar la estabilidad del pienso (oxidativa e hidrolítica) con un AGD, y para ello se eligió el de palma puesto que es uno de los más producidos a escala mundial. En ambos casos, se seleccionaron subproductos que procedieran de un origen botánico único, para así facilitar la comparación con los correspondientes aceites crudos y refinados que actuaron como piensos control.

Los piensos se fabricaron bajo diferentes condiciones (en forma de harina, granulado o extrusionado) y se almacenaron durante diferentes tiempos (0, 30 y 60 días) y a distintas temperaturas (temperatura ambiente y 35 °C).

Los piensos fueron fabricados en forma de harina inicialmente, y una parte de ellos fue sometida a un proceso que simulaba la granulación mediante un extrusor (al pienso en forma

de harina se añadió agua para lograr aproximadamente un 18% de humedad y las temperaturas del cono de extrusión se fijaron a 50, 55, 65 y 90 °C, siendo la temperatura media del pienso al salir del extrusor de 78,2 °C) mientras que otra parte se fabricó mediante el proceso de extrusión (en este caso, al pienso en forma de harina se añadió agua hasta conseguir aproximadamente un 20% de humedad, la presión se fijó a 28 bares y las temperaturas del cono de extrusión a 55, 170, 175 y 190 °C, siendo la temperatura media del pienso fabricado al salir del extrusor de 89,4 °C). Una vez elaborados todos los piensos, estos fueron homogeneizados y almacenados según el diseño experimental. Los piensos recién elaborados se envasaron al vacío y se congelaron a -20 °C hasta ser analizados. Lo mismo se hizo con los piensos almacenados durante 30 y 60 días a las dos temperaturas de conservación. Considerando todos estos factores del estudio el número de muestras fue de 108 piensos.

Las determinaciones analíticas en las fuentes grasas se realizaron por duplicado según lo descrito Varona et al. [146] (sección 5.1.1 y Anexo 8.1) y la metodología aplicada en el análisis de los piensos, también analizados por duplicado, exceptuando el análisis del contenido en compuestos volátiles, se detalla en la sección 5.1.4. La composición en AG fue determinada únicamente en los piensos elaborados como harinas a tiempo cero de almacenamiento. En el caso de la determinación del contenido de M, estos porcentajes fueron aplicados a las determinaciones del contenido de T y T3 y al contenido de compuestos volátiles, para obtener los resultados expresados sobre muestra seca, permitiendo así la comparación de resultados, dado que la humedad varió notablemente a lo largo del tiempo de almacenamiento en función del proceso de producción y de la temperatura de almacenamiento. Los métodos utilizados en las determinaciones fueron oficiales o procedentes de bibliografía ampliamente contrastada. En el caso de la determinación del contenido en compuestos volátiles se llevó a cabo una puesta a punto del método. El conjunto de determinaciones llevadas a cabo en las fuentes grasas y en los piensos fueron las siguientes:

Determinaciones en las fuentes grasas:

- Composición en ácidos grasos (AG)
- Contenido en tocoferoles y tocotrienoles (T y T3)
- Contenido en humedad y materia volátil (M)
- Contenido en impurezas insolubles (I)
- Contenido de materia insaponificable (U)
- Composición en clases lipídicas: compuestos poliméricos (POL), acilgliceroles (TAG, DAG y MAG) y ácidos grasos libres (AGL)

- Índice de peróxidos (PV)
- Índice de *p*-anisidina (*p*-AnV)

Determinaciones en los piensos:

- Contenido de humedad y materia volátil (M)
- Contenido en grasa cruda
- Composición en AG
- Contenido en tocoferoles (T) y tocotrienoles (T3)
- Composición en clases lipídicas: acilgliceroles (TAG, DAG, MAG) y ácidos grasos libres (AGL)
- Índice de peróxidos (PV)
- Contenido en compuestos volátiles (VC)

Los resultados en global se evaluaron estadísticamente mediante un ANOVA multifactorial (n = 108), estudiando tanto los efectos principales como las interacciones de los distintos factores considerados: la fuente grasa añadida (CS, AS, RS, P, PFAD y RP), el proceso tecnológico de fabricación del pienso (harina, granulado o extrusionado), el tiempo de almacenamiento (0, 30 y 60 días) y la temperatura de almacenamiento (temperatura ambiente y 35 °C). Cuando los factores principales mostraron un efecto significativo, las diferencias entre las medias agrupadas obtenidas del ANOVA multifactorial fueron evaluadas mediante el test post-hoc de Tukey. En todos los casos las diferencias fueron consideradas significativas con valores de $p \leq 0,05$. El análisis estadístico de los datos fue realizado con IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA).

Por último, para llevar a cabo el ***tercer objetivo*** planteado, se realizó un estudio *in vivo* con pollos de carne alimentados con AA o AGD además de con sus correspondientes grasas crudas y refinadas (CS, RS, CP, RP), tras el sacrificio de los cuales se tomaron las muestras de carne. La parte *in vivo* del estudio se llevó a cabo en el Servei de Granjes Experimentals de la Universitat Autónoma de Barcelona y todas las prácticas y procedimientos de manejo animal fueron aprobados por el Comité de Ética Animal (CEEAH) de la misma institución de acuerdo con la normativa europea. Para el estudio, se utilizaron 144 pollos hembras de engorde (Ross 308), los cuales fueron distribuidos aleatoriamente en 36 jaulas experimentales (4 animales por jaula), correspondientes a los seis tratamientos dietéticos (en forma de harina) suplementados al 6% con las mismas 6 fuentes grasas que el estudio de estabilidad de piensos (AA de soja y AGD de

palma, y las grasas crudas y refinadas de soja y palma), con 6 réplicas por tratamiento, considerando cada réplica una jaula. Los animales se alimentaron durante 37 días hasta que fueron sacrificados de acuerdo con los procedimientos comerciales. Tras el deshuesado manual, se obtuvieron muestras de muslo y contramuslo con piel de dos pollos de cada réplica. Las muestras de una misma réplica se homogeneizaron en una picadora y una parte se envasó al vacío y se congeló a -20 °C hasta el momento del análisis (carnes frescas, F). El resto de las muestras fueron cocinadas en un autoclave (hasta temperatura interna de 80°C) para después ser divididas en dos grupos: uno envasado directamente al vacío y almacenado a -20 °C hasta el análisis (carnes cocidas, C), mientras que el otro grupo se mantuvo en refrigeración (temperatura media de 4 °C) durante 17 días hasta que finalmente se envasó al vacío y se congeló a -20 °C hasta ser analizadas (carnes cocidas refrigeradas, CR).

En primer lugar, la caracterización de las 6 fuentes grasas utilizadas en este estudio se llevó a cabo según lo descrito en Varona et al. [146] (sección 5.1.1 y Anexo 8.1), y la metodología empleada para el análisis de los piensos aparece descrita en la sección 5.1.4.

Determinaciones en las fuentes grasas:

- Composición en ácidos grasos (AG)
- Contenido en tocoferoles y tocotrienoles (T y T3)
- Contenido en humedad y materia volátil (M)
- Contenido en impurezas insolubles (I)
- Contenido de materia insaponificable (U)
- Composición en clases lipídicas: compuestos poliméricos (POL), acilgliceroles (TAG, DAG y MAG) y ácidos grasos libres (AGL)
- Índice de peróxidos (PV)
- Índice de *p*-anisidina (*p*-AnV)

Determinaciones en los piensos:

- Contenido de humedad y materia volátil (M)
- Composición en ácidos grasos (AG)
- Contenido en tocoferoles (T) y tocotrienoles (T3)
- Índice de peróxidos (PV)
- Contenido en compuestos volátiles (VC)

Los diferentes parámetros de composición y calidad de la carne fueron analizados por duplicado, excepto la determinación de VC. La composición en AG fue únicamente determinada en la carne fresca. En la sección 5.1.5 se describen las determinaciones realizadas en carne, las cuales se nombran a continuación:

- Composición en ácidos grasos (AG)
- Contenido en tocoferoles (T) y tocotrienoles (T3)
- Contenido y formación de peróxidos lipídicos medidos mediante el método del naranja de xilenol tras incubaciones de 0,5 y 96 h (contenido LHP y valor final LHP, respectivamente). La medición a las 0,5 h de incubación estima el contenido de hidroperóxidos lipídicos de la muestra; mientras que la cantidad de hidroperóxidos formados a las 96 h de incubación mide la oxidabilidad (estabilidad oxidativa) de la muestra.
- Índice del ácido tiobarbitúrico (TBA)
- Contenido en compuestos volátiles (VC).

Los resultados se evaluaron estadísticamente mediante un ANOVA multifactorial (n = 108), estudiando tanto los efectos principales como las interacciones de los distintos factores considerados: el tratamiento dietético (CS, AS, RS, P, PFAD y RP) y el proceso tecnológico al que fue sometida la carne (fresca, cocida o cocida y almacenada en refrigeración. Cuando los factores principales mostraron un efecto significativo, las diferencias entre las medias agrupadas obtenidas del ANOVA multifactorial fueron evaluadas mediante el test post-hoc de Tukey. En todos los casos las diferencias fueron consideradas significativas con valores de $p \leq 0,05$. El análisis estadístico de los datos fue realizado con IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA).

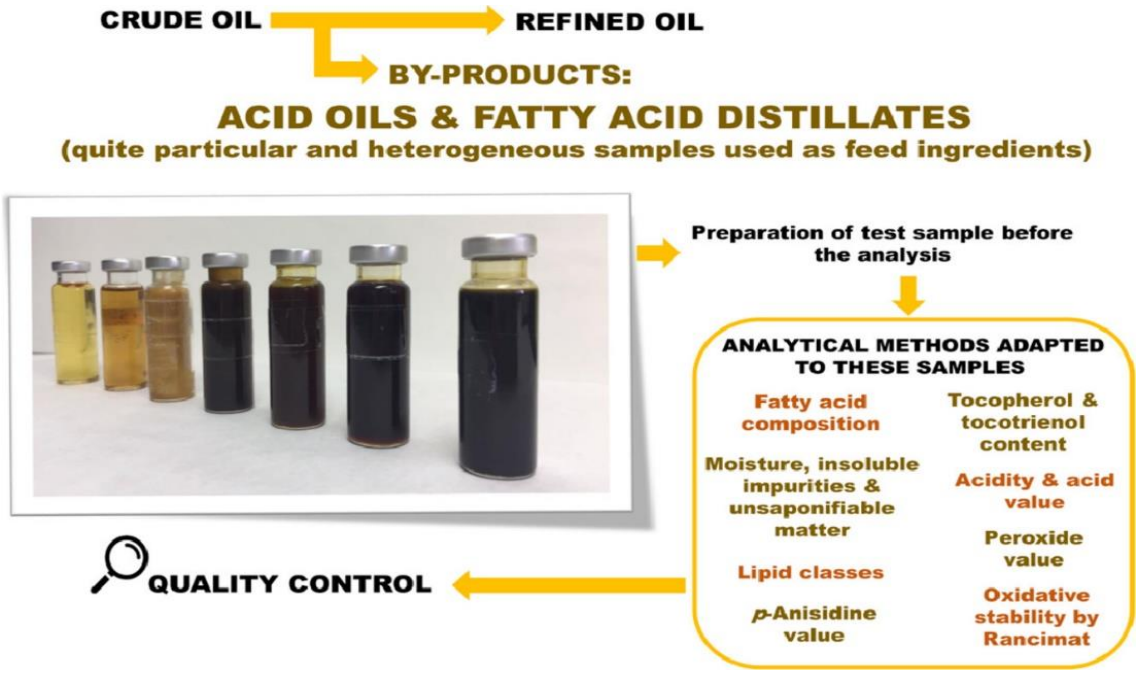
5. RESULTADOS Y DISCUSIÓN

5. Resultados y discusión

5.1. Publicaciones

5.1.1. Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding / Métodos para determinar la calidad de los aceites ácidos y los ácidos grasos destilados utilizados en alimentación animal

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F., *MethodsX*, 2021, 8, 101334. doi:10.1016/j.mex.2021.101334. (La información suplementaria de este artículo se corresponde a los protocolos de análisis, y se recoge en el Anexo 8.1).



Los aceites ácidos (AA) de refinación química y los ácidos grasos destilados (AGD) de refinación física son subproductos de la refinación de los aceites y grasas comestibles. Son utilizados como ingredientes de piensos, pero debido a su composición, la cual es muy variable, pueden verse afectados los parámetros productivos de los animales. Esto hace necesario su control de calidad y estandarización. Sin embargo, los métodos oficiales recomendados para grasas y aceites crudos y refinados deben modificarse para su aplicación a los AA y AGD. Esta publicación resume los inconvenientes durante la adaptación de los métodos analíticos y cómo se superaron para poder aplicarlos a los AA y AGD. Las determinaciones de composición de ácidos grasos, el contenido de tocoferol y tocotrienol, la materia insaponificable, la acidez y el índice de peróxidos, tuvieron que adaptarse mínimamente. Sin embargo, otras determinaciones como la humedad y materia volátil, las impurezas insolubles, las fracciones lipídicas y la *p*-

anisidina mostraron importantes inconvenientes que requirieron una adaptación más significativa. Todos ellas, de gran utilidad para mejorar el control de calidad de los AA y AGD.

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Method Article

Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding

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A B S T R A C T

Acid oils and fatty acid distillates are by-products from the refining of edible oils and fats. They are used as feed ingredients, but their highly variable composition sometimes affects the productive parameters of the animals. Thus, their quality control and standardization are necessary. The official methods recommended for crude and refined fats and oils must be modified to give reliable results when applied to acid oils and fatty acid distillates. This article summarizes the drawbacks that were encountered during the setup of the analytical methods and how were they overcome by adapting the methods to these type of fat samples. Some methods such as the determinations of fatty acid composition, tocopherol and tocotrienol content, unsaponifiable matter, acidity and peroxide value had to be minimally adapted. However, others such as the determinations of moisture and volatile matter, insoluble impurities, lipid classes and *p*-anisidine value showed important drawbacks that required a more significant adaptation.

- All the analytical methods have been successfully applied to acid oils and fatty acid distillates.
- A detailed description of the sample preparation for analysis and applied analytical methods is provided as a compendium of methods in the supplementary material.
- These methods will be extremely useful to improve the quality control of these heterogeneous feed ingredients.

Abbreviations: AO, acids oils; *p*-AnV, *p*-anisidine value; FA, fatty acids; FAD, fatty acid distillates; FAME, fatty acid methyl esters; FFA, free fatty acids; HPLC, high performance liquid chromatograph; I, insoluble impurities; M, moisture; MIU, sum of moisture, insoluble impurities and unsaponifiable matter; RSD, relative standard deviation; T, tocopherols; T3, tocotrienols; U, unsaponifiable matter; U/S ratio, unsaturated/saturated fatty acid ratio.

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Name and reference of original method:	*See* Table 2 *for this information*
Resource availability:	*N/A*

Introduction

The acid oils (AO) and fatty acid distillates (FAD) are fat by-products coming from refining of edible oils and fats. The AO come from the acidification of the soapstocks obtained during the neutralization step of the chemical refining; while the FAD come from the deodorization step of the physical refining, where the free fatty acids (FFA) and other volatile compounds are removed subjecting the oil to steam stripping at high temperatures under vacuum [1]. Thus, both by-products have a high content in FFA and they have been included in feeds to increase the dietary energy. Indeed, both AO and FAD are included in the European catalogue of feed materials [2]. However, the composition of AO and FAD has been reported to be very variable [3]. This is one of the reasons why nowadays, many feed producers and farmers are reluctant of using them routinely. In many cases, they even encounter differences in the productive parameters between batches from the same producer. Therefore, there is a need for quality control and standardization of these by-products before their use in animal feeding. This is important because an increased use of these by-products in animal feeding will lead to a more sustainable food chain.

In the quality control of the fatty ingredients of feeds is very important to determine the amount of compounds that can dilute their energy content, such as moisture (M), insoluble impurities (I) and unsaponifiable matter (U) (globally known as MIU value) or the non-elutable material that includes MIU plus oxidized and polymerized fatty acids and acylglycerols [4]. In addition, it is important to determine the FFA content and the U/S ratio (the ratio unsaturated fatty acids to saturated fatty acids) because it is widely recognized that these parameters affect the digestible and apparent metabolizable energy of the fatty ingredients [4,5] However, the fatty ingredients also provide liposoluble vitamins, such as vitamin E, and other essential lipid nutrients, such as linoleic and linolenic acids that can be easily oxidized.

An accurate determination of these parameters would contribute to obtain adequate conclusions on the nutritive value and stability of AO and FAD, which is essential to revalorize these by-products as animal feed ingredients. Nevertheless, analytical methods that are available to determine these parameters are intended for the analysis of crude or refined fats and oils. Compared to AO and FAD, crude and refined fats have a more homogenous composition, usually characterized by lower M, I, U and FFA contents, and higher triacylglycerol content. This sometimes makes it difficult to obtain accurate results when these analytical methods are applied to AO and FAD. Therefore, the objective of this article is to provide analytical protocols specifically adapted to the analysis of AO and FAD from different botanical origins. When available, the analytical methods selected were official methods for the analysis of crude or refined oils, but that were setup and sometimes modified to be adapted to these type of fat samples. The parameters for which no official methods were available were analyzed following in-house validated methods available in the scientific literature. These methods were applied to 92 samples, 79 AO and 13 FAD, coming from the refining of vegetable fats and oils and intended for animal feeding (Table 1). As these samples were very heterogeneous [5], the application of these methods presented several drawbacks that were overcome to obtain reliable results for all

Table 1
Samples classified according to the refining process and the botanical origin.

Refining process	Botanical origin	Subgroup: different mixtures	n	Total
Chemical refining (Acid oils, AO)	Blends of AO from seed oils, cocoa butter and palm oils[a]	Cocoa butter, rapeseed, soybean and palm oils (40/30/20/10)	2	12
		Cocoa butter, palm and seed oils	10	
	Blends of AO from seed and palm oils[a]	Soybean, rapeseed and palm oils (40/40/20)	2	5
		Sunflower, soybean, palm, corn and rapeseed oils	3	
	AO from olive pomace oil and blends of AO from olive pomace and olive oils	Olive pomace oil	13	18
		Olive pomace and olive oils (90/10)	5	
	Blends of AO from seed oils[a b]	Sunflower (80-90), rapeseed (20-10) and traces of palm and palm kernel oils and palm stearin	1	9
		Sunflower, corn and grapeseed oils (40/30/30)	3	
		Sunflower, soybean and corn oils	3	
		Sunflower, high oleic sunflower, soybean, corn and olive pomace oils	2	
	AO from sunflower oil	Sunflower oil	18	18
	Blends of AO from sunflower and soybean oils[a]	Sunflower and soybean oils	4	15
		Sunflower and soybean oils (10/90)	7	
		Sunflower and soybean oils (80/20)	2	
		Sunflower and soybean oils (90/10)	2	
	AO from soybean oil	Soybean oil	2	2
Physical refining (Fatty acid distillates, FAD)	FAD from coconut oil and blends of FAD from coconut and palm kernel oils (lauric FAD)[a]	Coconut and palm kernel oils	3	5
		Coconut oil	2	
	FAD from palm oil	Palm oil	6	6
	FAD from olive pomace and olive oils	Olive pomace oil	1	2
		Olive oil	1	

[a] For some blends the proportions were unknown.
[b] Some blends have traces of fruit oils.

types of samples. This article summarizes the drawbacks that were found during the setup of the analytical methods (Table 2) and how were they overcome by adapting the methods. In addition, a detailed description of the sample preparation for analysis and of the customized analytical methods is provided as a compendium of methods in the supplementary material. This compendium will be extremely useful to control the quality of these very particular and variable feed ingredients.

Setup and adaptation of the methods to the samples

The main difficulty found during the preparation of the sample for the analysis was to achieve the complete homogenization of the sample. The samples had very different nature and they had to be homogenized at temperatures that range from room temperature to 65 °C. The heating temperatures and times were always the minimum necessary to avoid any alteration of the samples by oxidation or polymerization (method S1, supplementary materials).

The fatty acid composition was determined after a double methylation in methanolic medium, first with sodium methoxide and later with boron-trifluoride, to ensure that FFA were completely methylated. Then, fatty acid methyl esters (FAME) were separated and identified by gas chromatography-flame ionization detector. The FAME were quantified by peak area normalization (the quantitative results are obtained by expressing the area of a given peak as a percentage of the sum of the areas of all the identified peaks). This method is based on the method described by Guardiola et al. [6] and it was adapted to the samples without any problem. To evaluate the

Table 2
Analytical methods that were adapted and used to control the quality of acid oils and fatty acid distillates.

Analytical method	Main reference[a]
Preparation of test sample	
Fatty acid composition	Guardiola et al. [6]
Tocopherol and tocotrienol content	Aleman et al. [7]
Moisture and volatile matter	AOCS official method Ca 2d-25 [8]
Insoluble impurities	ISO 663:2017 [12]
Unsaponifiable matter	AOCS official method Ca 6b-53 [13]
Acidity (free fatty acid content) and acid value	ISO 660:2009 [14]
Lipid classes (%): polymeric compounds, acylglycerols and free fatty acids	IUPAC method 2508 [15]
Peroxide value	Commission regulation (EEC) 2568/91 and its amendments [17]
p-Anisidine value	AOCS official method Cd 18-90 [18]
Oxidative stability by Rancimat instrument	AOCS official method Cd 12b-92 [19]

[a] For some methods other references have been used, see the supplementary materials for the complete list of references. No particular reference was followed for the preparation of the test sample.

intralaboratory repeatability, six determinations of two samples (one AO and one FAD) were carried out by the same analyst within a day, using the same reagents, equipment and instruments and the relative standard deviation (RSD, %) ranged from 0.13–1.31% for different fatty acids (method S2, supplementary materials).

The content in tocopherols (T) and tocotrienols (T3) was determined using a high performance liquid chromatograph (HPLC) equipped with a silica column and a fluorescence detector. In this procedure, prior to injection into the HPLC system, the sample was subjected to saponification and the unsaponifiable matter was extracted with petroleum ether, filtered, evaporated, and dissolved in n-hexane. The eight homologs of vitamin E: α-, β-, γ-, and δ-T and their corresponding T3 were quantified by external standard calibration and the results were expressed in mg/kg. This method is based on the method described by Aleman et al. [7] and it was adapted to the samples without any problem showing good intralaboratory repeatability for AO and FAD. To evaluate the repeatability, eight determinations were carried out for two samples (one AO and one FAD) and the RSD ranged from 4.5–13.5% for the four T. The recovery of the method was also good for all T, because even the recovery for δ-T in AO was quite low, in all cases the variability of the recovery was extremely low (method S3, supplementary materials).

The moisture, including other volatile matter at the conditions of the test, was determined by a vacuum oven method in which the sample was subjected to heat (58 °C) and vacuum (66 mbar, approximately 50 mm Hg) and the % loss in weight was reported as moisture and volatile matter. The method is based on the AOCS official method Ca 2d-25 [8], which showed some drawbacks when applied to these samples. Some AO samples can rise problems due to an excess of moisture, so they can explode producing splattering inside the vacuum oven. To avoid that, once the sample was weighed into the tared dish, the uncovered dish was kept overnight (16 h) into the desiccator applying vacuum progressively until a pressure of 10 mm Hg was reached to remove part of the moisture and volatiles from the sample. After this operation, the determination followed the procedure described in method S4 (supplementary materials). Another problem occurred with lauric FAD, which contain a significant amount of C6:0-C12:0 fatty acids. In the case of the lauric FAD coming from the physical refining of coconut or palm kernel oils, most of the fatty acids (65–82%) are in free form. It has been reported that some of these FFA, mainly C6:0 (boiling point at 50 mm Hg = 135 °C), can be volatilized in the vacuum oven. This volatilization under our working conditions (constant weight achievement) conducted to an overestimation of the moisture and volatile content. This overestimation was proven in several lauric FAD samples by comparing the results obtained as described above and following the one-component reagent volumetric Karl Fischer method (ISO 8534:2017) [9]. As it can be observed in Table 3, if constant weight was attained, the overestimation was considerably high; however, if only one 1 h-drying period was applied, the difference with the Karl Fischer results was minimum. These results agree with the fact that during the first drying period, water (boiling point at 50 mm

Table 3
Moisture (%) content in lauric fatty acid distillates determined by different methods (determinations in duplicate).

	Karl Fischer (ISO 8534:2017) [9]	Vacuum oven (method S4, supplementary materials)	
		One drying period (1 h)	Constant weight (5 drying periods of 1h)
Sample 1	0.28	0.32	0.47
Sample 2	0.16	0.14	0.36
Sample 3	0.18	0.21	0.57

Hg = 37.5 °C) and the most volatile compounds are lost. Thus, for lauric FAD only one drying period of 1 h was applied to obtain the results of moisture and volatile compounds. When other methods to determine moisture, such as the hot plate methods, AOCS official methods Ca 2b-38 [10] and Tb 1a-64 [11], were applied to lauric FAD, erroneous results were obtained due to the presence of a high amount of C6:0-C12:0 FFA. Once these drawbacks were solved, the intralaboratory repeatability of the method for AO and FAD was improved. To evaluate the repeatability, eight determinations were carried and the RSD obtained for an AO was 0.96% (mean of moisture and volatile matter = 0.98%), for a non-lauric FAD, 12.80% (mean = 0.07%) and for a lauric FAD, 8.43% (mean = 0.21%).

The insoluble impurities were determined by a procedure that implies dissolving the dried samples with an excess of light petroleum ether. Then, the obtained solution was filtered, and the filter and residue were washed with the same solvent and then dried at 103 °C in an air oven and weighed to determine the percentage of insoluble impurities retained in the filter. The results can be expressed on wet weight or on dry weight. The method determines dirt and other foreign substances insoluble in light petroleum ether and it is based on the ISO 663:2017 method [12]. The application of this method to the samples showed some drawbacks. For most samples, about 5 g of AO or FAD were accurately weighed into a tared moisture dish and the moisture and volatile matter were determined according method S4 (supplementary materials). Once the sample was dried, it was dissolved in petroleum ether and filtered to determine impurities (method S5, supplementary materials). However, samples with a high percentage of insoluble impurities may be difficult to filter. For such samples, 2 g can be weighted without significantly affecting the precision of the method. According to our results, it can be concluded that in AO samples with impurities percentages higher than 10% it is necessary to reduce the sample weight to 2 g instead of 5 g, in order to carry out the method without any problem in the filtration step nor in transferring the entire sample from the dish to the filter. In addition, in the case that some remains did not dissolve in the petroleum ether and remained in the moisture dish, they had to be weighed and this value must be added to the total weight for the calculation of the impurities. Once these drawbacks were overcome, the intralaboratory repeatability of the method for AO and FAD improved. To evaluate the repeatability, six determinations were carried for five different samples (four AO and one FAD) using different weights of sample and the RSD ranged from 7.72–12.75%.

The unsaponifiable matter content was determined by saponifying the sample with an ethanolic potassium hydroxide solution. In this method, the unsaponifiable matter was extracted from the soap solution with diethyl ether. The solvent was then evaporated, and the residue was weighed after drying and reported as percentage of unsaponifiable matter. The method is based on the AOCS official method Ca 6b-53 [13], which is a method recommended for samples having an unsaponifiable content higher than that usually found in fats and oils. Although the AOCS does not recommend this method for feed-grade fats, the method was successfully applied to AO and FAD (method S6, supplementary materials). The method showed a good intralaboratory repeatability for AO and FAD. To evaluate the repeatability, six determinations were carried for two samples (one AO and one FAD) and the RSD obtained for the AO was 5.35% (mean unsaponifiable matter = 5.02%) and for the FAD, 3.46% (mean = 1.22%). In fact, the repeatability RSD reported for a crude rapeseed oil having an unsaponifiable content of 1.43% in an interlaboratory study organized by IUPAC was quite high, 24.7% [13].

The acidity (or FFA content) and the acid value were determined by a volumetric titration in which the sample is dissolved in a mixture of ethanol 96% and toluene (1:1, v/v), and the acids present in

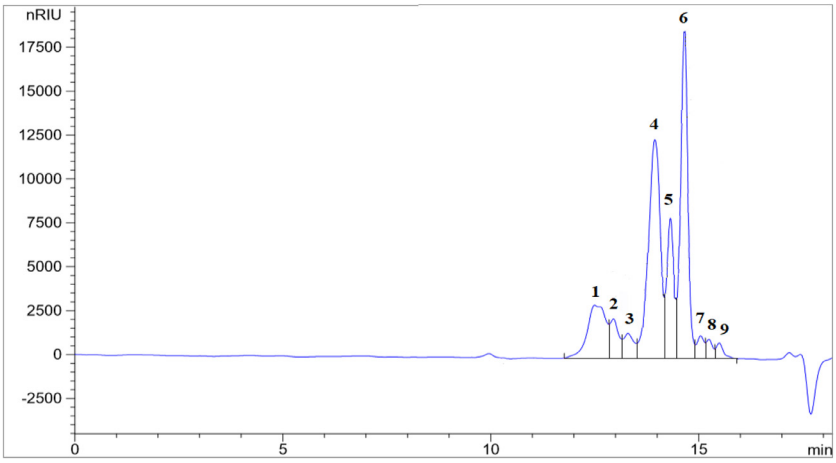

Fig. 1. Separation of the lipid classes from a lauric fatty acid distillate. Main compounds corresponding to peaks: 1, triacylglycerols; 2, diacylglycerols; 3, monoacylglycerols; 4, C16 and C18 free fatty acids; 5, myristic acid; 6, lauric acid; 7, capric acid; and 8 and 9, free fatty acids with less than 10 carbons.

the solution are then titrated with an 0.1 M ethanolic solution of potassium hydroxide. While the acidity or free fatty acid content is expressed as mass percent of lauric, palmitic, oleic or erucic acids, according to the fatty acid composition of the sample, the acid value is expressed as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in 1 g of fat. This method is based on the ISO 660:2009 method [14] and it was adapted to the samples without significant problems. As all samples were very rich in FFA and some of them were quite dark, to clearly observe the endpoint, only 0.5 g of sample was weighed. In these conditions, the method showed a good intralaboratory repeatability for AO and FAD (method S7, supplementary materials). To evaluate the repeatability, six determinations were carried for two samples (one AO and one FAD) and the RSD obtained for the AO was 4.04% (mean = 61.98 g oleic acid/100 g or 123.30 mg KOH/g of fat) and for the FAD, 1.06% (mean = 74.77 g lauric acid/100 g or 209.74 mg KOH/g of fat).

The lipid classes (polymeric compounds, triacylglycerols, diacylglycerols, monoacylglycerols and FFA) present in the samples were separated by size exclusion chromatography by means of two HPLC columns connected in series and determined through a refractive index detector. The lipid classes were quantified by peak area normalization (the quantitative results are obtained by expressing the area of a given peak as a percentage of the sum of the areas of all the peaks corresponding to the different lipid classes). The method is based on the IUPAC method 2508 [15] and is not applicable to fats and oils rich in medium- and/or short-chain fatty acids, such as lauric FAD, as their lipid classes have a wide range of molecular weights, which disables the size exclusion columns from separating them. We noted this problem using several triacylglycerol, monoacylglycerol and FFA standards with different molecular weights. For instance, monolaurin is included in the peak corresponding to C16 and C18 FFA (Fig. 1). This is because the molecular weights of monolaurin, palmitic and oleic acids are 274.4, 256.4 and 282.5 g/mol, respectively. For the same reason, tricaprin elutes together with the diacylglycerols in peak 2 (Fig. 1). Therefore, we discarded this method for lauric FAD since the separation between lipid classes cannot be achieved as the ranges of molecular weights of different lipid classes are clearly overlapped. On the contrary, the method separates well the lipid classes in fats and oils mainly composed of fatty acids with 16 and 18 carbons (Fig. 2). However, in some AO with low content of polymeric compounds, the peak corresponding to these compounds (peak 1, Fig. 2) appears as a shoulder before the peak corresponding to the triacylglycerols (peak 2). The method showed a good intralaboratory repeatability for AO and non-lauric FAD. To evaluate the repeatability, six determinations were carried for two samples (one AO and one non-lauric FAD). The results obtained for the AO sample were: 2.51% polymeric compounds (RSD = 11.38%);

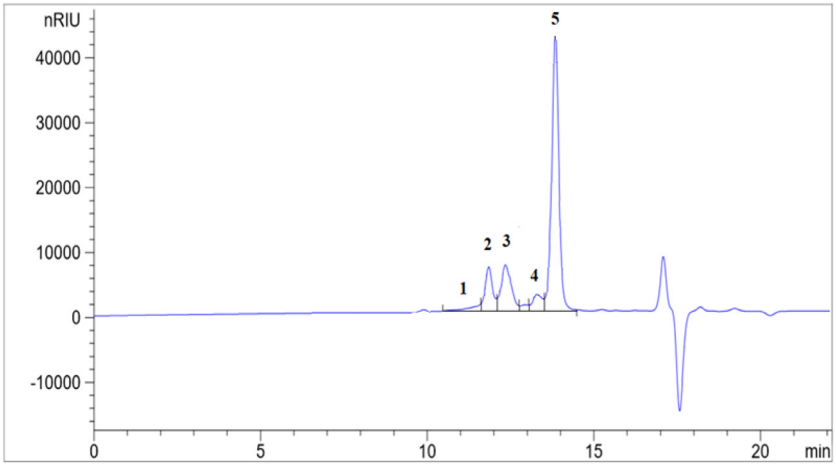

Fig. 2. Separation of the lipid classes from an acid oil coming from the refining of olive pomace oil. Main compounds corresponding to peaks: 1, polymeric compounds; 2, triacylglycerols; 3, diacylglycerols; 4, monoacylglycerols; and 5, C16 and C18 free fatty acids.

21.39% triacylglycerols (RSD = 0.79%); 17.98% diacylglycerols (RSD = 1.13%); 4.05% monoacylglycerols (RSD = 5.04%); and 54.07% FFA (RSD = 0.73%). For the non-lauric FAD the results were: 6.03% triacylglycerols (RSD = 6.79%); 5.88% diacylglycerols (RSD = 9.83%); 88.09% FFA (RSD = 0.93%); the polymeric compounds and the monoacylglycerols were not detected in this sample (method S8, supplementary materials). Moreover, to determine the lipid classes (sums of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids) in lauric FAD it is necessary to apply much more selective methods such as high-temperature gas chromatography-mass spectrometry [16]. With these highly selective methods, the different triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids can be determined individually at once and then added together in the different lipid classes. Of course, these selective methods will provide additional information, but they have not yet been applied to determine lipid classes in lauric fats and oils. This complex analytical problem should be addressed in the future since the relative quantity of triacylglycerols, diacylglycerols, monoacylglycerols and FFA affects the digestibility of fats in different animal species and therefore the dietary energy provided by these by-products, which is a crucial information to formulate feeds [5].

The peroxide value was determined by a volumetric titration in which the sample is dissolved in chloroform-acetic acid and treated with a solution of potassium iodide. The iodine liberated by oxidation of potassium iodide is then titrated with a standard volumetric sodium thiosulfate solution. The oxidant substances under these conditions are generally assumed to be peroxides. The peroxide value is the quantity of peroxides (expressed in milliequivalents of active oxygen per kg of fat) in the sample. This method is based on the method adopted by the European Union for olive oils [17] and it was adapted to the samples without significant problems. Since some AO samples were very dark and this made it difficult to observe the endpoint of the iodometric titration, no more than 2.5 g were weighed, even if the expected peroxide value of the sample was much lower than 10 (the 92 samples analyzed had peroxide values lower than 10 and the 95% had peroxide values between 0 and 3). The method applied in the conditions described in the supplementary material (method S9), paying special attention to the observation of the titration endpoint, showed a good intralaboratory repeatability. To evaluate the repeatability, six determinations were carried and the RSD obtained for a dark AO sample was 2.14% (mean = 2.63 milliequivalents of active oxygen per kg of fat, sample weight = 2.5 g) and for a non-dark FAD sample, 3.05% (mean = 1.27 milliequivalents of active oxygen per kg of fat, sample weight = 2.5 g).

The *p*-anisidine value (*p*-AnV) was determined by spectrophotometry. The method determines the amount of aldehydes (principally 2-alkenals and 2,4-dienals), which react with *p*-anisidine in an isooctane/acetic acid solution of the sample. The reaction products formed are measured at 350 nm. The intensity of the absorption depends not only on the amount of aldehydic compounds but also on their structure. A double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance at 350 nm four to five times, so especially 2-alkenals and 2,4-dienals will contribute substantially to the value found. The *p*-AnV is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1 g of the sample in 100 mL of a mixture of solvents and *p*-anisidine reagent. The method is based on the AOCS official method Cd 18-90 [18] and when applied to these samples, it showed some drawbacks. Most AO samples showed suspended particles after dissolution in isooctane and a filtration step was necessary to obtain a limpid solution before measuring its absorbance. Depending on the turbidity observed the filtration was carried using filters of 5 µm or 0.45 µm of pore and the sample weight was reduced (method S10, supplementary materials). On the contrary, limpid solutions were obtained for FAD samples and thus, the filtration step was not necessary. For all samples, the weight used depended on the linearity of the method. To check the linearity of the response, the *p*-AnV of various AO and FAD samples was determined in duplicate using different weights between 0.5 and 1.5 g. The linearity of the response was confirmed up to an absorbance after reaction with *p*-anisidine (As value) of 3.2, since until this As value the *p*-AnV of several samples was independent of the weight. Thus, in all cases the weight of the samples was adjusted in order to obtain As values equal or lower than 3.0. The method applied in the conditions described in the supplementary material (method S10) showed an improved intralaboratory repeatability for AO and FAD. To evaluate the repeatability, six determinations were carried for two samples (one AO and one FAD) and the RSD obtained for the AO sample was 2.96% (mean = 13.47, sample weight = 0.8 g) and for a the FAD sample, 6.98% (mean = 12.62, sample weight = 1.5 g).

The oxidative stability of the samples (induction time in hours) was measured by the Rancimat instrument from Metrohm (Herisau, Switzerland). In the Rancimat instrument, the sample is subjected to an air flow and a constant temperature which produce a deterioration of the sample. The air stream sweeps the highly volatile oxidation products such as alcohols, aldehydes, ketones and carboxylic acids, among others, which are transferred into the measuring vessel containing Milli-Q® water and the electrode that measures the conductivity. The conductivity is continuously registered and increases slowly to a sudden jump, at which point the oxidation accelerates and becomes high very rapid. The time elapsed until this rapid acceleration of the oxidation is a measure of the resistance to oxidation and is referred to as the induction time or induction period, which allows a reliable estimate of the oxidative stability of the sample. The computer software determines the break point of the conductivity curve by using the maximum of the second derivative of the curve and gives the induction time expressed in hours. The longer is this time, the higher is the oxidative stability of the sample. This method is based on the AOCS official method Cd 12b-92 [19] and its application to the samples required only slight modifications. However, in 10 AO samples the conductivity curve did not show a clear jump and the induction time could not be determined. The method applied in the conditions described in the supplementary material (method S11) performed as usual for the rest of samples (69 AO and 13 FAD) since the RSD was calculated for the duplicates of the 82 samples determined and the median of the RSD was 2.33%, similar to other types of samples.

Conclusion

Some methods such as the determinations of fatty acid composition, tocopherol and tocotrienol content, unsaponifiable matter, acidity and peroxide value, had to be minimally adapted from methods recommended for crude and refined fats and oils. However, others such as the determinations of moisture and volatile matter, insoluble impurities, lipid classes and *p*-AnV showed important drawbacks that required a more significant adaptation. Indeed, in the case of the determination of moisture and volatile matter, the method proposed varies depending on the botanical origin of the FAD, and in the case of the determination of the lipid classes, the method can only be applied to AO and FAD from non-lauric origin. After adaptations, in general, all the analytical methods have been

successfully applied to AO and FAD. A detailed description of all the methods is included in the supplementary material. These methods will be extremely useful to improve the quality control of these heterogeneous feed ingredients. In fact, the animal feed sector needs this information because the official methods recommended for the analysis of crude or refined fats and oils must be modified to give reliable results for these samples, which will help to accurately evaluate the energy and nutritive values of these feed ingredients.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101334.

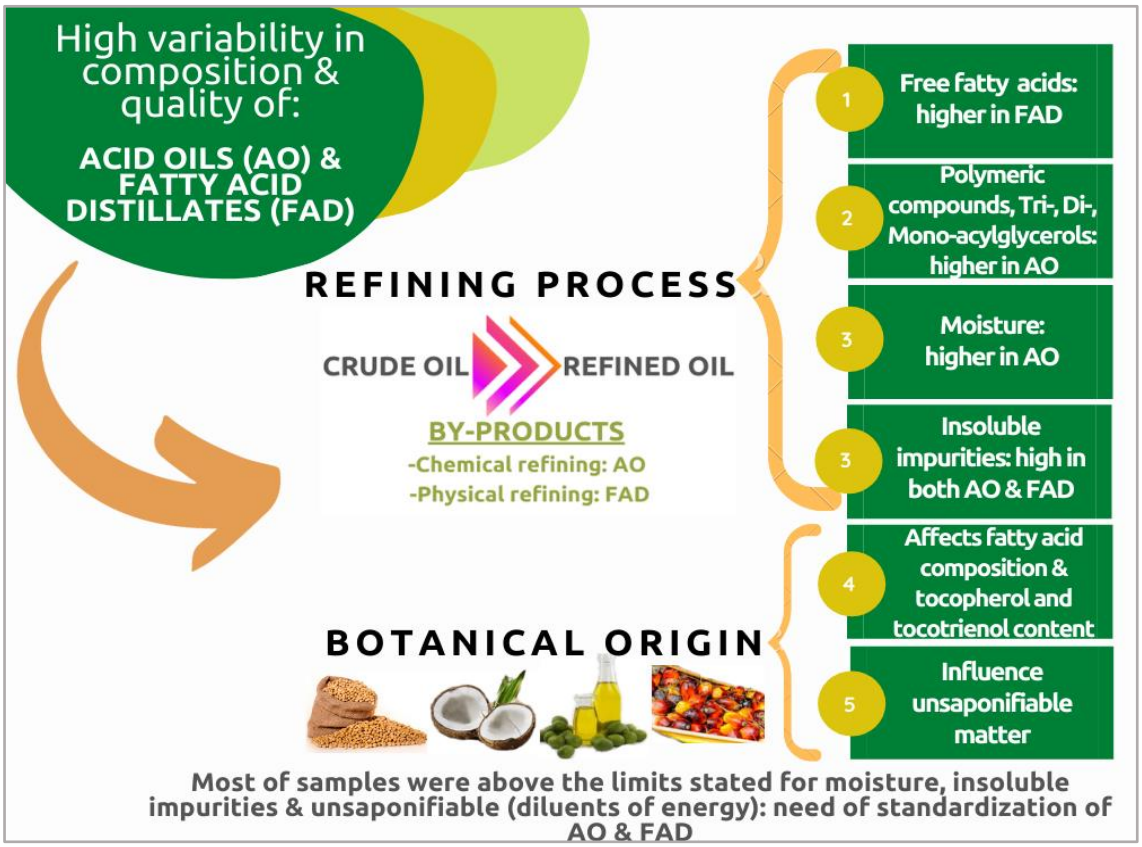
References

[1] FEDIOL, the EU vegetable oil and protein meal industry association, represents the interest of the European oilseed crushers, vegetable oil refiners. (http://www.fediol.eu, accessed 14 February 2021).
[2] Commission regulation (EU) 68/2013 and its ammendments on the Catalogue of feed materials, Off. J. Eur. Union L29 (2013) 1–64.
[3] C. Nuchi, F. Guardiola, R. Bou, P. Bondioli, L.Della Bella, R. Codony, Assessment of the levels of degradation in fat co-and byproducts for feed uses and their relationships with some lipid composition parameters, J. Agric. Food Chem. 57 (2009) 1952–1959, doi:10.1021/jf803369h.
[4] V. Ravindran, P. Tancharoenrat, F. Zaefarian, G. Ravindran, Fats in poultry nutrition: digestive physiology and factors influencing their utilisation, Anim. Feed Sci. Technol. 213 (2016) 1–21, doi:10.1016/j.anifeedsci.2016.01.012.
[5] E. Varona, A. Tres, M. Rafecas, S. Vichi, A.C. Barroeta, F. Guardiola, Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding, Animals 11 (2021) 196, doi:10.3390/ani11010196.
[6] F. Guardiola, R. Codony, M. Rafecas, J. Boatella, A. López, Fatty acid composition and nutritional value of fresh eggs, from large- and small-scale farms, J. Food Comp. Anal. 7 (1994) 171–188, doi:10.1006/jfca.1994.1017.
[7] M. Aleman, C.D. Nuchi, R. Bou, A. Tres, J. Polo, F. Guardiola, R. Codony, Effectiveness of antioxidants in preventing oxidation of palm oil enriched with heme iron: A model for iron fortification in baked products, Eur. J. Lipid Sci. Technol. 112 (2010) 761–769, doi:10.1002/ejlt.200900220.
[8] AOCS official method Ca 2d-25, Moisture and Volatile Matter, in Fats and Oils, Vacuum Oven Method, Official Methods and Recommended Practices of the American Oil Chemists' Society, 7th ed., AOCS Press, Champaign, IL, 2017.
[9] International Standard ISO 8534:2017, Animal and vegetable fats and oils, determination of water content, Karl Fischer method (pyridine free). 3rd ed. 2017.
[10] AOCS official method Ca 2b-38, Moisture and Volatile Matter in Butter, Fats, Margarines, and Oils, Hot Plate Method. Official Methods and Recommended Practices of the American Oil Chemists' Society, 7th ed., AOCS Press, Champaign, IL, 2017.
[11] AOCS official method Tb 1a-64, Moisture and Volatile Matter in Fatty Acids, Hot Plate MethodOfficial Methods and Recommended Practices of the American Oil Chemists' Society, 7th ed., AOCS Press, Champaign, IL, 2017.
[12] International Standard ISO 663:2017, Animal and vegetable fats and oils, determination of insoluble impurities content. 5th ed. 2017.
[13] AOCS official method Ca 6b-53, Unsaponifiable Matter, High Level MethodOfficial Methods and recommended practices of the American Oil Chemists' Society, 7th ed., AOCS Press, Champaign, IL, 2017.
[14] International Standard ISO 660:2009, Animal and vegetable fat and oils, determination of acid value and acidity. 3rd ed. 2009, reviewed and confirmed in 2014.
[15] International Union of Pure and Applied Chemistry (IUPAC)Determination of Polymerized Triglycerides in Oils and Fats by High Performance Liquid Chromatography (method 2508). In Standard Methods for the Analysis of Oils, Fats and Derivatives, 7th ed., IUPAC, Blackwell Scientific Publications, Oxford, UK, 1991.

[16] R. Michael-Jubeli, J. Bleton, A. Baillet-Guffroy, High-temperature gas chromatography-mass spectrometry for skin surface lipids profiling, J. Lipid Res. 52 (2011) 143–151, doi:10.1194/jlr.d008094.
[17] Commission regulation (EEC) 2568/91 and its amendments on the Characteristics of olive oil and olive-residue oil and on the relevant methods of analysis. Annex III - Determination of the peroxide value, Off. J. Eur. Communities L248 (1991) 1–83.
[18] AOCS official method Cd 18-90, *p*-Anisidine Value. Official Methods and Recommended Practices of the American Oil Chemists' Society, 7th ed., AOCS Press, Champaign, IL, 2017.
[19] AOCS official method Cd 12b-92, Oil Stability Index. Official Methods and Recommended Practices of the American Oil Chemists' Society, 7th ed., AOCS Press, Champaign, IL, 2017.

5.1.2. Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding / Composición y valor nutricional de aceites ácidos y ácidos grasos destilados utilizados en alimentación animal

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F., *Animals*, 2021, 11, 196. doi: 10.3390/ani11010196



Los aceites ácidos (AA) de refinación química y los ácidos grasos destilados (AGD) de refinación física son subproductos del refinado de aceites y grasas comestibles ricos en ácidos grasos libres. El objetivo de este estudio es su caracterización y la identificación de sus fuentes de variabilidad para que puedan ser estandarizados mejorando su uso como ingredientes de piensos. Se recogieron muestras (n = 92) representativas del mercado español y se analizaron diferentes parámetros como el valor de MIU (suma de humedad y materia volátil -M, impurezas insolubles -I y materia insaponificable-U), clases de lípidicas, composición de ácidos grasos (AG) y contenido de tocoles (T y T3). La composición de los AA y AGD fue muy variable incluso entre lotes de un mismo productor identificándose dos factores como posibles causas de dicha variabilidad: el proceso de refinación y el origen botánico del aceite crudo del que proceden los AA y AGD. Como los AGD se originan en la etapa de destilación de la refinación física, mostraron cantidades más elevadas de ácidos grasos libres (82,5 vs 57,0 g / 100 g, valores medios), mientras que los AA mantuvieron proporciones más altas de M, polímeros, tri-, di- y monoacilgliceroles.

En general, el valor de MIU fue mayor en AO (2,60-18,50 g / 100 g en AA frente a 0,63-10,44 g / 100 g en AGD), siendo la mayoría de los contenidos de I superiores a los de las guías de recomendaciones nutricionales. En cuanto a la composición de T, T3 y AG estuvo influenciada por el origen botánico del aceite crudo. Por otro lado, se calcularon los valores de energía alimentaria en los AA y AGD siendo de forma general más altos para AA. Estos valores de energía disminuyeron cuando se aplicó un factor de corrección considerando el MIU. En conclusión, el control analítico y la estandarización de estos subproductos es de suma importancia para revalorizarlos como ingredientes de piensos.

animals

MDPI

Article

Composition and Nutritional Value of Acid Oils and Fatty Acid Distillates Used in Animal Feeding

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Simple Summary: Acid oils and fatty acid distillates are by-products from the edible oil refining industry that are rich in free fatty acids. Their use as feed ingredients is a way to valorize them in order to increase the sustainability of the food chain; however, differences in the animal productive parameters when using them have been reported. The objective of this study is their characterization and the identification of their sources of variability. Results have revealed a high variability in their composition, being influenced both by the botanical origin of de crude oil and by the type of refining process. Thus, the analytical control and standardization of these by-products is of outmost importance to guarantee a standardized quality which would increase their value as feed ingredients. Remarkably, almost all samples showed some compositional values above the limits recommended by some feed fat guidelines, which suggests that the production of these by-products must be standardized and improved, and some of the thresholds should probably be revised.

Abstract: Acid oils (AO) and fatty acid distillates (FAD) are oil refining by-products rich in free fatty acids. The objective of this study is their characterization and the identification of their sources of variability so that they can be standardized to improve their use as feed ingredients. Samples (n = 92) were collected from the Spanish market and the MIU value (sum of moisture, insoluble impurities, and unsaponifiable matter), lipid classes, fatty acid composition, and tocol content were analyzed. Their composition was highly variable even between batches from the same producer. As FAD originated from a distillation step, they showed higher free fatty acid amounts (82.5 vs 57.0 g/100 g, median values), whereas AO maintained higher proportions of moisture, polymers, tri-, di-, and monoacylglycerols. Overall, the MIU value was higher in AO (2.60–18.50 g/100 g in AO vs 0.63-10.44 g/100 g in FAD), with most of the contents of insoluble impurities being higher than those in the guidelines. Tocol and fatty acid composition were influenced by the crude oil's botanical origin. The calculated dietary energy values were, in general, higher for AO and decreased when a MIU correction factor was applied. The analytical control and standardization of these by-products is of the outmost importance to revalorize them as feed ingredients.

Keywords: fat by-products; acid oils; fatty acid distillates; animal feed; nutritional value; poultry; pig; MIU value; energy

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1. Introduction

Many vegetable crude fats and oils need to be refined to agree with the established parameters in the regulations according to their intended use, to increase their shelf life and consumer acceptance [1]. Essentially, refining consists of removing free fatty acids (FFA) and other non-desirable components from crude oils, either by chemical or physical processes. Usually, chemical refining comprises various main steps, namely degumming, neutralization (when FFA are removed), winterization (optional), bleaching, and deodorization. The main steps of a physical refining process usually are degumming, winterization (optional), bleaching, and deodorization [2]. However, despite the well-controlled refining procedures, some components with positive effects such as essential fatty acids (FA), tocopherols (T), polyphenols, or sterols are concomitantly removed from crude oils and accumulated in the by-products. Acid oils (AO) from chemical refining and fatty acid distillates (FAD) from physical refining are by-products obtained from the refining steps where the FFA removal mainly takes place: (i) In the chemical refining, FFA are removed in the neutralization step in which an alkali (usually sodium hydroxide) is added to the degummed oil to precipitate FFA as soap-stocks that are then removed by centrifugation and acidulated to obtain AO; (ii) in physical refining, FFA are mainly removed by distillation in the deodorization step when oil is subjected to steam stripping at high temperatures under vacuum, with FAD being the by-product of this distillation [2,3]. Consequently, FFA are the major components in both AO and FAD and this provides them a high energetic value. They contain other compounds, such as lipid soluble vitamins, that are also removed during these refining steps. Because of this composition, they are valuable products for certain uses, and by this, they do not become waste products and harmful to the environment [4]. They present a growing interest in animal feeding, which is an important application to upcycle and valorize them [5,6]. Indeed, both AO and FAD are included in the European Catalogue of feed materials that shall be of voluntary use by the feed business operators [7]. Since AO and FAD are fat products, supplementing animal feeds with them would be a way to supply energy and fat-soluble vitamins to the animal diet [6,8].

The energy value of a fat depends on its quality and composition, as well as on the species, gender, and age of the animal [9]. Fat quality is often understood, simplified and in a practical way, as the amount of compounds that can dilute the energy content of fats, such as moisture and other volatile compounds (M), insoluble impurities (I), and unsaponifiable matter (U) that are globally known as the MIU value. The non-elutable material value (NEM) is another quality measure of fat that might contain M, I, U, and the oxidized and polymerized lipids [9]. According to previous studies, AO could be considered as potential cheap energy ingredients in poultry diets, as long as they maintain a minimum quality (pH $\geq$ 5 and low MIU content) [10,11]. However, AO and FAD quality is not always controlled or reported, nor are limits found for all these parameters in all feed fat regulations or guidelines. As an example, the European regulation states that M has to be reported in AO and FAD if it exceeds 1 g/100 g [7]. Some guidelines from associations for the development of animal feeding strategies state that I and MIU should be below 0.15 and 5 g/100 g, respectively, both for AO and FAD [12].

On the other hand, the fat composition, and the species, gender, and age of the animal, influence fat digestion and absorption, which are the main factors affecting the energy that animals can obtain from a fat. In this respect, the most relevant points of fat composition are the FFA content and the FA composition, especially the length of the FA carbon chain and the degree of saturation that are related to the oil source [13,14]. High FFA contents have been associated with digestibility impairments especially for saturated fats and for certain animal species and ages [15–17]. However, even if AO and FAD are rich in FFA, they have been suggested as valuable fat sources for feed [17,18].

However, there is a lack of characterization of AO and FAD, and to achieve it, analytical methods adapted to these particular products need to be used [19]. This means that detailed and representative information on their composition is scarcely present in the

feed ingredient composition tables [12,20]. Moreover, in studies that have dealt with some AO and FAD, their quality and composition have been reported to be very variable [4]. This is one of the reasons that, nowadays, many feed producers and farmers are reluctant to use them routinely. In many cases, they even encounter differences in the productive parameters between batches from the same AO or FAD producer. Therefore, determining the compositional parameters and the variability of these by-products, which is related to their nutritional value, is essential for the compliance of the minimum composition and quality requirements in terms of raw materials for animal feeding. Our hypothesis is that both the botanical origin of the crude oils as well as the refining process might affect the final AO and FAD composition, and that some compositional parameters might be more affected, leading to differences in the nutritional value of these by-products. Thus, the objective of this paper is to characterize AO and FAD available in the Spanish market, to detect and evaluate their sources of variability especially focusing on the parameters that determine the nutritional value of fat products, and to establish recommendations on the control of these parameters.

2. Materials and Methods

2.1. Samples

For this study, a total of 92 FFA-rich by-products of edible oil refining and intended for animal feeding were collected from the Spanish market: 79 samples were by-products from chemical refining (AO) and 13 from physical refining (FAD). The samples were obtained from edible oil refineries, AO producers (companies that buy soap-stocks to refineries and produce AO), and feed producers. Mostly, the samples were blends coming from the refining of different edible fats and oils, and only 43 samples came from a single fat or oil, reflecting the usual availability of these products (Table 1).

Table 1. Sample's classification according to the refining process and botanical origin.

Refining Process	Group	Botanical Origin	Subgroup: Different Mixtures	*N*	Total
Chemical refining (Acid oils, AO)	SCP	Blends of AO from seed oils, cocoa butter, and palm oil [1]	Cocoa butter, rapeseed, soybean, and palm oils (40/30/20/10)	2	12
			Cocoa butter, palm and seed oils	10	
	SP	Blends of AO from seed and palm oils [1]	Soybean, rapeseed, and palm oils (40/40/20)	2	5
			Sunflower, soybean, palm, corn, and rapeseed oils	3	
	O	AO from olive pomace oil and blends of AO from olive pomace and olive oils	Olive pomace oil	13	18
			Olive pomace and olive oils (90/10)	5	
	BS	Blends of AO from seed oils [1,2]	Sunflower (80–90), rapeseed (20–10) and traces of palm and palm kernel oils and palm stearin	1	9
			Sunflower, corn, and grapeseed oils (40/30/30)	3	
			Sunflower, soybean, and corn oils	3	
			Sunflower, high oleic sunflower, soybean, corn, and olive pomace oils	2	
	SU	AO from sunflower oil	Sunflower oil	18	18
	SU-SO	Blends of AO from sunflower and soybean oils [1]	Sunflower and soybean oils	4	15
			Sunflower and soybean oils (10/90)	7	
			Sunflower and soybean oils (80/20)	2	
			Sunflower and soybean oils (90/10)	2	
	SO	AO from soybean oil	Soybean oil	2	2

Table 1. *Cont.*

Refining Process	Group	Botanical Origin	Subgroup: Different Mixtures	*N*	Total
Physical refining (Fatty acid distillates, FAD)	LFAD	FAD from coconut oil and blends of FAD from coconut and palm kernel oils [1]	Coconut oil	2	5
			Coconut and palm kernel oils	3	
	PFAD	FAD from palm oil	Palm oil	6	6
	OFAD	FAD from olive pomace and olive oils	Olive pomace oil	1	2
			Olive oil	1	

[1] For some blends the proportions were unknown; [2] Some blends contained traces of fruit oils. Abbreviations: see the "Botanical origin" column for the definition of abbreviations.

Once samples arrived at the laboratory, they were melted, homogenized, and divided into smaller vials. Their head space was filled with N_2 and samples were stored at −20 °C until analysis. The melting conditions varied according to the botanical origin of each fat to avoid sample damage, using in all cases, the minimum temperature and heating time that guaranteed an optimal sample homogenization [19].

2.2. Methods

The analysis of M, I, U, acidity, FA composition, triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), FFA, polymeric compounds (POL), T, and T3 were conducted in duplicate in all AO and FAD samples (Table 2). When available, the official methods for the analysis of crude or refined oils were used, but due to the unusual characteristics of these type of fat samples, they had to be setup and sometimes modified as detailed [19]. The parameters for which no official methods were available were analyzed following standardized protocols available in the scientific literature.

Table 2. Analytical methods used to determine the quality parameters of acid oils and fatty acid distillates.

Analytical Method	Main Reference [1]
Sample preparation	Varona et al., under review [19]
M	AOCS Official Method Ca 2d-25 [21]
I	ISO 663:2017 [22]
U	AOCS Official Method Ca 6b-53 [23]
FFA-AC	ISO 660:2009 [24]
FA composition	Guardiola et al. [25]
POL, TAG, DAG, MAG and FFA-SE	IUPAC, standard method 2508 [26]
T and T3	Aleman et al. [27]
Dietary energy, calculated	Wiseman et al. [28]

[1] All protocols are detailed in [19]. Abbreviations: M, moisture and volatile matter; I, insoluble impurities; U, unsaponifiable matter; FFA-AC, acidity; FA, fatty acid; POL, polymeric compounds; TAG, triacylglycerols; DAG, diacylglcyerols; MAG, monoacylglycerols; FFA-SE, free fatty acids fraction; T, tocopherols; T3, tocotrienols. Note: the content of FFA was determined by titration (FFA-AC) and by size molecular exclusion chromatography (FFA-SE; IUPAC, standard method 2508)

Briefly, M (defined as the % of moisture and any other volatile matter under the conditions of the method) was determined by a vacuum oven method [19,21]. The International Standard ISO 663:2017 was adapted to determine I (g/100 g), in which I stands for the compounds (expressed on wet weight) that are not soluble in petroleum ether 40–60 °C [19,22]. The U content (g/100 g) was determined by saponification and extraction with diethyl ether according to the AOCS official method Ca 6b-53 [23] with some modifications [19]. The weight of the extracted U residue was corrected according to its FFA content (determined by titration with NaOH 0.01 M) expressed as mass of oleic acid.

The MIU values (g/100 g) were calculated by summing up M (g/100 g), I (g/100 g), and U (g/100 g) for each sample.

The International Standard ISO 660:2009 was used to measure acidity (FFA-AC), that is to say, FFA content by titration [24]. The FFA-AC was expressed as g of lauric acid/100 g of fat for FAD coming from coconut and palm kernel oils, as g of palmitic acid/100 g for PFAD and as g of oleic acid/100 g for the rest of samples as FA of 18 carbon atoms predominated [19]. To analyze the FA composition, FA methyl esters (FAME) were obtained by a double methylation [25], separated by GC-FID following the conditions described by Tres et al. [29], identified by means of comparison of retention times with those of an external standard mixture (Supelco 37 Component FAME Mix from Merck, Darmstadt, Germany), and quantified by internal area normalization (the quantitative results are obtained by expressing the peak area of a given FA as a percentage of the sum of the areas of all the identified FA peaks). The total saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (n-6 and n-3 PUFA) were calculated by the sum of the values of individual SFA (C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0 and C24:0), *cis*-MUFA (C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9) and *cis*-PUFA (C18:2 n-6, C18:3 n-3, C20:2 n-6, and C22:2). The unsaturated/saturated ratio (U/S ratio) used to predict dietary energy of these fat by-products was calculated according to (*cis*-MUFA + *cis*-PUFA)/SFA. To calculate this ratio, the *trans*-C18:1 isomers (sum of positional isomers) were considered as SFA and as recommended by Wiseman et al. [30] FA with 12 carbons or below were considered as unsaturated FA independently of their degree of saturation.

The TAG (%), DAG (%), MAG (%), FFA-SE (%), and POL (%) in AO and FAD were determined by size molecular exclusion chromatography according to the IUPAC 2508 method [26]. The results of each lipid class were expressed as internal area normalization in % (in relation to the sum of the peak areas of POL, TAG, DAG, MAG, and FFA). The method was applied to all AO, PFAD, and OFAD, but it could not be applied to LFAD samples because the wide range of different molecular weights of TAG, DAG, MAG, and FFA in LFAD meant that their separation by size exclusion columns was not possible [19]. Therefore, in this study, the content of FFA was determined by titration (FFA-AC) as explained above, and by size molecular exclusion chromatography (FFA-SE).

The amount of T and T3 was determined by HPLC-FLD after saponification [19,27]. Peaks were identified by means of comparison of retention times with those of α-, β-, γ-, and δ-T standards. Quantitation was done through calibration curves built with each T, and the curves were also used also for each corresponding T3. The vitamin E content (expressed as mg of α-T/kg) was calculated by multiplying the individual T and T3 amounts by their respective vitamin E activity conversion factors [31].

Last, the energy of these fat by-products (apparent metabolizable energy, AME, for broilers or the digestible energy, DE, for pigs) was calculated applying the equation suggested by Wiseman et al. [28]:

$$\text{AME (broilers) or DE (pigs)} = A + B\ (\text{FFA-AC}) + C\ e^{D\ (U/S)}, \quad (1)$$

where A, B, C, and D are the value of constants for different animal species and ages used in the corresponding prediction equations, with A being a positive coefficient and B, C, D, and E negative coefficients for young and old broilers and pigs; FFA-AC correspond to the FFA contents obtained by titration (acidity), expressed when introduced in this formula as FFA g/kg of fat; and U/S was the ratio unsaturated fatty acids to SFA. As commented above, as recommended by Wiseman et al. [30] to calculate U/S ratio, saturated fatty acids with a carbon chain length equal or shorter than 12 carbons were considered as unsaturated, because they have a similar digestibility. In addition, *trans*-C18:1 isomers were considered as saturated.

Also, energy was calculated by applying another prediction equation that had been based on the Wiseman's equation [28], but in which the energy was corrected by the MIU (g/100 g) [32]:

$$\text{AME (broilers) or DE (pigs)} = \left(A + B\,(FFA - AC) + C\,e^{D\,(U/S)}\right)\left(1 - \frac{MIU}{100}\right), \quad (2)$$

2.3. Statistics

First, the Shapiro–Wilk test was used to study if the results followed a normal distribution. As data did not follow a normal distribution, non-parametric tests were used for inferential analysis and the mean, standard deviation, median, minimum, and maximum values were considered as descriptive statistical parameters. Mann–Whitney U test was used to compare the distribution of M, I, U, MIU, FFA-AC, FA (SFA, MUFA, n-6 PUFA, n-3 PUFA, and PUFA), n-6/n-3 ratio, U/S ratio, POL, TAG, DAG, MAG, FFA-SE, and T, T3, T + T3, and vitamin E between AO and FAD sample groups.

Kruskal–Wallis test was applied to AO samples and to FAD samples separately to determine if the distribution of the variables was similar between sample groups of different botanical origins and the Stepwise Multiple Comparisons procedure was carried out to compare groups. The distribution of each parameter for AO and for FAD samples of a similar botanical origin was described by using Box-plot graphs. In all cases, $p < 0.05$ was considered significant. All univariate data analysis was performed with IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA).

Two Principal Component Analysis (PCA) were conducted on AO and on FAD samples to explore their natural distribution and grouping, to detect outlying samples and to investigate correlations among variables. This method reduces the number of variables to a specific number of principal components (PC), which are linear combinations of the initial variables. Data matrix used in this study consisted of 92 rows (samples) × 32 columns corresponding to the 32 variables for AO: M, I, U, MIU, FFA-AC, main individual FA (C6:0; C8:0; C10:0; C12:0; C14:0; C16:0; C16:1 n-9; C16:1 n-7; C17:0; C18:0; *trans*-C18:1 isomers; C18:1 n-9; C18:1 n-7; C18:2 n-6; C20:0; C18:3 n-3; C20:1 n-9; C22:0; C23:0; C24:0), U/S ratio, POL, TAG, DAG, MAG, FFA-SE, and T + T3 were included. The data matrix used for FAD included 27 variables as POL, TAG, DAG, MAG, and FFA-SE were excluded. All variables were mean centered and scaled to unit variance. The software used for PCA calculation was SIMCA v13.0 (Umetrics AB, Umea, Sweden).

3. Results

3.1. Variability of the Nutritional Parameters and Differences between Refining Process

Results evidenced a high variability for most of the analyzed parameters. For instance, M, I, and U showed a wide range of values, including samples with very high values both in AO and FAD (Table 3). Accordingly, the global MIU value also showed a high variability, with some FAD samples presenting very low values (0.63 g/100 g) up to some AO samples with MIU values above 18 g/100 g. A high variability was also observed for other parameters such as FA composition, lipid classes, and especially for T contents that in the case of AO ranged from 126.1 to 8464.4 mg/kg (Table 3).

Table 3. Mean, standard deviation, median, minimum, and maximum values obtained for acid oils (AO) and fatty acid distillates (FAD).

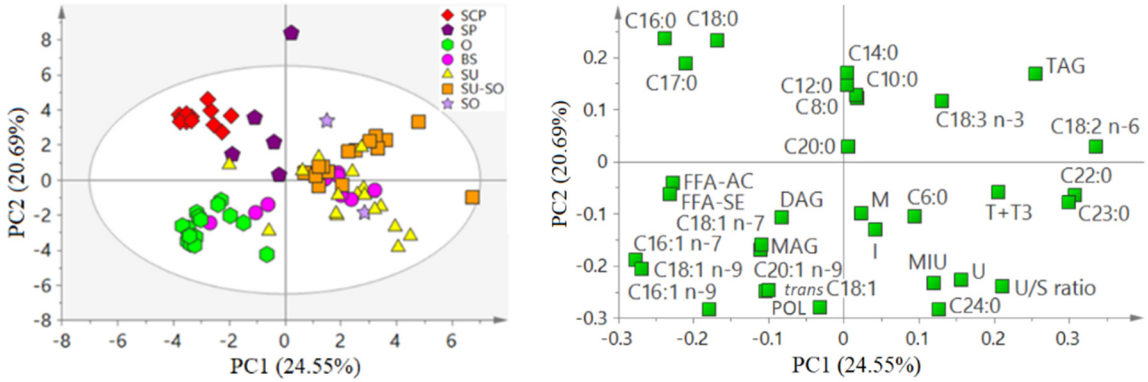
Parameter	Acid Oils (AO, *n* = 79)				Fatty Acid Distillates (FAD, *n* = 13)				p^1
	Mean ± SD	Median	Min	Max	Mean ± SD	Median	Min	Max	
M (g/100 g)	1.31 ± 1.25	0.97	0.17	8.32	0.12 ± 0.12	0.07	0.00	0.37	0.000
I (g/100 g)	1.95 ± 1.51	1.57	0.33	10.24	3.17 ± 2.65	2.85	0.05	8.74	0.228
U (g/100 g)	4.36 ± 1.89	4.20	1.67	10.06	2.07 ± 2.38	1.34	0.32	9.67	0.000
MIU (g/100 g)	7.62 ± 3.22	7.35	2.60	18.50	5.37 ± 3.00	5.11	0.63	10.44	0.032
FFA-AC (g/100 g) [2]	57.4 ± 8.06	57.0	36.4	74.7	79.7 ± 9.58	82.5	64.5	92.2	0.000
SFA (%) [3]	23.0 ± 10.62	18.5	14.2	49.1	59.8 ± 25.74	53.8	12.8	87.6	0.000
cis-MUFA (%) [3]	43.4 ± 15.72	38.9	22.8	70.7	33.2 ± 22.43	36.9	9.8	76.9	0.098
n-6 PUFA (%) [3]	32.2 ± 18.05	36.8	10.2	59.2	6.7 ± 3.39	8.5	2.3	10.9	0.000
n-3 PUFA (%) [3]	1.4 ± 1.03	1.1	0.2	5.3	0.3 ± 0.32	0.3	0.1	1.2	0.000
n-6/n-3 ratio	33.5 ± 43.15	18.5	9.3	312.7	27.6 ± 10.80	26.0	8.9	54.3	0.246
cis-PUFA (%) [3]	33.6 ± 18.52	37.3	11.2	60.7	7.0 ± 3.64	8.9	2.4	12.1	0.000
trans-C18:1 (%)	0.9 ± 0.82	0.66	N.D.	4.5	0.2 ± 0.10	0.2	0.1	0.5	0.000
U/S ratio [4]	4.0 ± 1.39	4.4	1.0	6.2	1.9 ± 1.90	1.4	0.8	6.8	0.001
POL (%) [5]	2.6 ± 1.58	2.5	ND	6.8	ND	ND	ND	ND	0.000
TAG (%) [5]	23.8 ± 8.61	24.6	9.3	54.2	5.3 ± 2.12	5.2	2.1	8.6	0.000
DAG (%) [5]	16.8 ± 3.25	16.9	6.5	28.2	4.4 ± 1.34	4.0	2.6	6.3	0.000
MAG (%) [5]	4.1 ± 0.98	4.2	ND	6.2	0.2 ± 0.57	0.0	ND	1.6	0.000
FFA-SE (%) [5]	52.7 ± 7.73	52.9	31.7	65.3	90.1 ± 2.85	90.0	87.2	93.6	0.000
T (mg/kg)	1167.1 ± 1234.52	813.1	126.1	8464.4	113.8 ± 117.93	65.6	1.8	350.1	0.000
T3 (mg/kg)	48.3 ± 80.21	21.8	ND	454.7	179.9 ± 194.13	89.3	ND	514.0	0.065
T+T3 (mg/kg)	1215.4 ± 1247.57	840.7	126.1	8514.1	293.7 ± 287.64	192.0	1.8	853.7	0.000
Vitamin E (mg/kg) [6]	707.8 ± 659.12	489.5	86.7	3855.9	95.7 ± 97.32	63.6	1.1	285.6	0.000

Abbreviations: M, moisture and volatile matter; I, insoluble impurities; U, unsaponifiable matter; MIU, sum of moisture, insoluble impurities and unsaponifiable matter; FFA-AC, free fatty acids determined by titration (acidity); SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6/n-3 ratio, n-6 polyunsaturated/n-3 polyunsaturated; U/S ratio, unsaturated/saturated ratio; POL, polymeric compounds; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA-SE, free fatty acids determined by size exclusion chromatography; T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; ND, not detected; [1] *p* values were obtained from Mann Whitney U test for independent samples to compare medians between both refining groups. $p \leq 0.05$ was considered significant; [2] FFA-AC (acidity) was expressed as g of oleic acid/100 g in all samples except for lauric FAD (g of lauric acid/100 g) and PFAD (g of palmitic acid/100 g); [3] Table shows the sums of fatty acids including all the identified and quantified FA, expressed as internal area normalization in %: C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C16:1 n-9, C16:1 n-7, C17:0, C18:0, *trans*-C18:1 (sum of positional isomers), C18:1 n-9, C18:1 n-7, C18:2 n-6, C20:0, C18:3 n-3, C20:1 n-9, C21:0, C20:2 n-6, C22:0, C23:0, C22:2, C24:0; [4] To calculate the U/S ratio to predict the dietary energy of these fat by-products through Wiseman's equation [28] the *trans*-C18:1 isomers were considered saturated and as recommended by Wiseman et al. [30] FA with 12 carbons or below were considered as unsaturated FA independently of their degree of saturation. [5] In the case of the variables corresponding to lipid classes (POL, TAG, DAG, MAG and FFA-SE) of FAD, *n* = 8 because these variables could not be determined in the lauric FAD samples (*n* = 5). In all cases, lipid fractions were expressed as internal area normalization in %; [6] The total vitamin E activity (expressed as mg of α-tocopherol/kg) was calculated using the activity conversion factors given by McLaughlin and Weihrauch [31] for each T and T3.

Most parameters significantly differed between AO and FAD, except for I, MUFA, n-6/n-3 ratio, and T3 (Table 3). For instance, M presented higher median and maximum values in AO samples than in FAD. Similarly, AO samples had the highest U median, but the maximum U values were similar between both groups. Consequently, the MIU amount was also higher in AO, although a wide range of MIU values was found in both groups. Also, TAG, DAG, MAG, and POL were higher in samples from chemical refining, and contrarily, FFA-AC and FFA-SE were higher in FAD. Regarding T and T + T3, we observed the highest medians in samples from chemical refining, also when expressed as vitamin E activity.

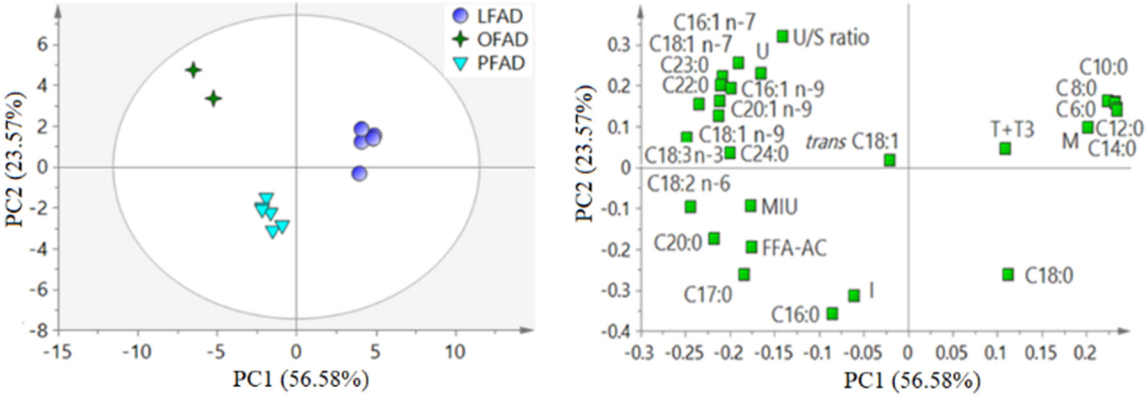
3.2. Sample Clustering According to Botanical Origin

For a global evaluation of the relationships between variables, to study natural clustering of samples and to detect outliers, a PCA was conducted for AO (n = 79, Figure 1) and FAD (n = 13, Figure 2) samples separately. The cumulative variance explained by the two firsts components of each PCA was 45.24% and 80.15% for the AO and the FAD PCA, respectively.



(**a**) (**b**)

Figure 1. Principal component analysis on the compositional parameters (32 variables, mean centered and scaled to unit variance) of acid oils from chemical refining (AO, n = 79). (**a**) Score plot colored according the botanical origin (see Table 1 for abbreviations); (**b**) loading plot (abbreviations: M, moisture; I, insoluble impurities; U, unsaponifiable matter; MIU, sum of M, I, and U; FFA-AC, free fatty acids determined by titration (acidity); POL, polymeric compounds; TAG, triacylglycerols DAG, diacylglycerols; MAG, monoacylglycerols; FFA-SE, free fatty acids determined by size exclusion chromatography; T + T3, sum of α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols) and U/S ratio, ratio of unsaturated to saturated fatty acids calculated as explained in Table 3.



(**a**) (**b**)

Figure 2. Principal component analysis on the compositional parameters (27 variables mean centered and scaled to unit variance, POL, TAG, DAG, MAG, and FFA-SE were excluded) of fatty acid distillates from physical refining (FAD, n = 13). (**a**) Score plot colored according the botanical origin (see Table 1 for abbreviations); (**b**) loading plot (see Figure 1 for abbreviations).

In the PCA developed with AO samples, SCP, SP, and O formed three groups (being SP more scattered) that were clearly distinguished from the rest (Figure 1a). Both SCP and SP groups agreed with a high contribution of various SFA such as C12:0, C14:0, C16:0, C17:0, or C18:0 (Figure 1b), while the O cluster agreed with a high contribution of various MUFA, mainly palmitoleic (C16:1 n-7) and oleic (C18:1 n-9) acids, together with FFA-SE and FFA-AC (Figure 1b). Samples from the other four botanical groups (BS, SU, SU-SO,

and SO) that mainly originated from seed oils, tended to be more scattered and overlapped ones with others (especially BS and SO) (Figure 1a). Their separation agreed with a high contribution of PUFA such as C18:3 n-3 (especially in various SU-SO samples) and C18:2 n-6, C22:0, U/S ratio, and T + T3 (especially in most SU samples). Also, the MIU variable, mainly influenced by U, contributed to the separation of the more unsaturated samples.

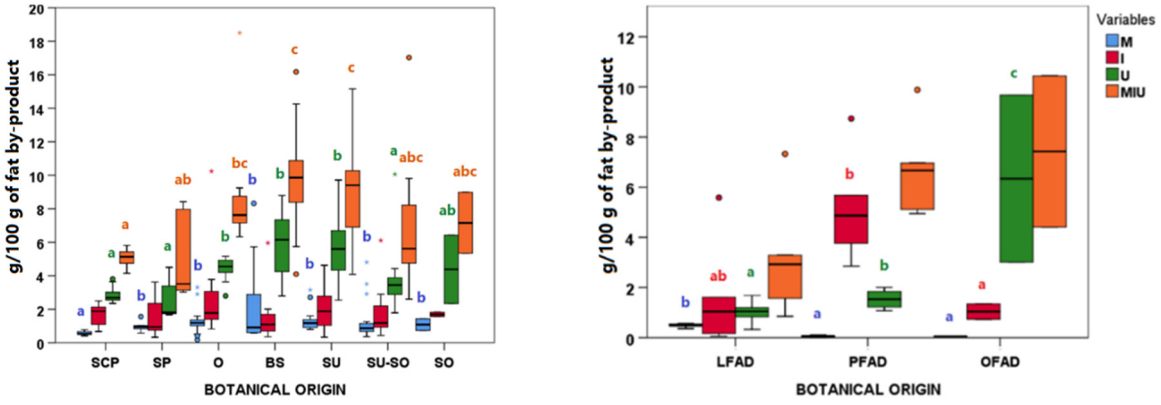
In the PCA developed with FAD samples, three sample groups were distinguished and agreed with the three main groups of crude oil's botanical origin (Figure 2). The two OFAD samples were plotted close to each other because of a high contribution of many unsaturated FA, U/S ratio, and U. Also, PFAD samples formed a cluster in agreement with a high contribution of I and C16:0, among others. The short and medium chain FA (C6:0, C8:0, C10:0, C12:0, C14:0) were correlated and contributed positively to the LFAD clustering, together with M and T + T3.

3.3. Differences between Botanical Groups within AO and FAD

Since the PCA results indicated that some clustering agreed with the botanical origin of the corresponding crude oils, we investigated the differences in the compositional and nutritional parameters between botanical groups within the same refining process. Data were visualized by using boxplot graphics.

3.3.1. M, I, U and MIU Values

In AO samples, the lowest M median was observed in the SCP group, which also showed a very low variability (Figure 3a). No significant differences were found between the other AO botanical groups, with the BS the group having the highest M variability, including one outlier with values above 8 g/100 g. Other groups such as O, SU, and SU-SO also showed some extreme outlier samples.



(**a**) (**b**)

Figure 3. Boxplots for moisture (M), insoluble impurities (I), unsaponifiable matter (U), and the sum of them (MIU) according to botanical groups for (**a**) acid oils from chemical refining (n = 79) and (**b**) fatty acid distillates from physical refining (n = 13) (see Table 1 for botanical group abbreviations). Within each type of refining and variable, botanical groups bearing different letters (a–c) are significantly different according to Kruskal-Wallis test and post-hoc comparisons ($p \leq 0.05$).

No significant differences were observed for I values among AO botanical groups (Figure 3a), although outliers with I values of 10.24, 6.11, and 5.95 g/100 g were observed in O, SU-SO, and BS groups, respectively.

Regarding U, not only a very high variability was observed within each botanical group, but also significant differences were found between groups (Figure 3a). In this case, SP, SCP, and SU-SO followed by SO were the groups with the lowest U. However, in the SU-SO group, one extreme outlier sample with a U value of 10.06 g/100 g was found. As in

general U was the factor that most contributed to the global MIU value, they behaved similarly. Outliers for MIU were found in the O, BS, and SU-SO groups (Figure 3a).

With respect to samples coming from physical refining (Figure 3b), differences between the botanical groups were observed for M, I, and U: The highest M median was found in LFAD, the highest I in PFAD, and the highest U in OFAD, which presented the highest variability. The global MIU showed a similar profile to U, but no significant differences were revealed.

3.3.2. Fatty Acid (FA) Composition

The effect of the botanical origin on the FA profile is shown in Figure 4. For SFA, differences were observed between botanical groups within AO samples, with SCP and SP being the groups with higher SFA (%) amounts (Figure 4a). The variability within each group was different, with SP being the one showing the widest range of SFA percentages. In FAD, SFA showed a narrower variability range and differences were also observed within groups (Figure 4b), with LFAD and PFAD being the richest in SFA.

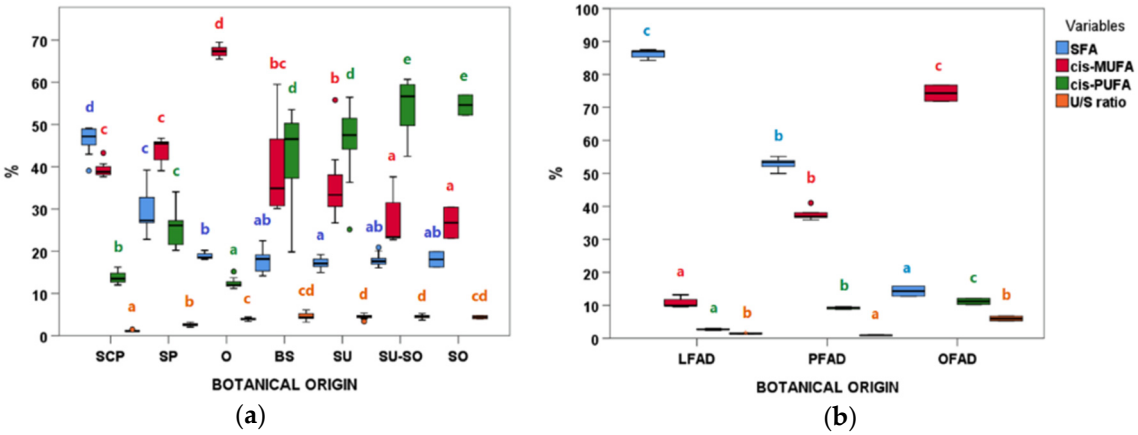


Figure 4. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and unsaturated/saturated ratio (U/S ratio) boxplots according to botanical groups for (**a**) acid oils from chemical refining (n = 79). and (**b**) fatty acid distillates from physical refining (n = 13) (see Table 1 for botanical group abbreviations). Within each type of refining and variable, botanical groups bearing different letters (a–e) are significantly different according to Kruskal–Wallis test and post-hoc comparisons ($p \leq 0.05$).

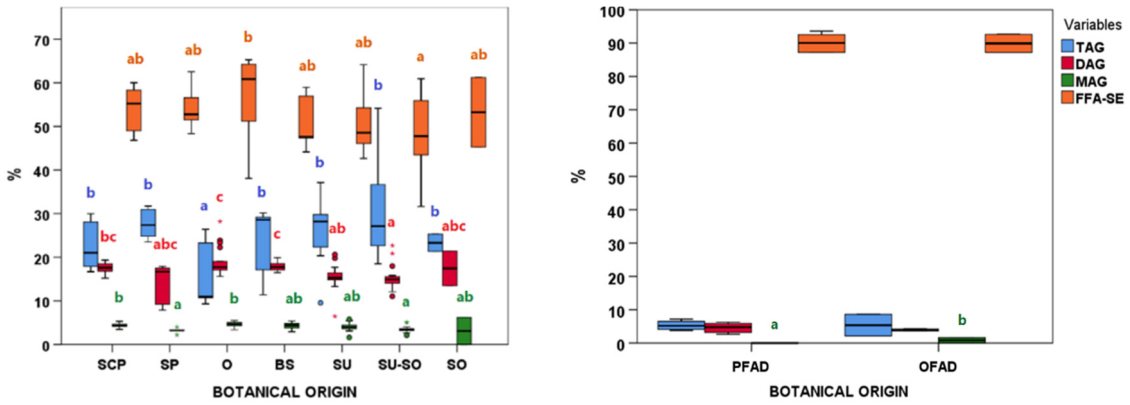
Regarding MUFA, samples from olive and olive pomace oil refining (O and OFAD) reached the highest values in both refining processes, followed by samples containing palm oil in their composition (SP, SCP, PFAD) (Figure 4). It was remarkable the wide MUFA ranges observed for the BS group, followed by SU-SO whose median values were very different (Figure 4a).

Regarding PUFA, AO samples (Figure 4a) coming mainly from seed oils (SU-SO, SO, SU, and BS) had higher and more variable values than the other groups. In FAD (Figure 4b), OFAD presented the highest PUFA median. Differences were observed for the n-6/n-3 ratio being higher and highly variable in BS, SU, SU-SO, and SO (Figure S1, Supplementary Material). The *trans*-C18:1 isomers were higher in AO than in FAD (Table 3). The lowest U/S ratios were found for SCP and SP, with BS, SP, and OFAD being the ones with the highest variability (Figure 4).

3.3.3. Lipid Classes (FFA-SE, MAG, DAG, TAG, and POL) and Acidity (FFA-AC)

In all cases, FFA-SE was the richest lipid fraction, followed by TAG, DAG, and MAG (Figure 5). They varied in wide ranges even within the same AO botanical group (Figure 5a). Even if the ranges of different AO groups were overlapped, some significant differences in their distributions were observed (Figure 5a). Regarding FFA-AC, it behaved similarly to FFA-SE (Figure S3, Supplementary Materials), as the main contributor to FFA-AC values

were FFA. For samples from physical refining, significant differences were found comparing MAG medians between PFAD and OFAD (Figure 5b). The lowest FFA-AC median within FAD was found in the LFAD group, which showed a higher variability that the other FAD groups.



(**a**) (**b**)

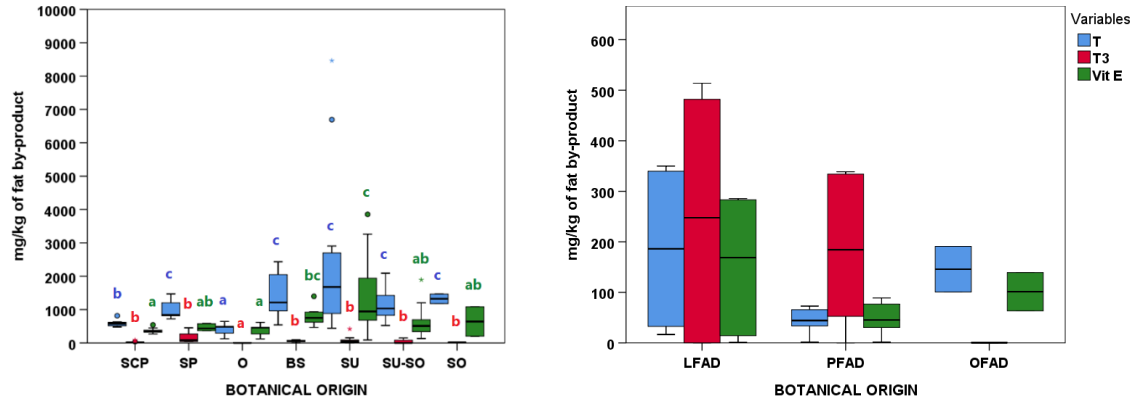
Figure 5. Triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), and free fatty acids (FFA-SE) boxplots according to botanical groups for (**a**) acid oils from chemical refining (n = 79) and (**b**) fatty acid distillates from physical refining (n = 8, lauric FAD group was excluded) (see Table 1 for botanical group abbreviations). Within each type of refining and variable, botanical groups bearing different letters (a–c) are significantly different according to Kruskal–Wallis test and post-hoc comparisons ($p \leq 0.05$).

Regarding POL, all AO groups presented relatively low medians (in all cases, below 4%), but again, a high variability (Figure S2, Supplementary Material). In many of them, samples with null values and also samples with relatively high values, even above 6% in O and SU, were found. As commented above, no POL were detected in FAD samples.

3.3.4. Tocopherol (T) and Tocotrienol (T3) Content

Among AO groups, O and SCP had lower T contents than the rest, while no differences were observed within FAD (Figure 6a). Differences were also found for the individual T and T3 (Table S1, Supplementary Material). For instance, O had the lowest γ-T and no α-T3 was found in O or OFAD groups. It was surprising that two SU samples presented very extreme T values of 6694.9 and 8464.4 mg/kg, especially compared to the other AO in the SU group and to sunflower oil [33]. The high variability in T contents was remarkable, especially for SU, BS, and SU-SO groups in AO, and in T and T3 contents in LFAD.

Overall, the vitamin E activity that was mainly determined by the α-T content (Table S1, Supplementary Material) was higher in the SU group followed by BS, SU-SO, SO, and SP groups (Figure 6). Similar to T results, vitamin E activity did not show significant differences between FAD groups. Again, the high variability observed for some groups such as SU, BS, or LFAD was remarkable.

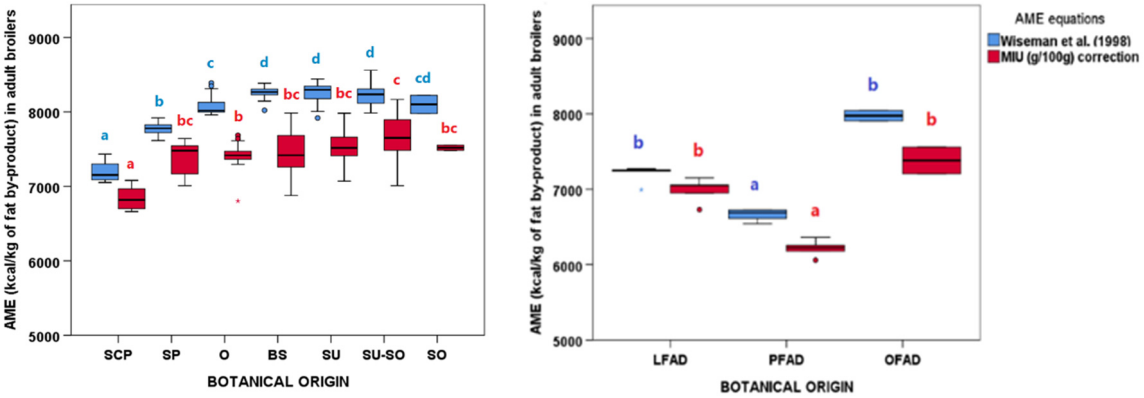


(**a**) (**b**)

Figure 6. Tocopherols (T), tocotrienols (T3), and Vitamin E content according to botanical groups for (**a**) acid oils from chemical refining (n = 79) and (**b**) fatty acid distillates from physical refining (n = 13) (see Table 1 for botanical group abbreviations). Within each type of refining and variable, botanical groups bearing different letters (a–c) are significantly different according to Kruskal–Wallis test and post-hoc comparisons ($p \leq 0.05$).

3.3.5. Dietary energy of These Fat by-products

The AME for broilers and DE for pigs of the fat by-products were calculated applying Wiseman's equation [28] and correcting it by the MIU content [32] (Figure 7 and Table S2, Supplementary Material). Differences were observed between groups, both for AO and for FAD. In AO, SCP samples showed the lowest calculated energy, followed by SP. In FAD, the lowest calculated energy was found for PFAD samples, followed by LFAD and OFAD. In all cases, these values decreased when the MIU correction was applied. It was also remarkable that after MIU correction, the ME and DE variability increased (Figure 7 and Table S2, Supplementary Material).



(**a**) (**b**)

Figure 7. Apparent metabolizable energy (AME) calculated for adult broilers according to Wiseman's equation [28] and by applying the MIU (g/100 g) correction to it [32] of (**a**) acid oils from chemical refining (n = 79) and (**b**) fatty acid distillates from physical refining (n = 13). See Table 1 for botanical group abbreviations. Within each type of refining and variable, botanical groups bearing different letters (a–d) are significantly different according to Kruskal–Wallis test and post-hoc comparisons ($p \leq 0.05$).

4. Discussion

Results have shown that some of the compositional parameters assessed in AO and FAD, such as FFA-AC, the lipid classes (TAG, DAG, MAG, and FFA-SE) or M, mainly varied depending on the refining process, while others such as T, T3, U, and FA composition, were more influenced by the botanical source of the oil originating these by-products.

4.1. Influence of the Refining Process

Both AO and FAD are the by-products from the steps of chemical and physical refining in which FFA are removed from crude oil, and thus both were rich in FFA, leading to high FFA-AC and FFA-SE. However, while in chemical refining (AO), FFA are mainly removed by neutralization and centrifugation and usually not exceeding 100 °C, in physical refining, they are removed during deodorization by distillation, applying vacuum and temperatures around 180–270 °C [1–3,34]. Thus, these different FFA removal processes might have also influenced AO and FAD composition, especially the FFA-AC and the FFA-SE contents that were much higher in FAD (78–94% for FFA-SE) than in AO (31–65% for FFA-SE), in agreement with Nuchi et al. [4]. On the contrary, TAG, DAG, MAG, POL, and M contents were higher in AO. Since FAD are originated from a distillation process, they might accumulate compounds such as FFA, as well as secondary oxidation compounds, T and T3, hydrocarbons, or sterols, that can distillate at the process conditions [3]. On the contrary, during the separation of soap-stocks from the refined oil by centrifugation in the neutralization step of chemical refining, a similar amount of neutral oil (50%) might be concomitantly washed away together with FFA, M, phospholipids, U compounds, proteins, and other mucilaginous substances [6,35]. However, as it will be discussed below, the botanical origin of the oil might also influence some of these parameters such as T and T3. On the other hand, the higher temperatures reached during deodorization in physical refining might have favored that water was less retained in FAD leading to median values of 0.97 g/100 g and 0.07 g/100 g for AO and FAD groups, respectively, and reaching values as high as 8.32 g/100 g in some outlying AO samples. The M values of AO could also be influenced by the presence of phospholipids. The presence of phospholipids in some AO samples could not be excluded as it is known that hydrated phospholipids from degumming step might be added to AO [36]. Thus, they could be contributing to higher water content in the AO.

4.2. Influence of Crude Oil Botanical Origin

As it has been explained above, process conditions in physical refining are usually more drastic than in chemical refining, especially when referring to process temperature that might favor lipid oxidation [1,3]. Consequently, chemical refining is usually preferred for unsaturated oils such as soybean, sunflower, or rapeseed, which in turn are also naturally richer in T than saturated fats [33], while physical refining is commonly used for less unsaturated fats such as palm or lauric oils [3,37]. Thus, although the processing conditions between chemical and physical refining differ, the different FA and T + T3 composition of crude oils (botanical origin) might have a major role in the FA composition and in T and T3 contents of AO and FAD. For instance, regarding tocols, it is known that, during refining, the tocol removal mainly occurs in the deodorization step followed by the neutralization step [1,38,39]. Thus, it would have been logical to expect higher tocols in FAD than in AO; but since AO were mainly obtained from seed oils and FAD from palm and lauric oils, AO were richer in tocols. Remarkably, tocols were higher in AO also when expressed as vitamin E, being from 2 to 20 times higher than in FAD. This, together with the higher POL in AO, contributed to the higher U contents in samples from chemical refining, and to the fact that U and tocols were correlated in the PCA.

The influence of the botanical origin of the crude oil also explained the higher SFA and lower *cis*-PUFA globally observed in FAD. Indeed, the influence of the crude oil botanical source was evident in the tocol and FA composition within the various AO and FAD groups. The highest *cis*-PUFA corresponded to AO coming from the most unsaturated

sources, such as soybean oils (SU-SO and SO) followed by other seed oils (SU and BS). All of them were richer in C18:2 n-6 and C18:3 n-3 than the other AO groups as these FA are present in high amounts in sunflower and soybean oils. The same trend was observed for T, in agreement with the high T contents in seed oils. Accordingly, SU, followed by BS, were the richest in vitamin E as they were also the richest in α-T, which is the tocol with the highest vitamin E activity. On the other hand, the highest *cis*-MUFA contents were observed in the olive oil by-products (compared to other AO and FAD), and also the separation of O and OFAD clusters in their respective PCAs was driven by C18:1 n-9, C16:1 n-9, and C20:1 n-9 that are FA characteristic of olive oil. Then, the second highest *cis*-MUFA in both refining processes corresponded to those groups that contained palm oil by-products (SCP, SP, PFAD) because although palm oil is frequently categorized as a saturated fat, it contains equal proportions of saturated (mainly palmitic acid) and unsaturated acids (mainly oleic acid) [33]. Differences between SFA were also related to the oil source: SCP group was the richest AO in total SFA and in palmitic and stearic acids as they are typical of cocoa butter while LFAD group was the richest FAD in SFA as a result of its high content in medium chain SFA reflecting the FA composition of coconut and palm-kernel oils [33].

The *trans*-C18:1 isomers were significantly higher in AO than in FAD (Table 3), which was unexpected as the formation of *trans* isomers during oil refining is mainly due to the high temperatures applied during deodorization. However, on the one hand, it needs to be taken into account that FAD showed lower *trans*-C18:1 isomers because they include LFAD samples (Table 1), which are poor in C18:1 n-9 and C18:1 n-7. On the other hand, seven out of the eight AO samples with a *trans*-C18:1 isomer content higher than 2% were AO from the refining of olive pomace oils. Olive pomace is usually obtained from the two-phase centrifugation method used in olive oil extraction (by large the method of olive oil extraction more used in Spain). It is a very wet pomace (called in Spain *alperujo*) that might contain up to 60 ± 5 g of moisture/100 g, and it must be dried down to 7–8 g of moisture/100 g to reach an optimum extraction yield of olive pomace oil. Usually, this drying is carried out using rotary dryers that consist of a large cylinder where hot and dry air enters at a high temperature (450 ± 50 °C) and leaves humid at 95 ± 5 °C [40,41], drying conditions that might have contributed to the formation of an important part of these *trans*-C18:1 isomers. The amount of *trans* FA in feed fats is of relevance as it might influence the final *trans* FA in meat of monogastric animals [42]. The current regulation in the EU has set a maximum limit in foods of 2 g (of *trans* FA, other than *trans* FA naturally occurring in fat of animal origin) per 100 g of fat [43]. Studies conducted in chickens fed feeds containing PFAD with a 0.5 or 5.2% of *trans* FA, which are values similar to the maximum values found for FAD and AO in this study, led to thigh meat (with skin) with 0.3 and 1.3 g of *trans* FA/ 100 g of fat, respectively [42], which are values below the current limit.

The U/S ratio showed similar results to PUFA in AO. The U/S ratio is a relevant parameter in the field of animal feeding because, generally, when U/S ratio increases, the fat digestibility increases as well [44,45]. Unsaturated FA not only have greater digestibility and absorption rates than SFA, but it is also believed that they might improve SFA digestibility by increasing the micelle formation when SFA are present in the diet [17,46]. However, the effect is not linear as it depends on the animal's age and species [46]. Indeed, it has been reported a better utilization of unsaturated FA than SFA in broiler chickens, as well as a better ability to digest and absorb fat when the animal age increases [14,17]. In the case of lauric oils, even if they have a high SFA content, they are highly digestible because of their high content in short chain FA [47]. This is why Wiseman et al. [30] recommended to include SFA with 12 carbon atoms or less as unsaturated FA in the U/S ratio for the energy calculation, and thus, this explained that within our FAD, the U/S ratio was lower for PFAD than for LFAD. Thus, considering AO and FAD results, it would be advisable to blend the more saturated products (part of SP samples, SCP, and PFAD) with a more unsaturated seed oil to increase the U/S ratio and improve digestibility. The optimal proportion of the blend needs to be set depending on the animal species, age, and blended

products, and it also needs to be determined if this blend is more appropriate to be done with an oil or with an oil by-product (such as OFAD, O, SU, SU-SO, SO or BS). However, it needs to be taken into account that high U/S ratios especially as a result of the presence of high PUFA contents, may lead to oxidative damage, predominantly in younger birds, and might affect broiler performance and meat quality [48]. However, on the other hand, some PUFA such as linoleic and linolenic acids are considered metabolically essential because birds are not able to synthesize them.

The presence of palm or corn oil by-products (especially in SP but also in SCP) also agreed with high T3 values, particularly γ-T3, which is a tocol analogue characteristic of palm oil. It was remarkable that T and T3 in LFAD reached even higher contents than those found in our PFAD or in coconut and palm kernel oils [33]. However, on the other hand, even if LFAD was the richest FAD in tocols and OFAD the lowest, in PCA, U was more correlated with OFAD. This would indicate that other U compounds such as squalene might have accumulated in OFAD during distillation. According to Psomiadou and Tsimidou [49] squalene is the major olive oil hydrocarbon, which could be present in more than 50% of the U content of the crude olive oil.

It is important to highlight the high variability observed for the FA composition, T + T3 contents and U, especially for the SU, BS, and LFAD groups. Various samples in the SU group had C18:3 n-3, γ-T, and T3 contents higher than those expected for a sunflower oil by-product, suggesting a presence of soybean by-products in some SU samples. On the other hand, the extremely high tocol contents of the two SU outliers could agree with the addition of tocol-rich products, such as deodistillates coming from the deodorization step in chemical refining [39]. However, deodistillates from chemical refining are not included among the products listed in the EU Catalogue of feed materials [7]. Regarding the variability observed for the LFAD group, it has to be taken into account that LFAD included two types of samples: Two pure coconut FADs of which T + T3 contents were below 100 mg/kg in agreement with the very low T3 amounts reported for coconut oil; and three blends of coconut and palm kernel FADs whose T + T3 contents widely differed (reaching up to 850 mg/kg) even if they had been provided by the same company. Unfortunately, for many AO and FAD blends (such as BS, LFAD, SCP, or SP), the exact proportion of each oil source in the blends declared by the producers was approximate or even unknown. In refineries that refine various types of oils, the same waste tank may collect soap-stocks from different oils that are later acidified and transformed into AO, and the same might happen with tanks collecting FADs. In addition, the homogeneity in these tanks is poor, especially in the case of soap-stocks, which are very viscous and form layers corresponding to the different refining batches. In other cases, fat producers may intentionally mix various AO to achieve a certain final composition for the blend. Hence, the natural variability of crude oils and the influence of process and storage conditions could lead to highly variable tocol amounts, and thus, it would be a challenge for AO and FAD producers to standardize the content of tocols and other nutrients. Nevertheless, the tocol content is an important point in animal feeding, because of its antioxidant properties and vitamin E activity [45]. Thus, this suggests that feed producers should be aware of this high variability, demanding this information to the producer so that when the expected amounts are not reached, they can be corrected by supplementing feeds with tocopherols.

4.3. Comparison with Fat Quality Thresholds in EU Regulations and Guidelines

Samples included in this study had been collected from the Spanish market and were expected to be compliant with the EU Catalogue of feed materials [7] and to follow the FEDNA guidelines [12]. The FEDNA guidelines state a maximum of 0.15 g/100 g for I measured by the ISO663:2017 method for various AO and FAD [12,22]. Even if in this study I was measured by this method, only one sample out of the 92 collected had an I content below this threshold. The high variability in I contents even between samples from similar botanical origins and refining process was remarkable. The presence of I in AO and FAD could be related to the I contents of crude oil, to processing contamination, or to

fragments of some gums such as lecithins that some refineries might add to AO to dispose them and to seek an improvement of fat utilization by the animal through their emulsifying properties [9,36]. Therefore, it is evident that this type of sample tends to accumulate high amounts of these I compounds as they are refining by-products. Considering that the median for I was 1.65 g/100 g, and the 90th percentile was 3.80 g/100 g, it would be advisable to revise the thresholds stated in the guidelines for this type of by-products.

Regarding M, it was above 1 g/100 g in 38 samples and thus, according to the EU Catalogue of feed materials, they were M amounts compulsory to declare [7]. The U or MIU contents are currently not specifically included in the EU regulation of feed fats [7], but the FEDNA guidelines recommend MIU values lower than 5 g/100 g both for AO and FAD [12]. However, only 23 samples agreed with this recommendation (17 AO and 6 FAD samples). Overall, only one sample was below the limits for M, I, or MIU in the EU Catalogue of feed materials or in the FEDNA guidelines [7,12]. Other parameters such as the FFA-AC content and the FA profile, including the U/S ratio, the n-6/n-3 ratio, and FA chain length, have also been described as nutritionally relevant for animal feeding as they might influence fat digestibility and, thus, energy utilization [46]. However, none of these parameters are specified in the EU Catalogue of feed materials [7].

4.4. Prediction of Dietary Energy Value of These Fat by-Products

The energy value of an animal diet can be calculated by the energy value of each ingredient reported in feeding tables from official organisms or be estimated through equations that relate the energy value to the chemical characteristics of the ingredients. Wiseman et al. [28] developed the most relevant and useful prediction equation so far for two species (broilers and pigs) and for two ages (young and old). The equation predicts AME (for broilers) and DE (for pigs) based on U/S ratio and fat FFA-AC content, derived through curvilinear regression analysis. As explained above, the U/S ratio is relevant for the energy value of a fat and it depends on its botanical origin. Thus, this caused the energy of AO and FAD to be higher in the more unsaturated groups (SU, SU-SO, SO, BS, and O and OFAD). The lowest calculated energy values were found for PFAD, followed by LFAD that were rich in SFA of 12 C atoms or less [47]. As these FA are highly digestible, they were considered as unsaturated in the U/S ratio as reported by Wiseman et al. [30] in order to avoid underestimating the energy of lauric oils as no other equations have been described for this type of oils so far.

The estimation of the energy value also considers the FFA-AC content, which is one of the most distinct compositional traits between these by-products. Various studies relate FFA with impairments of energy value, fat digestion, and absorption. For instance, back into 1979, Sklan et al. [50] reported that feeding oils rich in FFA led to both a reduction of MAG contents in the intestinal lumen and also of endogenous bile secretion, which resulted in a poorer energy utilization because MAG is needed to solubilize and absorb FFA. Later, Wiseman, and Salvador [13] established that FFA progressively reduced the AME of AO and FAD, and that also the U/S ratio influenced it, especially in young birds. Later, Vilà and Esteve-Garcia [51] found no significant differences in feed efficiency between the use of oils rich in FFA and refined oils, and recently, Rodriguez-Sanchez et al. [14,17] found that SFA had a greater impact on FA absorption than FFA, and that the FFA utilization improved with the age of the animal.

Apart from this, also the dilution role of oxidized FA and POL on AME has been recognized, and these and some other U and NEM compounds have been negatively correlated with AME [52,53]. Therefore, it can be concluded that U/S ratio and FFA alone would not be a good measure of ME in broilers and that increases in energy diluent compounds might lead to a reduction of the dietary energy [51,52]. Thus, modifications of the Wiseman's equation have been suggested, for instance to correct the energy by the MIU content [32]. When the energy of AO and FAD was corrected for their MIU contents, the energy of the most unsaturated groups (BS, SU, SU-SO, SO, and O) decreased to values similar to those of SP group, all still being higher than that of SCP. In FAD, the decrease

was more pronounced in OFAD and PFAD, with PFAD still being the one with the lowest energy values. Thus, the decrease depended on the oil origin and refining process, and it could be as high as 806 kcal/kg in MIU rich samples. This highlights that a proper energy calculation by equations should include the values of the energy diluents, especially when working with MIU rich by-products such as AO and FAD, so that feed formulations can be adjusted accordingly.

It also needs to be taken into account that the content of most of these components in these by-products is sometimes erratic even between batches from the same company as they depend on the natural variability of the raw materials and on the refining process conditions. Even if the refining process is optimized to obtain a standardized refined oil, any effort towards the standardization of these by-products could contribute to increase the confidence of animal nutritionists in them. This standardization could imply the optimization of the by-product processing, or the correction of the contents of some nutritional relevant compounds such as essential FA or tocols.

5. Conclusions

The use of fat by-products from edible oil refining, such as AO and FAD, as feed fats might be a way to upcycle and valorize these by-products, and to increase feed energy and its content of liposoluble vitamins and other essential lipid nutrients. However, the different way of removing FFA by chemical or physical refining also caused other compounds to be concomitantly washed away (or not) from the refined oil, thus affecting the global composition of AO and FAD. Overall, our results reflect a relationship between the main components of these by-products and the production technology. As FAD originated from a distillation step, they showed higher FFA-AC and FFA-SE, whereas AO maintain higher proportions of POL, TAG, DAG, and MAG from the initial oil as they are concomitantly washed away with FFA during alkali neutralization. Thus, depending on the obtention process, the ratio between FFA and the rest of the lipid fractions might vary considerably. However, the content of other compounds with nutritional relevance, such as FA and tocols, was more dependent on the botanical origin of the corresponding crude oils. More interestingly, for most of these compounds there was a great variability in their values, even between batches from the same producer, which might make it difficult for animal nutritionists to get a standardized product that they can easily include in feed formulations. Furthermore, it would be advisable to revise the limit stated for I in the guidelines for these by-products, as almost all samples were above it. In many samples (69 out of 92), MIU values were also above those recommended by FEDNA guidelines (5 g/100 g) [12], and MIU values as high as 18 g/100 g could be reached. Thus, it would be advisable to estimate the energy of fats considering the diluent effect of MIU compounds, because otherwise the calculation might be overestimated even by 806 kcal/kg depending on the type of refining process and the botanical origin of the oil. Therefore, it is necessary to standardize these fat by-products to increase animal nutritionists' confidence in them, and the industry should be encouraged to optimize their production practices to achieve products with less variable composition.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/11/1/196/s1, Figure S1: n-6 Polyunsaturated/n-3 polyunsaturated fatty acid ratio (n-6/n-3 ratio) boxplots according to botanical groups; Figure S2: Polymeric compounds (POL) boxplots according to botanical groups; Figure S3: Acidity (FFA-AC) boxplots according to botanical groups; Table S1: Median values for the individual tocopherols, tocotrienols and vitamin E content (mg/kg) according to the refining process and botanical origin; Table S2: Median values of apparent metabolizable energy (AME, kcal/kg) for broilers and digestible energy (DE, kcal/kg) for pigs of different ages (young and adult) of acid oils and fatty acid distillates. AME and DE values were obtained according to Wiseman et al. [28] equation and by applying the MIU (g/100 g) correction to it as suggested by Bierinckx [32].

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References

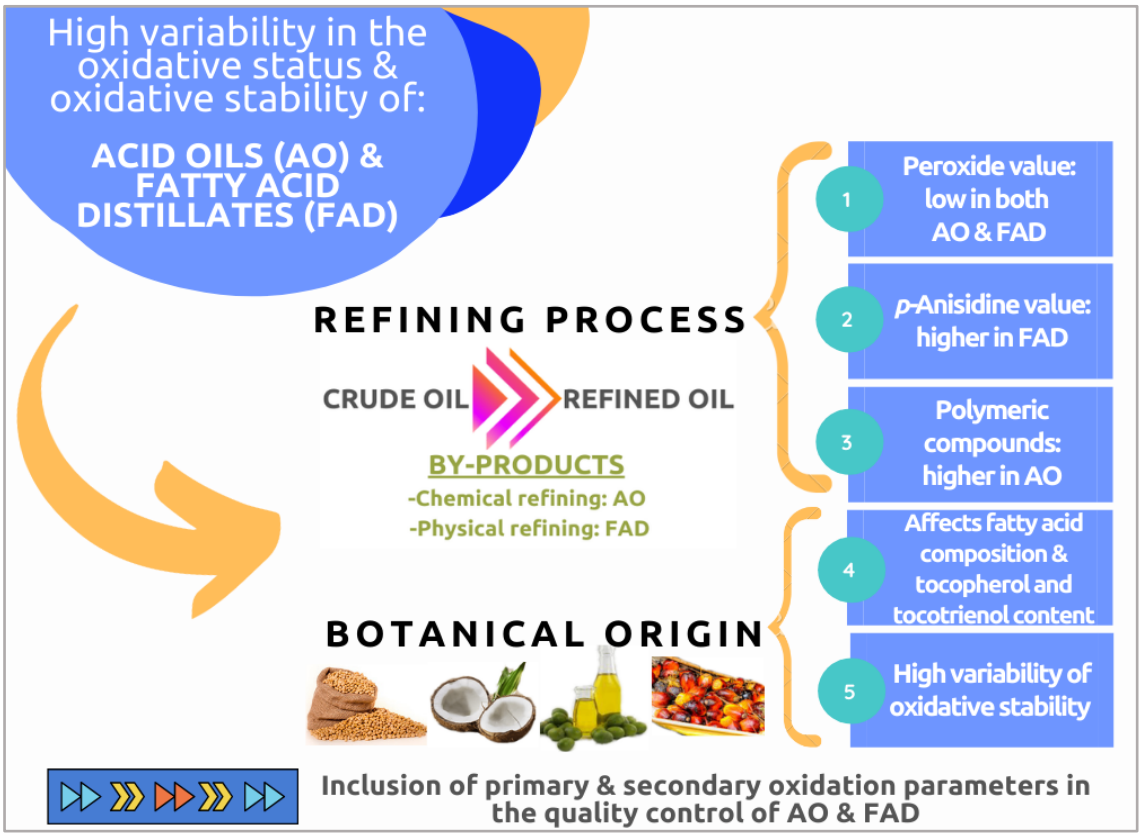
1. Ghazani, S.M.; Marangoni, A.G. Minor components in canola oil and effects of refining on these constituents: A review. *JAOCS, J. Am. Oil Chem. Soc.* **2013**, *90*, 923–932. [CrossRef]
2. FEDIOL, The European Vegetable Oil and Protein Meal Industry Association—Refining of Edible Oils. Available online: www.fediol.eu/web/refining/1011306087/list1187970096/f1.html (accessed on 30 October 2020).
3. Gibon, V.; De Greyt, W.; Kellens, M. Palm oil refining. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 315–335. [CrossRef]
4. Nuchi, C.D.; Guardiola, F.; Bou, R.; Bondioli, P.; Della Bella, L.; Codony, R. Assessment of the levels of degradation in fat co-and byproducts for feed uses and their relationships with some lipid composition parameters. *J. Agric. Food Chem.* **2009**, *57*, 1952–1959. [CrossRef]
5. FEDNA (Fundación Española para el Desarrollo de la Nutrición Animal). *Tablas FEDNA de Composición y Valor Nutritivo de Alimentos para la Fabricación de Piensos Compuestos*, 3rd ed.; Blas, C.; Mateos, G.G.; García-Rebollar, P. Eds; Madrid, Spain. 2010. Available online: http://www.fundacionfedna.org/ingredientes-para-piensos (accessed on 20 September 2020).
6. Dumont, M.J.; Narine, S.S. Soapstock and deodorizer distillates from North American vegetable oils: Review on their characterization, extraction and utilization. *Food Res. Int.* **2007**, *40*, 957–974. [CrossRef]
7. Commission Regulation (EU) 68/2013 of 16 January 2013 and its amendments on the Catalogue of feed materials. *Off. J. Eur. Union* **2013**, *29*, 1–64. Available online: http://data.europa.eu/eli/reg/2013/68/oj. (accessed on 10 September 2020).
8. Baião, N.; Lara, L. Oil and fat in broiler nutrition. *Braz. J. Poult. Sci.* **2005**, *7*, 129–141. [CrossRef]
9. Ravindran, V.; Tancharoenrat, P.; Zaefarian, F.; Ravindran, G. Fats in poultry nutrition: Digestive physiology and factors influencing their utilisation. *Anim. Feed Sci. Technol.* **2016**, *213*, 1–21. [CrossRef]
10. Mateos, G.G.; Serrano, M.P.; Berrocoso, J.; Pérez-Bonilla, A.; Lázaro, R. Improving the Utilization of Raw Materials in Poultry Feeding: New Technologies and Inclusion Levels. In XXIV World's Poultry Congress, Salvador Bahia, Brazil; 2002. Available online: www.facta.org.br/wpc2012-cd/pdfs/plenary/Gonzalo_Mateos.pdf (accessed on 29 October 2020).
11. Vieira S., L.; Ribeiro, A.M.L.; Kessler, A.M.; Fernandes, L.M.; Ebert, A.R.; Eichner, G. Energy utilization of broiler feeds formulated with acidulated soybean soapstock. *Braz. J. Poult. Sci.* **2002**, *4*, 1–13. [CrossRef]
12. FEDNA (Fundación Española para el Desarrollo de la Nutrición Animal). Normas FEDNA de Control de Calidad de Ingredientes para Piensos- Especificación Técnica de Materia Prima, Grasas y Aceites. 2002. Available online: www.fundacionfedna.org/sites/default/files/8_Grasas_y_aceites.pdf (accessed on 30 October 2020).
13. Wiseman, J.; Salvador, F. The influence of free fatty acid content and degree of saturation on the apparent metabolizable energy value of fats fed to broilers. *Poult. Sci.* **1991**, *70*, 573–582. [CrossRef] [PubMed]
14. Rodriguez-Sanchez, R.; Tres, A.; Sala, R.; Guardiola, F.; Barroeta, A.C. Evolution of lipid classes and fatty acid digestibility along the gastrointestinal tract of broiler chickens fed different fat sources at different ages. *Poult. Sci.* **2019**, *98*, 1341–1353. [CrossRef]
15. Wiseman, J.; Salvador, F.; Craigon, J. Prediction of the apparent metabolizable energy content of fats fed to broiler chickens. *Poult. Sci.* **1991**, *70*, 1527–1533. [CrossRef] [PubMed]
16. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. II. Effect of free fatty acid content and degree of saturation of free fatty acids and neutral fat on fatty acid digestibility. *Br. Poult. Sci.* **1996**, *37*, 119–130. [CrossRef]
17. Rodriguez-Sanchez, R.; Tres, A.; Sala, R.; Garcés-Narro, C.; Guardiola, F.; Gasa, J.; Barroeta, A.C. Effects of dietary free fatty-acid content and saturation degree on lipid-class composition and fatty-acid digestibility along the gastrointestinal tract in broiler starter chickens. *Poult. Sci.* **2019**, *98*, 4929–4941. [CrossRef] [PubMed]
18. Palmquist, D.L. Palm fats for livestock feeding. *Palm Oil Dev.* **2004**, *40*, 10–16.

19. Varona, E.; Tres, A.; Rafecas, M.; Barroeta, A.C.; Guardiola, F. Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding. *Methods X*, under review.
20. Federatie Nederlandse Diervoederketen (FND). CVB Feed Table. Chemical Composition and Nutritional Values of Feedstuffs. 2019. Available online: www.cvbdiervoeding.nl/bestand/10601/cvb-feed-table-2019.pdf.ashx (accessed on 29 October 2020).
21. AOCS official method Ca 2d-25. Moisture and volatile matter, vacuum oven method. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 7th ed.; AOCS Press: Champaign, IL, USA, 2017.
22. International Standard ISO 663:2017. *Animal and Vegetable Fats and Oils, Determination of Insoluble Impurities Content*, 5th ed.; ISO: Geneva, Switzerland, 2017.
23. AOCS official method Ca 6b-53. Unsaponifiable matter. In *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 7th ed.; AOCS Press: Champaign, IL, USA, 2017.
24. International Standard ISO 660:2009. *Animal and Vegetable Fats and Oils, Determination of Acid Value and Acidity*, 3rd ed.; ISO: Geneva, Switzerland, 2009; Reviewed and confirmed in 2014.
25. Guardiola, F.; Codony, R.; Rafecas, M.; Boatella, J.; López, A. Fatty acid composition and nutritional value of fresh eggs, from large- and small-scale farms. *J. Food Comp. Anal.* **1994**, *7*, 171–188. [CrossRef]
26. IUPAC (International Union of Pure and Applied Chemistry). Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography (method 2508). In *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 7th ed.; IUPAC, Blackwell Scientific Publications: Oxford, UK, 1991.
27. Aleman, M.; Nuchi, C.D.; Bou, R.; Tres, A.; Polo, J.; Guardiola, F.; Codony, R. Effectiveness of antioxidants in preventing oxidation of palm oil enriched with heme iron: A model for iron fortification in baked products. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 761–769. [CrossRef]
28. Wiseman, J.; Powles, J.; Salvador, F. Comparison between pigs and poultry in the prediction of the dietary energy value of fats. *Anim. Feed Sci. Technol.* **1998**, *71*, 1–9. [CrossRef]
29. Tres, A.; Tarnovska, N.; Varona, E.; Quintanilla-Casas, B.; Vichi, S.; Gibert, A.; Vilchez, E.; Guardiola, F. Determination and comparison of the lipid profile and sodium content of gluten-free and gluten-containing breads from the Spanish market. *Plant. Foods Hum. Nutr.* **2020**, *75*, 344–354. [CrossRef]
30. Wiseman, J.; Blanch, A. The effect of free fatty acid content on the apparent metabolisable energy of coconut/palm kernel oil for broiler chickens aged 12 and 52 days. *Anim. Feed Sci. Technol.* **1994**, *47*, 225–236. [CrossRef]
31. Mclaughlin, P.J.; Weihrauch, J.L. Vitamin E content of foods. *J. Am. Diet. Assoc.* **1979**, *75*, 647–665.
32. Bierinckx, K. Variation in dietary lipids potentially influences your poultry production. *Int. Poult. Prod.* **2020**, *28*, 27–28. Available online: http://www.positiveaction.info/pdfs/articles/pp28_3p27.pdf. (accessed on 5 October 2020).
33. Codex Alimentarius. Standard for named vegetable oils (CXS 210-1999). In *Codex Alimentarius, International Food Standards*; Codex Alimentarius: Rome, Italy, 1999; Adopted in 1999. Revised in 2001, 2003, 2009, 2017, 2019. Amended in 2005, 2011, 2013, 2015, 2019.
34. Henry, J. Processing, manufacturing, uses and labelling of fats in the food supply. *Ann. Nutr. Metab.* **2009**, *55*, 273–300. [CrossRef] [PubMed]
35. Wang, T. Soybean oil. In *Vegetable Oils in Food Technology: Composition, Properties and Uses*, 2nd ed.; Gunstone, F.D., Ed.; Blackwell Publishing Ltd.: Oxford, UK, 2011; pp. 59–106. [CrossRef]
36. 'Brien, R.D. Fats and oils processing. In *Fats and Oils: Formulating and Processing for Applications*, 2nd ed.; CRC Press LLC: Boca Raton, FL, USA, 2004.
37. Vaisali, C.; Charanyaa, S.; Belur, P.D.; Regupathi, I. Refining of edible oils: A critical appraisal of current and potential technologies. *Int. J. Food Sci. Technol.* **2015**, *50*, 13–23. [CrossRef]
38. Verleyen, T.; Sosinska, U.; Ioannidou, S.; Verhé, R.; Dewettinck, K.; Huyghebaert, A.; Greyt, W. Influence of the vegetable oil refining process on free and esterified sterols. *J. Am. Oil Chem. Soc.* **2002**, *79*, 947–953. [CrossRef]
39. Chen, Y.; Thiyam-Hollander, U.; Barthet, V.J.; Aachary, A.A. Value-added potential of expeller-pressed canola oil refining: Characterization of sinapic acid derivatives and tocopherols from byproducts. *J. Agric. Food Chem.* **2014**, *62*, 9800–9807. [CrossRef]
40. Petrakis, C. Olive oil extraction. In *Olive Oil Chemistry and Technology*, 2nd ed.; Boskou, D., Ed.; AOCS Press: Champaign, IL, USA, 2006.
41. Gómez-de la Cruz, F.J.; Casanova-Peláez, P.J.; Palomar-Carnicero, J.M.; Cruz-Peragón, F. Modeling of olive-oil waste rotary dryers: Green energy recovery systems. *Appl. Therm. Eng.* **2015**, *80*, 362–373. [CrossRef]
42. Tres, A.; Nuchi, C.D.; Magrinya, N.; Guardiola, F.; Bou, R.; Codony, R. Use of palm-oil by-products in chicken and rabbit feeds: Effect on the fatty acid and tocol composition of meat, liver and plasma. *Animal* **2012**, *6*, 1005–1017. [CrossRef]
43. Commission Regulation (EU) 2019/649 of 24 April 2019 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards trans fat, other than trans fat naturally occurring in fat of animal origin. *Off. J. Eur. Union* **2019**, L 110/17. Available online: http://data.europa.eu/eli/reg/2019/649/oj (accessed on 8 September 2020).
44. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. I. Influence of age, rate of inclusion and degree of saturation on fat digestibility and metabolisable energy of acid oils. *Br. Poult. Sci.* **1996**, *37*, 105–117. [CrossRef]
45. Leeson, S.; Summers, J.D. *Commercial Poultry Nutrition*, 3rd ed.; Nottingham University Press: Nottingham, UK, 2008.
46. Doppenberg, J.; Van der Aar, P.J. *Facts about Fats, A Review of the Feeding Value of Fats and Oils in Feeds for Swine and Poultry*; Wageningen Academic Publishers: Wageningen, The Netherlands, 2010. [CrossRef]

47. Ketels, E.; De Groote, G. Effect of ratio of unsaturated to saturated fatty acids of the dietary lipid fraction on utilization and metabolizable energy of added fats in young chicks. *Poult. Sci.* **1989**, *68*, 1506–1512. [CrossRef]
48. Amaral, A.B.; Silva, M.V.; Lannes, S.C.S. Lipid oxidation in meat: Mechanisms and protective factors—a review. *Food Sci. Technol.* **2018**, *38*, 1–15. [CrossRef]
49. Psomiadou, E.; Tsimidou, M. On the role of squalene in olive oil stability. *J. Agric. Food Chem.* **1999**, *47*, 4025–4032. [CrossRef] [PubMed]
50. Sklan, D. Digestion and absorption of lipids in chicks fed triglycerides or free fatty acids: Synthesis of monoglycerides in the intestine. *Poult. Sci.* **1979**, *58*, 885–889. [CrossRef] [PubMed]
51. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. III. Effect of chemical composition on metabolisable energy of by-products of vegetable oil refining. *Br. Poult. Sci.* **1996**, *37*, 131–144. [CrossRef]
52. Wiseman, J.; Edmunds, B.K.; Shepperson, N. The apparent metabolisable energy of sunflower oil and sunflower acid oil for broiler chickens. *Anim. Feed Sci. Technol.* **1992**, *36*, 41–51. [CrossRef]
53. DeRouchey, J.M.; Hancock J., D.; Hines R., H.; Maloney C., A.; Lee D., J.; Cao, H.; Dean, D.W.; Park, J.S. Effects of rancidity and free fatty acids in choice white grease on growth performance and nutrient digestibility in weanling pigs. *J. Anim. Sci.* **2004**, *82*, 2937–2944. [CrossRef]

5.1.3. Oxidative quality of acid oils and fatty acid distillates used in animal feeding / Calidad oxidativa de aceites ácidos y ácidos grasos destilados utilizados en alimentación animal

Varona, E., Tres, A., Rafecas, M., Vichi, S., Sala, R., Guardiola, F., *Animals*, 2021, 11, 2559. doi:10.3390/ani11092559



Los aceites ácidos (AA) de refinación química y los ácidos grasos destilados (AGD) de refinación física son subproductos de la refinación de aceites y grasas comestibles, respectivamente. Su alto valor energético hace que su reciclaje sea interesante como alternativa sostenible a las grasas convencionales en la alimentación animal. El objetivo de este estudio es caracterizar su calidad oxidativa y guiar en las recomendaciones sobre su control con fines en alimentación animal. Se analizaron parámetros respectivos al estado de oxidación como el índice de peróxidos (PV), el índice de *p*-anisidina (*p*-Anv), el % de compuestos poliméricos (POL), también se evaluó la estabilidad oxidativa (tiempo de inducción medido por el Rancimat a 120 °C (IT)), la composición de ácidos grasos (AG) y se analizó el contenido de tocoferoles (T) y tocotrienoles (T3) de 92 muestras de AA y AGD representativas del mercado español. Tanto los AA como los AGD mostraron un PV bajo (0,8 y 1 meq O_2/ kg); sin embargo, el *p*-AnV fue mayor en los AGD (36,4 vs 16,4 en los AA), y POL fue mayor en los AA (2,5% vs no detectado en AGD), como consecuencia del tipo de proceso de refinado. El origen botánico del aceite crudo del que

procedían los AA y los AGD influyó en la composición de AG y tocoles, e influyó también en la IT. Se observó una alta variabilidad para la mayoría de los parámetros analizados, lo que refuerza la necesidad de estandarizar los AA y AGD para obtener ingredientes para alimentación animal confiables e incluyendo parámetros oxidativos primarios y secundarios dentro de su control de calidad.

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Oxidative Quality of Acid Oils and Fatty Acid Distillates Used in Animal Feeding

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Simple Summary: Feed producers and farmers seek alternative and economical fat ingredients to supply animals with energy and beneficial components, such as natural antioxidants. Some byproducts from the edible oil refining industry, such as acid oils (AO) and fatty acid distillates (FAD), fulfil these requirements, but differences in the animal performance have been reported as their main drawback, which might be due to their high variable composition, including their oxidation status. Therefore, the valorization of these byproducts as feed ingredients requires ensuring standardized products with adequate quality in terms of oxidation parameters. In this study, 92 AO and FAD were characterized, finding a huge variability in their oxidation status and stability. They all showed low primary oxidation values (peroxide values). The content of secondary oxidation compounds was higher in FAD (which are released from physical refining processes) than in AO (which originate from chemical refining), while polymeric compounds were higher in the latter. The fatty acid and tocol compositions that were related with the botanical origin influenced their oxidative stability. Thus, in the quality control of these products, apart from the compositional parameters, it is recommended to include the evaluation of the oxidation status, both by primary and secondary oxidation parameters.

Abstract: Acid oils (AO) and fatty acid distillates (FAD) are byproducts from chemical and physical refining of edible oils and fats, respectively. Their high energy value makes their upcycling interesting as alternatives to conventional fats in animal feeding. The objective of this study is to characterize their oxidative quality and to provide recommendations about their evaluation for animal feeding purposes. The oxidation status (peroxide value (PV), *p*-Anisidine value (*p*-AnV), % polymeric compounds (POL)), the oxidative stability (induction time by the Rancimat at 120 °C (IT)), the fatty acid composition (FA), and tocopherol and tocotrienol content of 92 AO and FAD samples from the Spanish market were analyzed. Both AO and FAD showed low PV (0.8 and 1 meq O_2/kg); however, *p*-AnV was higher in FAD (36.4 vs. 16.4 in AO) and POL was higher in AO (2.5% vs. not detected in FAD) as a consequence of the type of refining process. The botanical origin of AO and FAD influenced FA and tocol composition, and they influenced IT. A high variability was observed for most analyzed parameters, reinforcing the need for standardizing AO and FAD to obtain reliable feed ingredients and to include primary and secondary oxidative parameters within their quality control.

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1. Introduction

Fats are relevant feed ingredients because of their high energetic and nutritional values, as they supply vitamins and essential fatty acids (FA), such as linoleic (C18:2 n-6) and linolenic acids (C18:3 n-3) [1]. However, when lipid oxidation occurs in these fat ingredients, several deteriorative changes in their chemical composition, sensorial properties, and nutritional value also take place. Lipid oxidation depends on availability of substrates (oxygen and oxidable compounds, such as polyunsaturated fatty acids (PUFA)) and on the balance between antioxidants (such as tocopherols (T), among others) and pro-oxidants (such as certain mineral elements). Thus, the oxidation process is influenced by factors inherent to fats and oils (e.g., FA profile, free fatty acid (FFA) content, antioxidants, and pro-oxidants), and by external factors, such as processing and storage conditions (light exposure, temperature, and oxygen availability) [2,3].

When lipid oxidation occurs in a fat, it might be accompanied by a reduction in its nutritional value, related to the loss of PUFA and antioxidants, and to an increase in oxidation compounds, which implies a loss of energy value. Several detrimental biological effects have been described for some oxidation compounds formed in vivo or absorbed from the diet [4,5]. Kanner [6] reported that many of these lipid oxidation products may be absorbed by animals after the dietary intake of oxidized lipids, especially if they are secondary oxidation compounds rather than hydroperoxides, as the latter in the stomach may decompose to alcohols and aldehydes, which will be further partly absorbed through the intestines. Moreover, digestibility impairments at high polymer contents have been reported, resulting from a lower efficiency of pancreatic lipase over them [7,8]. Feeding poultry with different oxidized oils has been described to induce metabolic oxidative stress and negatively affect growth performance [9]. Nevertheless, it seems that the effects on the animal performance vary depending on the degree of oil oxidation, the type of oxidation compounds, the length of the feeding period [10,11], the FA profile, the dietary fat inclusion rate, and the inherent presence of antioxidants/pro-oxidants, among others [12]. Moreover, diets containing oxidized oils might cause a reduction in the T content, which could affect meat T content, resulting in a decrease in its oxidative stability [13–17]. Nevertheless, according to Billek [18] feeding oxidized oils only led to toxic effects in animals when these oils were extremely overheated and had a very high content of oxidized compounds.

The presence of lipid oxidation products in feeds may be a consequence of their formation during feed manufacturing and storage, or it may come from some of the feed ingredients, such as the added fat. Crude oils are usually used as feed fats, but feed producers and farmers are interested in finding more economical and sustainable feed fats, being the co- and byproducts of the fat industry among the candidates [19]. For instance, acid oils (AO) and fatty acid distillates (FAD) are fat byproducts from the refining processes of edible fat and oils, and are included in the European Catalogue of feed materials [20]. Concretely, they are obtained from the steps meant to remove FFA from the crude oil: AO from the alkali neutralization step in chemical refining (after the acidification of the soap stocks), and FAD from the deodorization step in physical refining [21–23]. Soap stocks and FAD are released from the refining processes at different proportions depending on the level of FFA in the crude oils; in alkali neutralization, the soap stocks usually represent 1.6–2% of the crude oil, while the palm FAD (PFAD) represents 4–5% [24,25]. Thus, one of the ways to avoid them becoming waste products is to upcycle them, incrementing their use as high-energy ingredients for feeds. This is a more sustainable application than their use by the oleochemical industry to obtain different derivative products, since, for this latter purpose, a subsequent chemical and/or physical processing is always necessary. Thus, they could be an alternative to conventional fat

and oils used in animal nutrition due to their lower prices and their suitable nutritional performance [26]. However, for this purpose, their quality, energy, and nutritional value should be guaranteed to assure competitive productive parameters and an adequate quality of the foods obtained. The main constituents of AO and FAD are FFA, which are higher in FAD than in AO [1,19]. They also contain variable amounts of neutral oil, moisture, impurities, and minor compounds, such as T, among others [1,27,28]. Despite high FFA contents being related to a reduction in FA digestibility and absorption, especially for SFA, and also, consequently, to a reduction in the dietary energy value of the fat sources [29,30], several studies have demonstrated the potential use of these FFA-rich byproducts (AO and FAD) without impacting FA utilization by animals, especially in adults, as long as the diets do not exceed a determined FFA content and unsaturated/saturated fatty acid (U/S) ratio [31,32] (for instance, in grower–finisher broilers, diets of up to 35% FFA using saturated or unsaturated fat sources [31], or up to 30% FFA and a U/S ratio of 2.61 using PFAD [32]). On the other hand, these byproducts show a non-standardized quality, because refining conditions are constantly being improved to increase yields and quality of refined oils and this, consequently, influences the composition and quality of the final byproducts [33,34]. Furthermore, the management of these byproducts in refineries seeks simplicity, and often, when several oils are refined, soap stocks or FAD are wasted in the same tank, which complicates the standardization of the final product [1]. Thus, the final composition of AO and FAD is highly variable, and feed producers and farmers are reluctant to use them as reliable feed ingredients.

To overcome this, a standardization of the AO and FAD present in the market would be necessary, including the evaluation of their oxidation status. However, despite the effects described above when feeding oxidized oils, feed fat regulations or guidelines usually do not include any lipid oxidation parameter [20,35], or only recommend the evaluation of the peroxide value (PV) [36], which is a primary oxidation parameter and, therefore, it is completely insufficient as a global measure of fat oxidation [19,37–39]. Moreover, AO and FAD quality is not always controlled or reported, nor are the sources of variability of their composition and oxidative stability known. In Varona et al. [1], AO and FAD from the Spanish market were collected and characterized to evaluate the parameters relevant for their energy value, finding some differences in their composition, such as for moisture and FFA content, that were mainly related to the refining process, while others (unsaponifiable matter, FA, and T composition) were more related to the botanical origin. Furthermore, all AO and FAD showed a high content of insoluble impurities [1]. Therefore, our hypothesis is that these differences in composition could lead to differences in the oxidative status and susceptibility to oxidation of these fats, and that the refining process and botanical origin of the crude oil might determine the type and amount of oxidation compounds accumulated in these byproducts. Thus, the main objective of this study is to assess the oxidation status and the oxidative stability of these byproducts to determine if they are influenced by the refining process and botanical origin of the oil, and to establish recommendations on the control of the oxidation parameters.

2. Materials and Methods

2.1. Samples

The same sample set used in Varona et al. [1] to evaluate the nutritional value of AO and FAD was used for this study. It included a total of 92 FFA-rich byproducts of edible oil refining intended for animal feeding and representative of the Spanish market. Of them, 79 samples were byproducts from chemical refining (AO) and 13 from physical refining (FAD). They were collected from edible oil refineries, AO producers (companies that buy AO and soap stocks to refineries and produce custom AO for different animal species), and feed producers. As a result of the usual availability of these products in the market, only 43 samples came from a single fat or oil, while most of the samples were blends obtained from the refining of different edible fats and oils (Table 1).

Table 1. Samples' classification according to the refining process and botanical origin.

Refining Process	Group [1]	Botanical Origin	Subgroup: Different Mixtures	*N*	Total
Chemical refining (Acid oils, AO)	SCP	Blends of AO from seed oils, cocoa Butter, and palm oil	Cocoa butter, rapeseed, soybean, and palm oils (40/30/20/10)	2	12
			Cocoa butter, palm, and seed oils [2]	10	
	SP	Blends of AO from seed and palm oils	Soybean, rapeseed, and palm oils (40/40/20)	2	5
			Sunflower, soybean, palm, corn, and rapeseed oils [2]	3	
	O	AO from olive pomace oil and blends of AO from olive pomace and olive oils	Olive pomace oil	13	18
			Olive pomace and olive oils (90/10)	5	
	BS	Blends of AO from seed oils	Sunflower (80–90), rapeseed (20–10), and traces of palm and palm kernel oils and palm stearin [3]	1	9
			Sunflower, corn, and grapeseed oils (40/30/30)	3	
			Sunflower, soybean, and corn oils [2]	3	
			Sunflower, high oleic sunflower, soybean, corn, and olive pomace oils [2]	2	
	SU	AO from sunflower oil	Sunflower oil	18	18
	SU-SO	Blends of AO from sunflower and soybean oils	Sunflower and soybean oils [2]	4	15
			Sunflower and soybean oils (10/90)	7	
			Sunflower and soybean oils (80/20)	2	
			Sunflower and soybean oils (90/10)	2	
	SO	AO from soybean oil	Soybean oil	2	2
Physical refining (Fatty acid distillates, FAD)	LFAD	FAD from coconut oil and blends of FAD from coconut and palm kernel oils (lauric oils)	Coconut oil	2	5
			Coconut and palm kernel oils [2]	3	
	PFAD	FAD from palm oil	Palm oil	6	6
	OFAD	FAD from olive pomace and olive oils	Olive pomace oil	1	2
			Olive oil	1	

[1] See the "Group" and "Botanical origin" columns for the definition of abbreviations. [2] For some blends the proportions were unknown. [3] Some blends have traces of fruit oils.

When the samples arrived at the laboratory, they were melted, homogenized, divided into smaller vials whose head space was filled with N_2, and stored at −20 °C until analysis. The melting conditions were adapted to the botanical origin of each fat according to Varona et al. [40], selecting the minimum temperature and heating time that guaranteed an optimal sample homogenization and minimized sample damage.

2.2. Methods

The analysis of PV, *p*-Anisidine value (*p*-AnV), polymeric compounds (POL), induction time measured by Rancimat at 120 °C (IT), FA composition and T and tocotrienol (T3) contents were conducted in duplicate in all AO and FAD samples. All detailed analytical protocols can be found in Varona et al. [40] and corresponded to the official methods or methods available in the scientific literature for the analysis of crude and refined oils that were set up or adapted to AO and FAD analysis. Briefly, PV (expressed in milliequivalents of active oxygen per kg of fat) was determined by a volumetric titration in which the sample is dissolved in chloroform–acetic acid and treated with a solution of potassium iodide [40]. The *p*-AnV is a spectrophotometric method that measures a chromophore formed after the reaction in an iso-octane/acetic acid solution of the aldehydic compounds (mainly 2-alkenals and 2,4-alcadienals) present in the sample and the *p*-Anisidine [40]. POL (%) was determined by size molecular exclusion chromatography [40]. It was quantified by internal peak area normalization (%) considering the other lipid classes (triacylglycerols, diacylgycerols, monoacylglycerols, and FFA [40]). The evaluation of the oxidative stability

by Rancimat (Metrohm, Herisau, Switzerland) at 120 °C is a measurement of its resistance to oxidation when it is subjected to air flow at a constant temperature. It consists of the measurement of the time elapsed until a rapid oxidation of the sample occurs (which agrees with a sudden jump in the conductivity in the vessel where the highly volatile oxidation products are collected), which is named as induction time (IT) [40]. The FA composition and tocol contents (α-, β-, γ-, and δ-T; and α-, β-, γ-, and δ-T3) were determined by GC-FID and by HPLC-FLD, respectively, as described in Varona et al. [40]: FA were quantified by internal peak area normalization (%), and tocols were quantified through calibration curves built with α-, β-, γ-, and δ-T, also applying the curves for each corresponding α-, β-, γ-, and δ-T3.

2.3. Statistics

As data did not follow a normal distribution (Shapiro–Wilk test, $p \leq 0.05$), nonparametric tests were used for inferential analysis, and the mean, standard deviation, median, minimum, and maximum values were considered as descriptive statistical parameters, and boxplots were used to compare the distribution of the results between the groups. Mann–Whitney U-test was used to compare the PV, *p*-AnV, POL, IT, FA, T, T3, and T + T3 between AO and FAD sample groups (n = 92 for all variables, except for IT, for which 82 were included, as the measures of 10 AO samples did not provide a clear IT).

The distribution of each parameter for AO and for FAD samples from a similar botanical origin was described by using boxplot graphs. Kruskal–Wallis test was applied to AO samples and to FAD samples separately to determine if the variables were different between sample groups from different botanical origins, and the differences between groups were assessed by the stepwise multiple comparisons procedure of SPSS. In all cases, $p \leq 0.05$ was considered significant. All univariate data analysis was performed with IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA).

Principal component analysis (PCA) was conducted on AO and FAD as a multivariate data analysis, to explore the natural distribution and grouping of samples, to detect outlying samples, and to investigate correlations among variables. The data matrix used in this study consisted of 92 rows (samples) × 25 columns corresponding to the 25 variables: PV, *p*-AnV, POL, T, T3, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C16:1 *n*-9, C16:1 *n*-7, C17:0, C18:0, *trans*-C18:1, C18:1 *n*-9, C18:1 *n*-7, C18:2 *n*-6, C20:0, C18:3 *n*-3, C20:1 *n*-9, C22:0, C23:0, and C24:0 (all variables were mean-centered and scaled to unit variance). The software used for PCA calculation was SIMCA v13.0 (Umetrics AB, Umea, Sweden). As IT could not be measured for all samples, it was not included in the PCA. Thus, its relationship with the rest of the variables was assessed through the Spearman's correlation coefficients, calculated for AO (n = 69) and for FAD (n = 13) separately, considering $p \leq 0.05$ as significant (IBM SPSS Statistics, v23, IBM, Armonk, NY). Significant correlation coefficients were graded as negligible (0.00–0.09), weak (0.10–0.39), moderate (0.40–0.69), strong (0.70–0.89), or very strong (0.90–1.00) according to the stratifications outlined by Schober et al. [41].

3. Results

3.1. Variability of the Oxidation Parameters and Differences between AO and FAD

All parameters significantly differed between AO and FAD, except for PV, *cis*-MUFA, and T3. Moreover, some of them showed a high variability in AO and FAD (Table 2).

FA composition and T contents were very variable, even within AO or FAD groups. Comparing the medians of both groups, SFA (C12:0, C14:0, and C16:0) were higher in FAD, while PUFA (C18:2 *n*-6 and C18:3 n-3) and T were higher in AO, resulting in a higher UFA/SFA in AO than in FAD (Table 2).

Primary oxidation measured by PV was low, both in the AO and FAD groups, with median values around 1 meq O_2/kg of fat, even including samples with null values in both groups (Table 2). Secondary oxidation was assessed by measuring two parameters: *p*-AnV and POL. The amount of secondary oxidation aldehydes (measured through *p*-AnV, a parameter that principally determines 2-alkenals and 2,4-dienals) had significantly higher

median values in the FAD group, while POL was higher in the AO group (Table 2). Indeed, POL were not detected in FAD. Regarding the oxidative stability measured by Rancimat at 120 °C (IT), globally, it was significantly higher in AO, reaching the highest maximum and median value compared to FAD, but its values varied in wider ranges than those of FAD samples (Table 2). Moreover, for some of the AO (10 samples), the IT could not be determined because the conductivity curve did not show a clear and sudden jump, even when the conductivity was measured for a long time, up to 200 h. Apart from this, it is remarkable the high variability observed for *p*-AnV and IT, both for AO and for FAD samples. In both groups, the IT was positively correlated with SFA and negatively correlated with UFA/SFA, both correlations being weak in AO and strong in FAD [41] (Table 3). Moreover, IT for AO was negatively correlated with POL and *trans*-C18:1 (weak correlation), while, for FAD, a significant negative correlation was observed with *p*-AnV (moderate correlation), *cis*-MUFA, and PUFA (both strong correlations) (Table 3).

Table 2. Mean, standard deviation, median, minimum, and maximum values obtained for acid oils (AO) from chemical refining and fatty acid distillates (FAD) from physical refining.

Parameter	Acid Oils (AO, *n* = 79)				Fatty Acid Distillates (FAD, *n* = 13)				*p* [1]
	Mean ± SD	Median	Min	Max	Mean ± SD	Median	Min	Max	
C12:0 (%)	0.1 ± 0.91	0.0	0.0	8.1	13.1 ± 17.07	0.3	0.0	36.4	<0.001
C14:0 (%)	0.2 ± 0.40	0.1	0.1	3.6	8.0 ± 9.63	1.2	0.0	24.0	<0.001
C16:0 (%)	13.6 ± 4.14	13.0	8.1	25.2	29.1 ± 16.44	18.9	10.3	47.5	0.001
C18:0 (%)	6.9 ± 6.93	3.9	2.3	25.0	4.5 ± 1.74	4.5	1.8	8.2	0.594
C18:1 n-9 (%)	40.5 ± 14.74	36.6	21.1	67.1	31.7 ± 21.05	35.7	9.3	71.6	0.173
C18:2 n-6 (%)	32.1 ± 18.07	36.8	10.2	59.2	6.6 ± 3.37	8.5	2.3	10.8	<0.001
C18:3 n-3 (%)	1.4 ± 1.03	1.1	0.2	5.3	0.3 ± 0.32	0.3	0.0	1.2	<0.001
SFA (%) [2]	23.0 ± 10.62	18.5	14.2	49.1	59.8 ± 25.74	53.8	12.8	87.6	<0.001
cis-MUFA (%) [2]	42.5 ± 15.31	38.1	22.7	69.5	33.0 ± 22.43	36.7	9.6	76.8	0.121
trans-C18:1 (%) [2]	0.9 ± 0.82	0.7	0.0	4.5	0.2 ± 0.10	0.2	0.1	0.5	<0.001
n-6 PUFA (%) [2]	32.2 ± 18.05	36.8	10.2	59.2	6.7 ± 3.39	8.5	2.3	10.9	<0.001
n-3 PUFA (%) [2]	1.4 ± 1.03	1.1	0.2	5.3	0.3 ± 0.32	0.3	0.0	1.2	<0.001
UFA/SFA ratio [3]	4.0 ± 1.38	4.4	1.0	6.1	1.4 ± 2.13	0.9	0.1	6.8	<0.001
T (mg/kg)	1167.1 ± 1234.52	813.1	126.1	8464.4	113.8 ± 117.93	65.6	1.8	350.0	<0.001
T3 (mg/kg)	48.3 ± 80.21	21.8	0.0	454.7	179.9 ± 194.13	89.3	0.0	513.9	0.065
T + T3 (mg/kg)	1215.4 ± 1247.57	840.7	126.1	8514.0	293.7 ± 287.64	192.0	1.8	853.7	<0.001
PV (meq O_2/kg)	1.0 ± 1.12	0.8	0.0	6.5	1.0 ± 0.88	1.0	0.0	3.3	0.791
p-AnV	18.4 ± 9.39	16.4	6.2	54.5	36.1 ± 20.31	36.4	11.1	62.1	0.006
POL (%) [4]	2.6 ± 1.58	2.5	ND	6.8	ND	ND	ND	ND	<0.001
IT (h) [5]	31.1 ± 36.83	20.5	1.0	192.9	8.1 ± 17.26	0.7	0.3	61.9	<0.001

Abbreviations: SFA, saturated fatty acids; *cis*-MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UFA/SFA ratio, unsaturated/saturated ratio; T, sum of α-, β-, γ-, and δ-Tocopherol; T3, sum of α-, β-, γ-, and δ-Tocotrienol; T + T3, sum of tocopherols and tocotrienols; PV, peroxide value; *p*-AnV, *p*-Anisidine value; POL, polymeric compounds; IT, induction time; SD, standard deviation; ND, not detected. [1] *p* values were obtained from Mann–Whitney U-test for independent samples to compare medians between AO and FADs. $p \leq 0.05$ was considered significant. [2] Table shows the sums of fatty acids, including all the identified and quantified FA, expressed as internal area normalization in %. SFA includes: C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, and C24:0; cis-MUFA includes C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7, and C20:1 n-9; n-6 PUFA includes C18:2 n-6 and C20:2 n-6; n-3 PUFA includes C18:3 n-3; *trans*-C18:1 includes a sum of positional isomers. [3] To calculate the UFA/SFA ratio, the following relationship was considered: the sum of *cis*-MUFA, *cis*-PUFA, and *trans*-C18:1/ SFA. [4] POL was quantified by internal peak area normalization in % (in relation to the sum of the peak areas of all lipid classes POL, triacylglycerols, diacylglycerols, monoacylglycerols, and free fatty acids). In FAD, *n* = 8 for POL, as lipid classes could not be determined in the lauric FAD samples. [5] In AO, *n* = 69 for IT, as IT value could not be determined for 10 AO samples.

3.2. Sample Clustering According to Botanical Origin

A PCA was conducted for AO (*n* = 79) and FAD (*n* = 13) samples to study natural clustering of samples, detecting possible outliers, and to reveal relationships between variables (Figure 1). The cumulative variance explained by the two first components was 52.1%.

The PCA score plot revealed a tendency of AO and FAD samples to cluster separately, and they were quite scattered (Figure 1a,b). By observing the loading plot (Figure 1c), it was evidenced that, in general, the separation of AO samples agreed with a high contribution of POL, T, some *cis*-MUFA, such as C16:1 n-7, C18:1 n-9, and C20:1 n-9, some *cis*-PUFA, such as C18:2 n-6 and C18:3 n-3, and long-chain SFA, such as C24:0 and C22:0. On the other hand, FAD were more related to *p*-AnV, some short- and medium-chain SFA (such as C6:0, C8:0, C10:0, C12:0, C14:0, and C16:0), and T3.

Table 3. Spearman's correlation coefficients (Spearman's Rho) between the induction time (IT) and the main variables determined in acid oils (AO) from chemical refining and in fatty acid distillates (FAD) from physical refining.

Parameter	AO (n = 69) [1]	FAD (n = 13)
PV	NS	NS
p-AnV	NS	–0.577 *
POL	–0.375 **	NA
SFA	0.507 **	0.819 **
cis-MUFA	NS	–0.819 **
trans-C18:1	–0.264 *	NS
n-6 PUFA	NS	–0.758 **
n-3 PUFA	NS	–0.830 **
UFA/SFA ratio	–0.507 **	–0.819 **
T	NS	NS
T3	NS	NS
T + T3	NS	NS

See Table 2 for abbreviations; NA, not available, because the content of POL was null in FAD. [1] For 10 AO samples, no IT could be determined. NS, not significant coefficient ($p > 0.05$). * Statistically significant coefficient ($p \leq 0.05$). ** Statistically significant coefficient ($p \leq 0.01$).

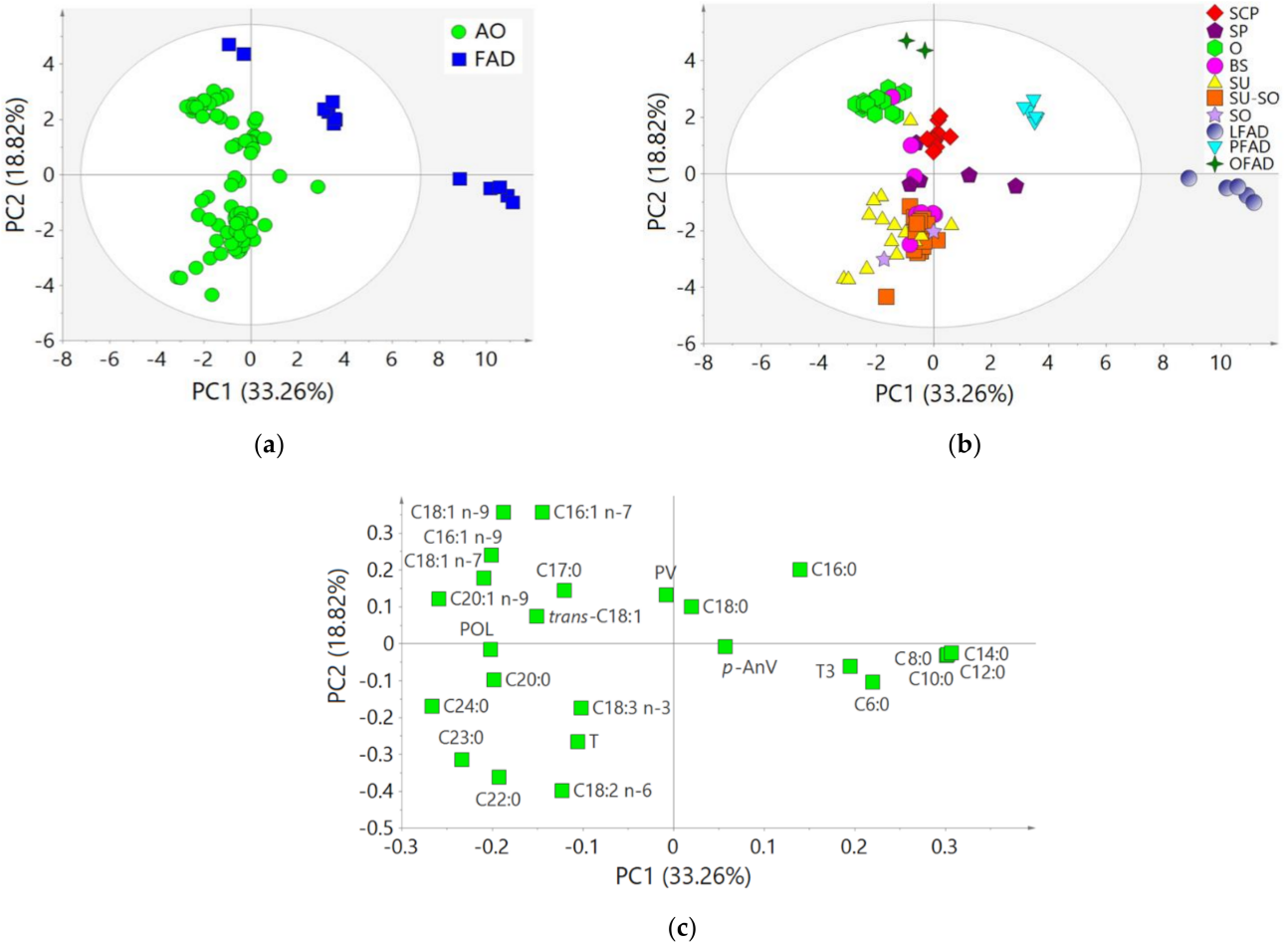


Figure 1. Principal component analysis (PCA) on the compositional and oxidation parameters (25 variables, mean-centered and scaled to unit variance) of acid oils from chemical refining (AO, n = 79) and fatty acid distillates from physical refining (FAD, n = 13). (**a**) Score plot colored according to the refining process, (**b**) score plot colored according to the botanical origin, and (**c**) loading plot. See Table 1 for abbreviations.

Furthermore, three different FAD clusters (corresponding to LFAD, PFAD, and OFAD group samples) and some AO clusters (corresponding to SCP and O group samples) were clearly distinguishable in the score plot (Figure 1). Short- and medium-chain FA and T3

contributed to the clustering of LFAD and C16:0 to that of PFAD. The two FAD samples plotted within the AO group corresponded to FAD from the refining of olive and olive pomace oils (OFAD). Indeed, the two OFAD samples laid close to the O cluster, and they all agreed with a high contribution of C18:1 n-9 and C16:1 n-7, while POL seemed to contribute more to O than to OFAD clustering (Figure 1). Other AO groups, such as SU or BS, were more scattered due to a high variability in their compositional parameters, especially due to the highly variable T (Figure 1).

Regarding the oxidation parameters, PV loadings were low, and basically no contribution to the explained variance was observed, especially for PC1. On the contrary, the *p*-AnV loadings were also relatively low but positive for PC1, contributing to the separation of the FAD group, while POL showed a high negative loading in PC1, contributing to the separation of some AO, especially of O and some SU (Figure 1).

3.3. Differences between Botanical Groups within AO and FAD

As the PCA revealed some clustering according to the botanical origin of the corresponding crude oils, we studied the differences in the compositional and oxidation parameters between the botanical groups within AO and FAD by using boxplot graphics.

3.3.1. Oxidation and Oxidative Stability Parameters

Regarding primary oxidation that was assessed by means of the PV, O presented the highest median value, while SU showed the lowest median (Figure 2a). Nevertheless, certain variability was observed in various groups, such as SCP, BS, SU, and SU-SO, where PV ranged from null to outlier or extreme values in some cases. No significant differences were observed for PV among FAD botanical groups, although LFAD tended to show lower values (Figure 2b).

Differences in *p*-AnV between AO botanical groups were also encountered, SP, BS, SU, and SU-SO being the groups with the highest median values, as well as the highest variability, while SCP showed the lowest median (Figure 2c). Outliers for *p*-AnV were found in the SP, BS, SU, and SU-SO groups. Regarding FAD, PFAD presented the highest and the most variable *p*-AnV values (Figure 2d).

With respect to POL, a high variability was observed within all the AO groups (Figure 2e). Moreover, SCP, SP, and SU-SO groups presented POL values significantly lower than O, BS, and SU groups. As commented above, no POL were detected in FAD samples.

Regarding the oxidative stability (IT) measured by Rancimat at 120 °C, significant differences were observed between botanical origin groups from both AO and FAD (Figure 3). In AO, the most stable samples were SCP and SP, but, in general, a high variability was observed, including some outliers in O, SU, and SU-SO (Figure 3a). Regarding FAD, the IT values observed for LFAD were higher and more variable than those of PFAD and OFAD (Figure 3b).

3.3.2. Fatty Acid (FA) Composition

The variations in SFA, MUFA, and PUFA between botanical groups were reported in Varona et al. [1]. Here, the detailed FA composition is reported, including n-6 and n-3 PUFA, as they are substrates of lipid oxidation (Figure 4 and Tables S1–S3 in Supplementary Materials).

Briefly, regarding FA composition within AO, the SCP group was the richest in SFA, followed by SP (which, besides, showed the highest variability), while SU showed the lowest SFA median value (Figure 4a). Considering *trans*-C18:1 % in AO, the O group showed the highest values. The highest MUFA % was observed in O samples, followed by SP, SCP, and BS, which contained palm oil in their blends. n-6 PUFA medians in SU-SO and SO groups were the highest, followed by SU and BS, with all these groups (except for SO) presenting a high variability. Considering n-3 PUFA %, the highest values were found in SU-SO, SU, and SP groups. Overall, the UFA/SFA ratio was only significantly different for SCP and SP groups, which showed the lowest ratios.

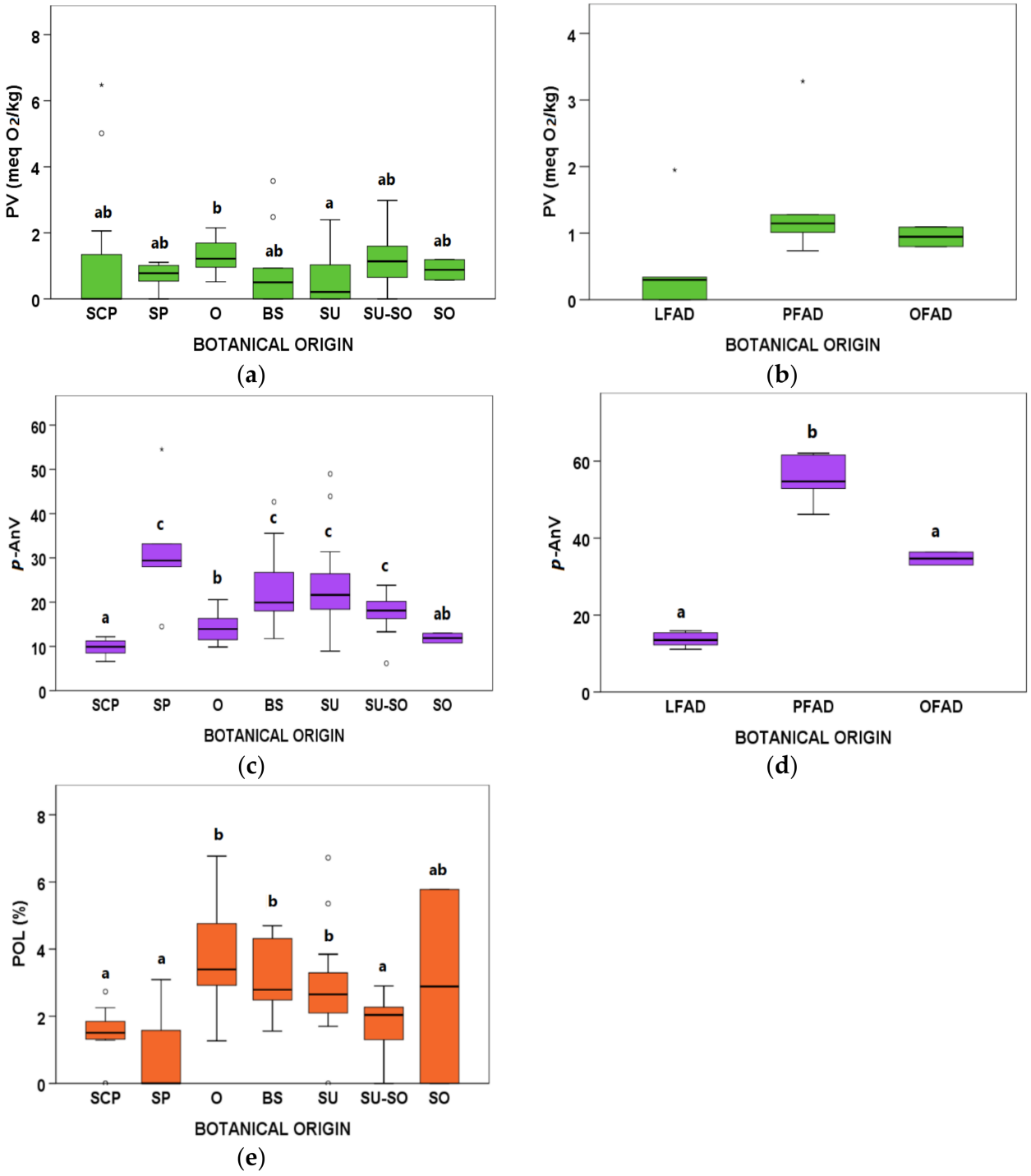


Figure 2. Peroxide value (PV), *p*-Anisidine value (*p*-AnV), and polymeric compounds (POL) boxplots according to botanical groups for (**a**,**c**,**e**) acid oils (AO) from chemical refining ($n = 79$), and (**b**,**d**) fatty acid distillates (FAD) from physical refining ($n = 13$) (see Table 1 for abbreviations of botanical groups). Within each type of byproduct (AO or FAD) and variable, botanical groups bearing different letters (a–c) show significantly different medians ($p \leq 0.05$) according to Kruskal–Wallis test and stepwise multiple comparisons for independent samples. ° Outliers: samples with values between (Q3 + 1.5 × IQR) and (Q3 + 3 × IQR) or between (Q1 − 1.5 × IQR) and (Q1 − 3 × IQR). * Extreme outliers: samples with values above (Q3 + 3 × IQR) or below (Q1 − 3 × IQR). No POL were detected in FAD.

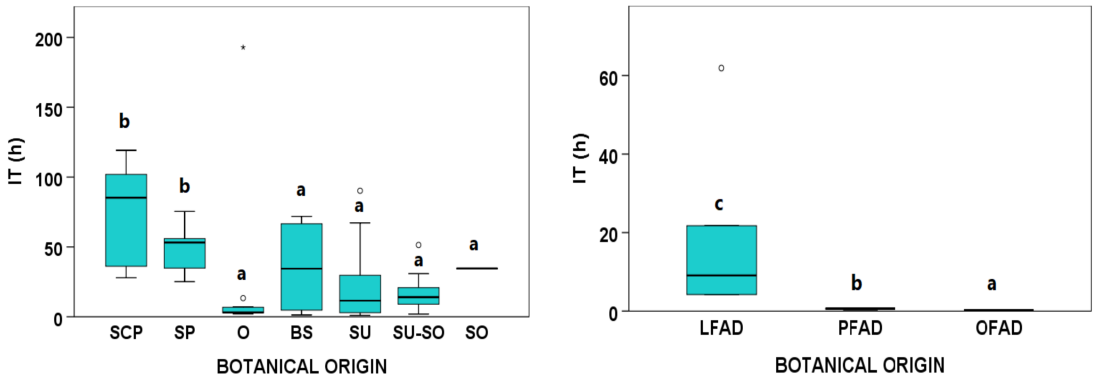


Figure 3. Boxplots for induction time (IT) measured by Rancimat at 120 °C according to botanical groups for (**a**) acid oils (AO) from chemical refining (n = 69, because in 10 AO samples the IT could not be determined) and (**b**) fatty acid distillates (FAD) from physical refining (n = 13) (see Table 1 for abbreviations of botanical groups). Within each type of byproduct (AO or FAD), botanical groups bearing different letters (a–c) show significantly different medians ($p \leq 0.05$) according to Kruskal–Wallis test and stepwise multiple comparisons for independent samples. ° Outliers: samples with values between (Q3 + 1.5 × IQR) and (Q3 + 3 × IQR) or between (Q1 − 1.5 × IQR) and (Q1 − 3 × IQR). * Extreme outliers: samples with values above (Q3 + 3 × IQR) or below (Q1 − 3 × IQR).

Within FAD, differences between FA composition were also observed but, in this case, the intragroup variabilities were lower. The lowest SFA median was found in OFAD group (Figure 4b). No differences were observed for *trans*-C18:1 medians (0.15% for LFAD, 0.21% for PFAD, and 0.22% for OFAD), while OFAD group presented the highest % of *cis*-MUFA, n-6 PUFA, and n-3 PUFA, followed by PFAD. Thus, the UFA/SFA ratio also showed the highest values in OFAD samples, followed by PFAD, and then by LFAD.

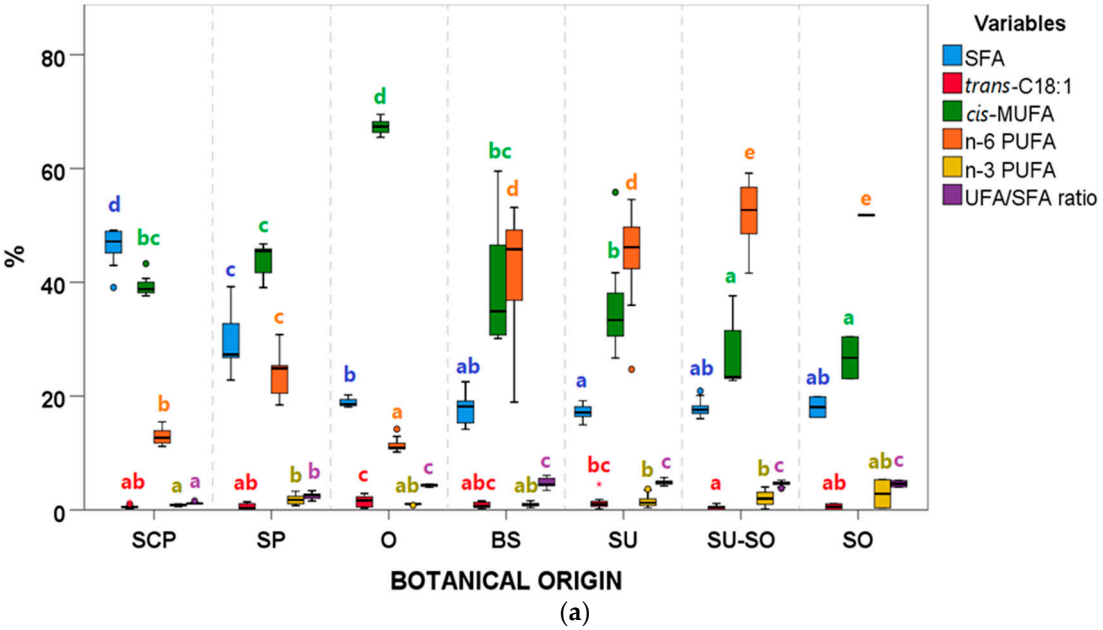
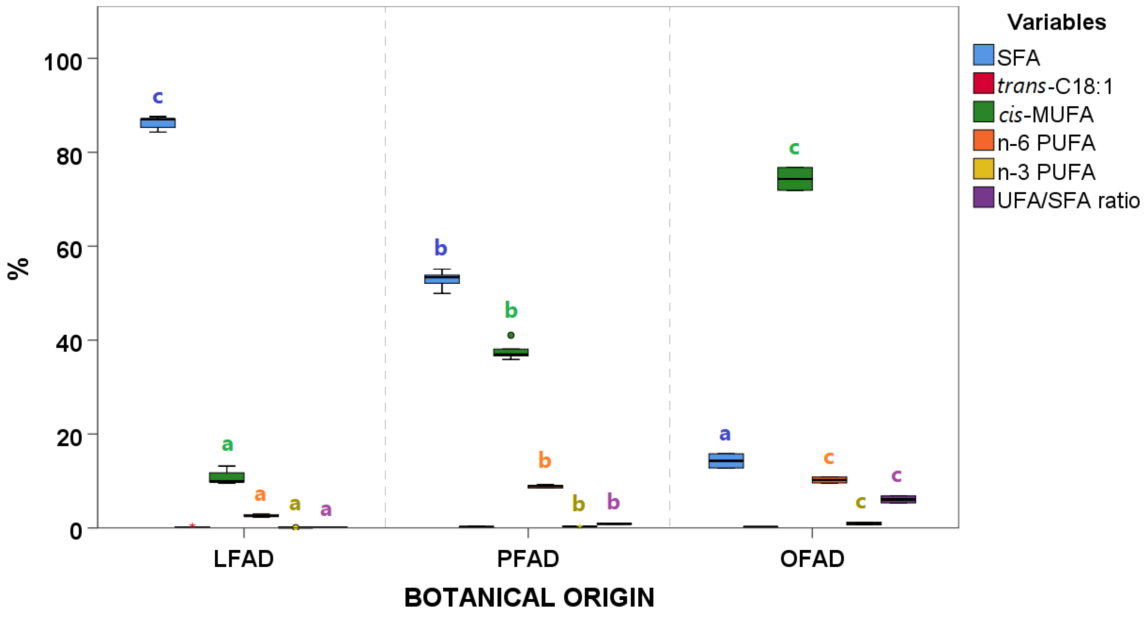


Figure 4. *Cont.*

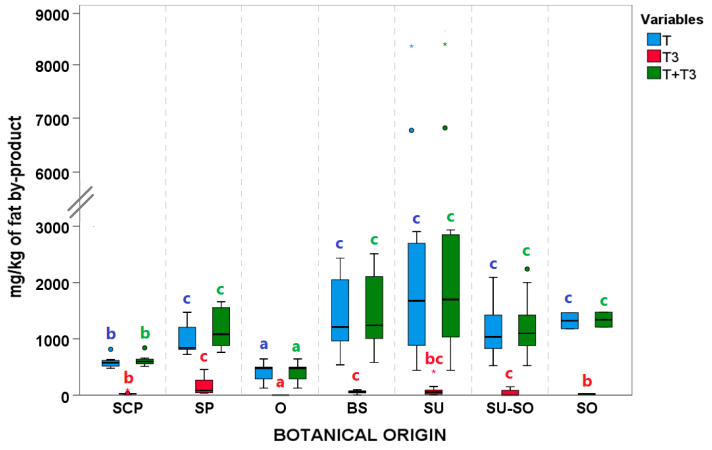


(**b**)

Figure 4. Saturated fatty acids (SFA), trans-C18:1, cis-monounsaturated fatty acids (cis-MUFA), polyunsaturated fatty acids (n-6 and n-3 PUFA), and boxplots according to botanical groups for (**a**) acid oils (AO) from chemical refining (n = 79) and (**b**) fatty acid distillates (FAD) from physical refining (n = 13) (see Table 1 for abbreviations of botanical groups). Within each type of byproduct (AO or FAD) and variable, botanical groups bearing different letters (a–e) show significantly different medians ($p \leq 0.05$) according to Kruskal–Wallis test and stepwise multiple comparisons for independent samples. Tables S2 and S3 in the Supplementary Materials report the numeric results of the FA composition. ° Outliers: samples with values between (Q3 + 1.5 × IQR) and (Q3 + 3 × IQR) or between (Q1 − 1.5 × IQR) and (Q1 − 3 × IQR).

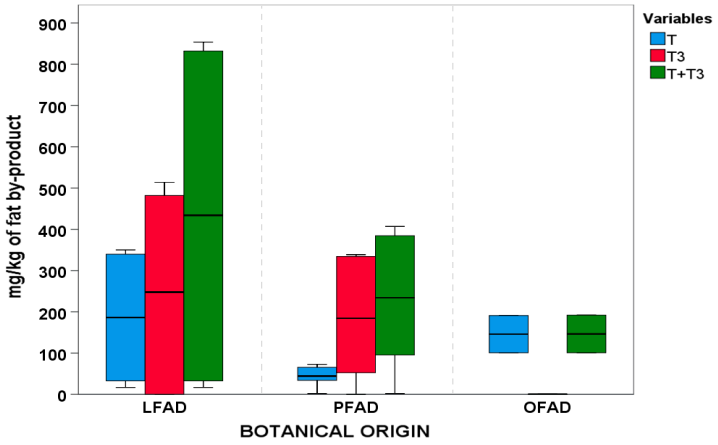
3.3.3. Tocopherol (T) and Tocotrienol (T3) Content

Considering AO, differences were observed for the content of tocols depending on the botanical origin (Figure 5a). The lowest T content was found for samples from the refining of olive and olive pomace oils, closely followed by SCP blends. They both also presented T values that varied within narrow ranges. The rest of botanical groups presented higher T values. Interestingly, two SU samples presented extreme T values (6694.9 and 8464.4 mg/kg).



(**a**)

Figure 5. *Cont.*



(**b**)

Figure 5. Tocopherols (T), tocotrienols (T3), and T + T3 contents according to botanical groups for (**a**) acid oils (AO) from chemical refining (n = 79) and (**b**) fatty acid distillates (FAD) from physical refining (n = 13) (see Table 1 for abbreviations of botanical groups). Within each type of byproduct (AO or FAD) and variable, botanical groups bearing different letters (a–c) show significantly different medians ($p \leq 0.05$) according to Kruskal–Wallis test and stepwise multiple comparisons for independent samples. ° Outliers: samples with values between (Q3 + 1.5 × IQR) and (Q3 + 3 × IQR) or between (Q1 − 1.5 × IQR) and (Q1 − 3 × IQR). * Extreme outliers: samples with values above (Q3 + 3 × IQR) or below (Q1 − 3 × IQR).

About the global sum of T + T3 in AO, it showed a similar profile to T, as the sum of tocopherols (T) was the main contributor to the sum of T + T3 (Figure 5a). Regarding FAD, no significant differences between medians were observed between botanical origins for T, nor for T3 (Figure 5b).

4. Discussion

The results of this study revealed a high variability in the content of oxidation compounds and in the oxidative stability of AO and FAD, even within samples from similar origins. This fact is in addition to the high variability also encountered for their energy value [1], reflecting a lack of standardization of these feed fat ingredients.

Overall, primary oxidation (assessed by means of the PV) was low in all samples, and no relationship with the refining process or botanical origin could be clearly defined. Regarding secondary oxidation, *p*-AnV and POL were clearly influenced by the type of refining, rather than by the botanical origin of the crude oil, the aldehydes content being higher in FAD and the POL content higher in AO. On the contrary, T and T3 contents and FA composition were more related to the botanical origin of the crude oils.

4.1. Influence of the Refining Process (AO vs. FAD)

First, regarding primary oxidation, no differences were encountered between AO and FAD groups, and neither of them presented samples with high PV, which agreed with those results previously found by Nuchi et al. [19] for similar byproducts. Moreover, Kuntom et al. [42] found PV values in PFAD below 5 meq O_2/kg in most samples, except for two outliers with PV above 10 meq O_2/kg. As a primary oxidation parameter, PV measures lipid hydroperoxides, which are unstable at high temperatures. In the case of chemical refining, temperatures reached during the neutralization step, centrifugation, and acidification to obtain AO usually rise to 80–90 °C, while, during the deodorization step in physical refining, higher temperatures (180–270 °C) and low pressure (0.05–0.8 kPa) are applied to remove FFA by distillation [21,43]. Therefore, it can be expected that AO and FAD samples that are recently produced might show PV close to 0 due to peroxide decomposition at the temperatures reached during their obtention. Once AO and FAD are

obtained, PV will start to increase during storage, the rate and magnitude of the increase being dependent on the oxidizability of the sample and storage conditions.

However, even if, in these AO and FAD, the PV values were low, the oxidation degree was high in some of them due to the presence of secondary oxidation compounds assessed by both *p*-AnV and POL. Secondary oxidation compounds in these byproducts might originate by peroxide decomposition during sample storage, but they can also come from the initial crude oils (to a greater or lesser extent, depending on their degree of oxidation) or be formed during the heat treatments applied during the refining of the crude oils and the obtaining of the AO and FAD. Regarding *p*-AnV, wide ranges were found in both AO and FAD, as also reported by Nuchi et al. [19]. The *p*-AnV is a parameter that is particularly sensible to 2-alkenals and 2,4-alcadienals, which are easily formed from the breakdown of peroxides from PUFA, such as linolenic and linoleic acids. Although these PUFA were mainly present in AO, the *p*-AnV values in FAD samples were surprisingly higher. This was found also in OFAD compared to O, even though both were obtained from olive oils, but, as only two OFAD samples were included, this comparison is somehow tentative. The highest *p*-AnV found in FAD agreed with the higher temperatures usually applied in the physical refining process [27,43]. Moreover, since FAD are obtained by distillation, during this process, the secondary oxidation aldehydes will also be distilled and accumulated in FAD together with other distillable compounds, such as FFA and T [44–46]. On the contrary, high molecular weight compounds, such as POL, might not be distilled at all, or at very low amounts, explaining the null POL contents found in the FAD samples. However, in AO, POL might be washed out from the oil and accumulated in AO after the separation of the soap stocks in the neutralization step. The higher POL content in AO compared to FAD was also reflected when the two OFAD samples were compared with the O samples. POL mainly consist of triacylglycerol polymers created by the reaction of triacylglycerol radicals and accumulated in the advanced stages of oxidation [2]. In general, the formation of POL is higher in oils with higher PUFA contents, because they are more prone to form radicals [47]. In fact, SCP and SP, as the more saturated groups in AO, presented POL values that were significantly lower than the rest, whereas, remarkedly, SU-SO also showed significant lower values, despite this group being more unsaturated.

4.2. Influence of the Botanical Origin of the Crude Oil

The FA and tocol composition of crude oils influence the FA composition and T + T3 contents of these refining byproducts. According to Varona et al. [1], the significant differences found for FA and tocols when AO and FAD were compared could be explained by the fact that the type of refining (chemical or physical) is usually selected according to the composition of the crude oil, which depends on its botanical origin. As more drastic conditions are usually applied in physical refining, it is preferred for more saturated crude oils, such as coconut, palm kernel, and palm oils, obtaining FAD with FA and tocol profiles typical of these fats. On the contrary, chemical refining is usually preferred for crude oils rich in unsaturated FA, which are more prone to oxidation [44–46]. Therefore, the FA composition of AO is usually richer in PUFA, such as C18:2 n-6 and C18:3 n-3, and in T, reflecting the FA and T profile of the unsaturated crude oils (soybean, sunflower, grapeseed, corn oils, etc.) [48]. This influence of the botanical origin of the crude oil was also evident even within different types of AO and FAD, as reported in Varona et al. [1]: the SU-SO and SO groups, followed by SU and BS, were the richest in n-6 PUFA (mainly represented by C18:2 n-6) and in T, as they come from seed oils; the O and OFAD groups, followed by the groups containing palm oil byproducts (SCP, SP, PFAD), were the richest in C18:1 n-9, as they come from olive or palm oils; PFAD were the richest FAD in palmitic acid and in T3, as they are byproducts from palm oil refining; SCP blends were the richest AO in palmitic and stearic acids, as SCP included AO from the refining of cocoa butter (which is rich in C16:0 and C18:0); and LFAD showed a high content of medium-chain SFA, which are typical FA in coconut and palm kernel oils [23,48] (see also Table S1, Supplementary Material). The presence of corn and palm AO in SP and SCP blends was also reflected in

their γ-T3 content. In addition, as discussed in Varona et al. [1], the two samples in the SU group with extremely high T contents could agree with the addition of tocol-rich products, such as deodistillates obtained from the deodorization step in chemical refining.

Thus, the botanical origin determines the final FA and T composition of AO and FAD, which could have a role in the oxidative stability (IT) of these byproducts. However, even though, globally, AO presented higher IT medians than FAD, this depended on the botanical group, as different IT medians were observed between various botanical groups. Knowing that FA oxidizability mainly depends on their degree of unsaturation, in order to relate the IT with the FA composition, the UFA/SFA ratio was calculated, including all unsaturated FA in UFA and all saturated FA in SFA (regardless of the configuration of the double bond). Thus, this calculation is slightly different from the U/S ratio usually used to predict the energy value of fats and that was reported in Varona et al. [1], in which SFA with 12 carbons or below were considered as unsaturated fatty acids (thus, in the numerator –U- of that ratio), and *trans*-FA were considered as SFA (thus, in the denominator –S- of the ratio). The botanical group with the lowest UFA/SFA median was SCP, which also led to the highest IT median, even if it was the group with the lower T content. Then, it was followed by SP group and by AO groups, such as SU and SU-SO, that presented higher UFA/SFA. This resulted in a negative correlation between IT and UFA/SFA for AO. The FA composition influence could also explain the negative correlation between IT and POL in AO, as it agreed with a concomitant negative correlation between POL and SFA; since SFA are less prone to oxidation, they might have not only led to higher IT, but also to lower POL formation.

Nevertheless, some of the AO groups presented longer IT than the FAD groups, even if AO were richer in n-6 and n-3 PUFA that are more prone to lipid oxidation reactions. This could be explained by the higher T content in AO that would be acting as an antioxidant, delaying the onset of oxidation of these AO. Moreover, the addition of synthetic antioxidants to some AO could not be excluded and would explain some of the extremely high IT values found for certain AO. In fact, the sample suppliers reported the addition of synthetic antioxidants in 16 AO samples, while they did not do so in any of the FAD samples.

The T and T3 content of these byproducts was highly variable, even within botanical groups [1]. Interestingly, two SU samples deserved special attention as they were revealed as outliers in the boxplot. Apart from the very high T amounts (8464.39 and 6694.94 mg/kg) that they presented, they also had very high *p*-AnV (49.0 and 43.9, respectively). Actually, these *p*-AnV were more in the range of *p*-AnV found for FAD samples than those found for AO, which supports the hypothesis of the possible addition of deodistillates from the deodorization step to these AO [1]. In AO samples, no significant correlation ($p = 0.326$) between T content (major tocols) and IT was observed, which could be related to the addition of synthetic antioxidants in some samples. In contrast, in FAD samples, where the suppliers did not report antioxidant addition, an almost significant correlation ($p = 0.071$) was observed between T3 content (major tocols) and IT.

4.3. Comparison with Fat Quality Thresholds in EU Regulations and Guidelines

EU regulations regarding feed ingredients do not include any parameter to evaluate the oxidation status of feed fats, nor to assess primary or secondary oxidation [20]. Some animal nutrition associations include PV assessment in their guidelines for fat sources used in feeds, setting its threshold at 10 meq O_2/kg for various AO and FAD [36]. In the present study, no AO or FAD samples were above this limit. However, since peroxides can decompose into secondary oxidation compounds or radicals can form POL [2,49], the measurement of PV is totally insufficient as a global evaluation of fat oxidation and should be evaluated together with a secondary oxidation parameter [2,19,38]. However, the evaluation of secondary oxidation is not usually included in the quality guidelines [36], nor routinely performed by the producers.

The type of secondary oxidation products formed depends on the FA composition of the fat, as well as on the oxidation conditions (O_2 availability, temperature, light exposure).

Thus, the parameter to evaluate the secondary oxidation in a feed fat should be accordingly selected to obtain a realistic evaluation of the oxidation status. In this study, we have chosen the *p*-AnV, as it is especially adequate to evaluate 2-alkenals and 2,4-alcadienals that are characteristic breakdown products of lipid hydroperoxides formed from C18:2 n-6, C18:3 n-3, and more unsaturated FA, and at a lesser extent, from C18:1 n-9 [49,50]. Therefore, it is a parameter suitable for most vegetable oils and for some animal fats, and has been successfully applied to feed fat ingredients [19,38]. In other studies, TBA value has been selected instead of *p*-AnV [51]. However, TBA value mainly reacts with malondialdehyde, which is especially formed from PUFA with more than two double bonds, and not from MUFA [4,38]. According to this, Nuchi et al. [19] reported a good application of TBA value for fish oils, with it not being sensitive enough to evaluate secondary oxidation of the rest of feed fat byproducts, for which *p*-AnV was preferred. Moreover, considering that *p*-AnV is a simple and rapid analytical determination, it could be a suitable parameter to be included in feed quality control guidelines for vegetable fats and oils. The values obtained in this study were very similar to those reported by Nuchi et al. [19] for AO and FAD. However, unfortunately, since it is not currently included in the guidelines, the results of this study cannot be compared with an established threshold. In fact, comparing the absolute values of this index to draw conclusions about the degree of oxidation of different fats would only make sense when fats have a similar FA composition, since the formation of aldehydes depends on that.

Regarding POL, it is neither included in feed fat regulations or guidelines. Even its determination is not as simple and economic as that of *p*-AnV; it has been suggested as a complementary control parameter for feed fats [19]. It would provide additional information to that of *p*-AnV or TBA, and, moreover, as it has been commented above, POL have been related with digestibility effects. However, as FAD have shown not to accumulate POL, its determination would not be a priority for this type of fat byproduct. However, POL content has been suggested to control the feed fats submitted for long heating periods to high temperatures [19,38], such as recycled cooking oils (if permitted in feeds). In AO, the POL content correlated positively with the UFA/SFA ratio ($p = 0.016$) and negatively with the IT ($p = 0.002$). Thus, this parameter could also be interesting for the quality control of AO.

Overall, considering the repercussions described above for the occurrence of oxidation in feed fats, the evaluation of fat oxidation should be included in a global evaluation of feed fat quality, including proper primary and secondary parameters. Unfortunately, currently this is not the case in regulations, feed fat quality guidelines, or tables on the chemical composition and nutritional value of feedstuff [35,36]. As a consequence, some producers usually do not report these values in the products' technical sheets, or they only report PV, lacking a complete information of the oxidative status of the fat. The high variability in the oxidation status observed in this study for AO and FAD, sometimes even within batches from the same producer, supports the inclusion of these parameters in the routine characterization of the products. Their values might depend on the composition of the crude oil, the refining and byproduct obtention processes, and storage time and conditions. Any efforts towards the reduction in the global oxidation, or at least towards its standardization, would contribute to increasing the farmers' confidence in them, as was already recommended for the parameters determining the energy value of the fats [1]. In this respect, various actions could be considered, ranging from the standardization of the byproduct collection in the waste tank by avoiding mixing AO or FAD from various sources to the optimization of the distillation step in physical refining by adjusting the time, temperature, and stripping agent, or the post-treatment of AO and FAD to standardize their composition or reduce their oxidation compounds.

5. Conclusions

AO and FAD from the refining of edible oils and fats provide energy, liposoluble vitamins, and essential nutrients to an animal diet. Results from this study have shown

that the oxidative status and oxidative stability of these byproducts can be highly variable, even between samples from similar botanical origins. Since oxidation aldehydes are easily distillable in the deodorization step, FAD presented higher *p*-AnV, while AO presented higher POL. Primary oxidation was low, both in AO and FAD, due to the instability of peroxides at the high temperatures usually reached during the obtention of these byproducts. Increases in PV would be the result of the storage conditions over time. Moreover, wide ranges for some of the studied oxidation parameters were observed even when the AO or the FAD had been obtained from the same type of crude oils. The botanical origin of the crude oil also affected the FA and the tocol contents. All of these variations led to highly variable oxidative stability for all types of samples. Therefore, the inclusion of adequate primary and secondary oxidation parameters in the quality control of these byproducts is necessary to guarantee a constant composition and stability and, thus, proper quality. This could contribute to farmers obtaining more consistent production results when animals are fed these fats, which is essential to upcycle and to valorize these byproducts.

Supplementary Materials: The following are available online https://www.mdpi.com/article/10.3390/ani11092559/s1, Table S1: Median values for the individual fatty acids according to the type of by-product and the botanical origin; Table S2: Mean, standard deviation, median, minimum and maximum values for the different sums of fatty acids identified and quantified by peak area normalization (%) according to the botanical origin of the acid oils (AO); Table S3: Mean, standard deviation, median, minimum and maximum values for the different sums of fatty acids identified and quantified by peak area normalization (%) according to the botanical origin of the fatty acid distillates (FAD).

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References

1. Varona, E.; Tres, A.; Rafecas, M.; Vichi, S.; Barroeta, A.C.; Guardiola, F. Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding. *Animals* **2021**, *11*, 196. [CrossRef] [PubMed]
2. Velasco, J.; Dobarganes, C. Oxidative stability of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 661–676. [CrossRef]
3. Fang, B.; Zhang, M.; Shen, Y.M. Importance of the higher retention of tocopherols and sterols for the oxidative stability of soybean and rapeseed oils. *J. Food Sci. Technol.* **2017**, *54*, 1938–1944. [CrossRef]
4. Esterbauer, H. Cytotoxicity and genotoxicity of lipid-oxidation products. *Am. J. Clin. Nutr.* **1993**, *57*, 779S–786S. [CrossRef] [PubMed]
5. Guardiola, F.; Dutta, P.C.; Codony, R.; Savage, G.P. *Cholesterol and Phytosterol Oxidation Products: Analysis, Occurrence, and Biological Effects*; AOCS Press: Champaign, IL, USA, 2002.
6. Kanner, J. Dietary advanced lipid oxidation endproducts are risk factors to human health. *Mol. Nutr. Food Res.* **2007**, *51*, 1094–1101. [CrossRef]
7. Márquez-Ruiz, G.; García-Martínez, M.C.; Holgado, F. Changes and effects of dietary oxidized lipids in the gastrointestinal tract. *Lipid Insights* **2008**, *2*, 11–19. [CrossRef]

8. Olivero David, R.; González-Muñoz, M.J.; Benedí, J.; Bastida, S.; Sánchez-Muniz, F.J. Thermally oxidized palm olein exposure increases triglyceride polymer levels in rat small intestine. *Eur. Food Res. Technol.* **2010**, *112*, 970–976. [CrossRef]
9. Lindblom, S.C.; Gabler, N.K.; Bobeck, E.A.; Kerr, B.J. Oil Source and peroxidation status interactively affect growth performance and oxidative status in broilers from 4 to 25 d of age. *Poult. Sci.* **2019**, *98*, 1749–1761. [CrossRef]
10. Liu, P.; Chen, C.; Kerr, B.J.; Weber, T.E.; Johnston, L.J.; Shurson, G.C. Influence of thermally oxidized vegetable oils and animal fats on energy and nutrient digestibility in young pigs. *J. Anim. Sci.* **2014**, *92*, 2980–2986. [CrossRef]
11. Zhang, Q.; Saleh, A.S.M.; Chen, J.; Shen, Q. Chemical alterations taken place during deep-fat frying based on certain reaction products: A review. *Chem. Phys. Lipids* **2012**, *165*, 662–681. [CrossRef]
12. Hung, Y.T.; Hanson, A.R.; Shurson, G.C.; Urriola, P.E. Peroxidized lipids reduce growth performance of poultry and swine: A meta-analysis. *Anim. Feed Sci. Technol.* **2017**, *231*, 47–58. [CrossRef]
13. Bou, R.; Guardiola, F.; Tres, A.; Barroeta, A.C.; Codony, R. Effect of dietary fish oil, α-tocopheryl acetate, and zinc supplementation on the composition and consumer acceptability of chicken meat. *Poult. Sci.* **2004**, *83*, 282–292. [CrossRef] [PubMed]
14. Jensen, C.; Engberg, R.; Jakobsen, K.; Skibsted, L.H.; Bertelsen, G. Influence of the oxidative quality of dietary oil on broiler meat storage stability. *Meat Sci.* **1997**, *47*, 211–222. [CrossRef]
15. Sheehy, P.J.A.; Morrissey, P.A.; Flynn, A. Consumption of thermally-oxidized sunflower oil by chicks reduces α-tocopherol status and increases susceptibility of tissues to lipid oxidation. *Br. J. Nutr.* **1994**, *71*, 53–65. [CrossRef]
16. Tres, A.; Bou, R.; Codony, R.; Guardiola, F. Moderately oxidized oils and dietary zinc and α-tocopheryl acetate supplementation: Effects on the oxidative stability of rabbit plasma, liver, and meat. *J. Agric. Food. Chem.* **2010**, *58*, 9112–9119. [CrossRef] [PubMed]
17. Silva-Guillen, Y.V.; Arellano, C.; Boyd, R.D.; Martinez, G.; Van Heugten, E. Growth performance, oxidative stress and immune status of newly weaned pigs fed peroxidized lipids with or without supplemental vitamin E or polyphenols. *J. Anim. Sci. Biotechnol.* **2020**, *11*, 22. [CrossRef]
18. Billek, G. Health aspects of thermoxidized oils and fats. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 587–593. [CrossRef]
19. Nuchi, C.; Guardiola, F.; Bou, R.; Bondioli, P.; Bella, L.D.; Codony, R. Assessment of the levels of degradation in fat co-and byproducts for feed uses and their relationships with some lipid composition parameters. *J. Agric. Food Chem.* **2009**, *57*, 1952–1959. [CrossRef]
20. Commission Regulation (EU) 68/2013 of 16 January 2013 and its amendments on the Catalogue of feed materials. *Off. J. Eur. Union* **2013**, *29*, 1–64.
21. Bockisch, M. *Fats and Oils Handbook*, 1st ed.; Academic Press and AOCS Press: Champaign, IL, USA, 1998.
22. FEDNA (Fundación Española para el Desarrollo de la Nutrición Animal). *Tablas FEDNA de Composición y Valor Nutritivo de Alimentos para la Fabricación de Piensos Compuestos*, 4th ed.; Blas, C., Mateos, G.G., García-Rebollar, P., Eds.; FEDNA: Madrid, Spain, 2019; Available online: http://www.fundacionfedna.org/ingredientes-para-piensos (accessed on 20 March 2021).
23. Gunstone, F.D. *Vegetable Oils in Food Technology: Composition, Properties and Uses*, 2nd ed.; Blackwell Publishing Ltd.: Oxford, UK, 2011.
24. Pawar, D.M.; Marathe, A.B. Critical review on value added products from cracking of waste vegetable oil. *Int. J. Sci. Eng. Res.* **2015**, *6*, 1063–1068.
25. Chang, A.S.; Sherazi, S.T.H.; Kandhro, A.A.; Mahesar, S.A.; Chang, F.; Shah, S.N.; Laghari, Z.H.; Panhwar, T. Characterization of palm fatty acid distillate of different oil processing industries of Pakistan. *J. Oleo Sci.* **2016**, *65*, 897–901. [CrossRef]
26. Bondioli, P. Refining by-products as a source of compounds of high-added value. *Grasas y Aceites* **2006**, *57*, 116–125. [CrossRef]
27. Gibon, V.; De Greyt, W.; Kellens, M. Palm oil refining. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 315–335. [CrossRef]
28. Dumont, M.J.; Narine, S.S. Soapstock and deodorizer distillates from north american vegetable oils: Review on their characterization, extraction and utilization. *Food Res. Int.* **2007**, *40*, 957–974. [CrossRef]
29. Wiseman, J.; Salvador, F.; Craigon, J. Prediction of the apparent metabolizable energy content of fats fed to broiler chickens. *Poult. Sci.* **1991**, *70*, 1527–1533. [CrossRef] [PubMed]
30. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. II. Effect of free fatty acid content and degree of saturation of free fatty acids and neutral fat on fatty acid digestibility. *Br. Poult. Sci.* **1996**, *37*, 119–130. [CrossRef]
31. Rodriguez-Sanchez, R.; Tres, A.; Sala, R.; Garcés-Narro, C.; Guardiola, F.; Gasa, J.; Barroeta, A.C. Effects of dietary free fatty-acid content and saturation degree on lipid-class composition and fatty-acid digestibility along the gastrointestinal tract in broiler starter chickens. *Poult. Sci.* **2019**, *98*, 4929–4941. [CrossRef]
32. Jimenez-Moya, B.; Barroeta, A.C.; Tres, A.; Soler, M.D.; Sala, R. Soybean oil replacement by palm fatty acid distillate in broiler chicken diets: Fat digestibility and lipid-class content along the intestinal tract. *Animals* **2021**, *11*, 1035. [CrossRef]
33. Oey, S.B.; Van der Fels-Klerx, H.J.; Fogliano, V.; Van Leeuwen, S.P.J. Mitigation strategies for the reduction of 2- and 3-MCPD esters and glycidyl esters in the vegetable oil processing industry. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18*, 349–361. [CrossRef]
34. Xie, D.; Zhou, H.; Jiang, X. Effect of chemical refining on the levels of bioactive components and hazardous substances in soybean oil. *J. Food Meas. Charact.* **2019**, *13*, 1423–1430. [CrossRef]
35. Rostagno, H.S.; Albino, L.F.T.; Hannas, M.I.; Donzele, J.L.; Sakomura, N.K.; Perazzo, F.G.; Saraiva, A.; de Abreu, M.L.T.; Rodrigues, P.B.; de Oliveira, R.F.; et al. *Tablas Brasileñas para Aves y Cerdos: Composición de Alimentos y Requerimientos Nutricionales*, 4th ed.; Rostagno, H.S., Ed.; Universidad Federal de Viçosa: Viçosa, Brasil, 2017; ISBN 978-85-8179-122-7.

36. FEDNA (Fundación Española para el Desarrollo de la Nutrición Animal). Normas FEDNA de Control de Calidad de Ingredientes para Piensos-Especificación Técnica de Materia Prima, Grasas y Aceites. 2002. Available online: http://www.fundacionfedna.org/sites/default/files/8_Grasas_y_aceites.pdf (accessed on 15 March 2021).
37. Tres, A.; Magrinyà, N.; Bou, R.; Guardiola, F.; Nuchi, C.D.; Codony, R. Impact of the oxidative quality of fish oils in feeds on the composition and oxidative stability of chicken and rabbit meat. *Anim. Feed Sci. Technol.* **2014**, *196*, 76–87. [CrossRef]
38. Winkler–Moser, J.K.; Hwang, H.S.; Kerr, B.J. Changes in markers of lipid oxidation and thermal treatment in feed-grade fats and oils. *J. Sci. Food Agric.* **2020**, *100*, 3328–3340. [CrossRef]
39. Shurson, G.C.; Kerr, B.J.; Hanson, A.R. Evaluating the quality of feed fats and oils and their effects on pig growth performance. *J. Anim. Sci. Biotechnol.* **2015**, *6*, 10. [CrossRef]
40. Varona, E.; Tres, A.; Rafecas, M.; Vichi, S.; Barroeta, A.C.; Guardiola, F. Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding. *MethodsX* **2021**, *8*, 101334. [CrossRef]
41. Schober, P.; Boer, C.; Schwarte, L.A. Correlation coefficients: Appropriate use and interpretation. *Anesth. Analg.* **2018**, *126*, 1763–1768. [CrossRef]
42. Kuntom, A.; Siew, W.L.; Tan, Y.A. Characterization of palm acid oil. *J. Am. Oil Chem. Soc.* **1994**, *71*, 525–528. [CrossRef]
43. FEDIOL—The European Vegetable Oil and Protein Meal Industry Association. Refining of Edible Oils. Available online: https://www.fediol.eu/web/refining/1011306087/list1187970096/f1.html (accessed on 14 April 2021).
44. Ghazani, S.M.; Marangoni, A.G. Minor components in canola oil and effects of refining on these constituents: A review. *J. Am. Oil Chem. Soc.* **2013**, *90*, 923–932. [CrossRef]
45. Vaisali, C.; Charanyaa, S.; Belur, P.D.; Regupathi, I. Refining of edible oils: A critical appraisal of current and potential technologies. *Int. J. Food Sci. Technol.* **2015**, *50*, 13–23. [CrossRef]
46. O'Brien, R.D. Fats and oils processing. In *Fats and Oils: Formulating and Processing for Applications*, 2nd ed.; CRC Press LLC: Boca Raton, FL, USA, 2004.
47. Gertz, C.; Aladedunye, F.; Matthäus, B. Oxidation and structural decomposition of fats and oils at elevated temperatures. *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 1457–1466. [CrossRef]
48. Codex Alimentarius. Standard for named vegetable oils (CXS 210-1999). In *Codex Alimentarius, International Food Standards*; Codex Alimentarius: Rome, Italy, 1999; Adopted in 1999; Revised in 2001, 2003, 2009, 2017, 2019; Amended in 2005, 2011, 2013, 2015, 2019.
49. Choe, E.; Min, D.B. Comprehensive reviews in food science and food safety mechanisms and factors for edible oil oxidation. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 169–186. [CrossRef]
50. Snyder, J.M.; Frankel, E.N.; Selke, E. Capillary gas chromatographic analyses of headspace volatiles from vegetable oils. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1675–1679. [CrossRef]
51. Wealleans, A.L.; Bierinckx, K.; Witters, E.; Benedetto, M.; Wiseman, J. Assessment of the quality, oxidative status and dietary energy value of lipids used in non-ruminant animal nutrition. *J. Sci. Food Agric.* **2021**, *101*, 4266–4277. [CrossRef] [PubMed]

5.1.4. Effect of processing and storage conditions on lipid hydrolysis and oxidative stability of feeds containing various fats and fat byproducts /Efecto de las condiciones de procesado y almacenamiento sobre la hidrólisis lipídica y la estabilidad oxidativa de piensos formulados con diferentes grasas y subproductos grasos

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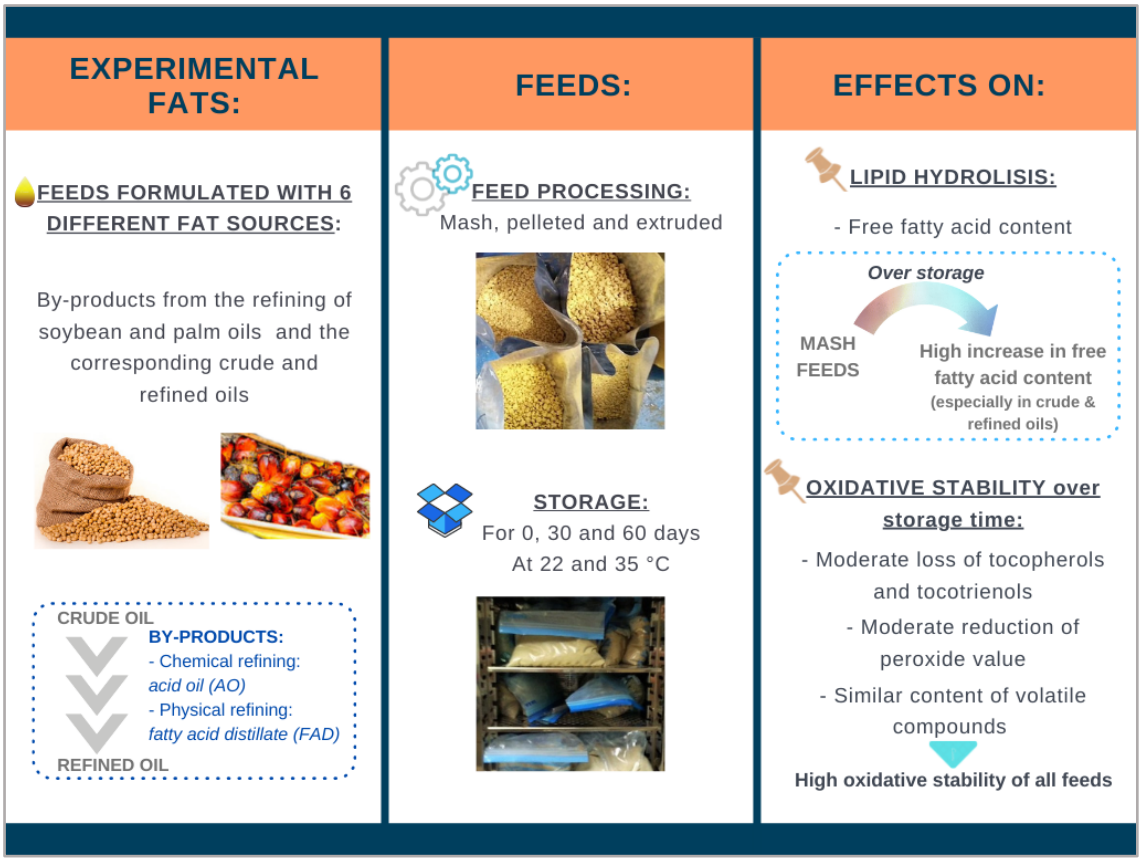

Se estudió el efecto de las condiciones de procesamiento y almacenamiento sobre la hidrólisis de lípidos y la estabilidad oxidativa de piensos que contienen diversas grasas y subproductos de grasas. Para ello, se agregaron seis fuentes de grasas (6%) a la misma dieta basal en forma de harina: aceites de soja y palma crudos y refinados, y dos subproductos ricos en ácidos grasos libres (AGL), específicamente el aceite ácido obtenido durante la refinación química del aceite de soja (AS) y el destilado de ácidos grasos de palma (PFAD) obtenido durante la refinación física del aceite de palma. Así, se obtuvieron 6 piensos elaborados con diferentes fuentes de grasa en forma de harina. De estos piensos, una parte se mantuvo como harina, otra se granuló y otra se extruyó. Los 18 piensos obtenidos se almacenaron durante 0, 30 y 60 días a 22 y 35 °C.

Se observó que los AGL aumentaron muy rápidamente en los piensos en forma de harina, pero no en los piensos granulados y extrusionados. Los tratamientos térmicos de la granulación y la extrusión podrían inactivar las lipasas endógenas y prevenir la hidrólisis de triacilglicerol. El aumento de AGL en harinas durante el almacenamiento fue más pronunciado en los piensos que contenían aceites crudos y refinados que en los que contenían los subproductos (AS y PFAD). Los subproductos ya son ricos en AGL (> 61%) y pobres en triacilgliceroles (<26%), los sustratos de las lipasas. Como era de esperar, los tratamientos térmicos que tuvieron lugar durante la granulación y extrusión de los piensos produjeron una disminución de los tocoferoles y tocotrienoles y un aumento en el índice de peróxidos de los piensos recién preparados, pero la estabilidad oxidativa de los piensos granulados y extrusionados durante el almacenamiento no disminuyó en comparación con las harinas. Así, en estas condiciones de almacenamiento, la formulación de piensos con subproductos ricos en AGL no planteó un problema relevante de hidrólisis de lípidos o estabilidad oxidativa, mientras que la formulación con aceites crudos podría resultar en un contenido de AGL más alto de lo esperado en piensos en harina, lo que podría implicar un deterioro en la digestibilidad y absorción de los ácidos grasos en animales jóvenes.

Effect of processing and storage conditions on lipid hydrolysis and oxidative stability of feeds containing various fats and fat by-products

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ABSTRACT

The effect of processing and storage conditions on lipid hydrolysis and oxidative stability of feeds containing various fats and fat by-products was studied. For this purpose, six fat sources were added (6%) to the same basal diet in mash form. The fat sources assayed were crude and refined soybean and palm oils, and two by-products rich in free fatty acids (FFA), specifically the acid oil obtained during the chemical refining of soybean oil (AS) and the palm fatty acid distillate (PFAD) obtained during the physical refining of palm oil. Thus, 6 feeds with different fat sources were obtained in mash form. From these feeds, a part was kept as mash, another was pelleted, and another was extruded. The resulting 18 feeds were stored for 0, 30 and 60 days at 22 and 35 °C.

Regarding the lipid hydrolysis, it was observed that FFA increased very quickly in mash feeds, but not in pelleted and extruded feeds. Thus, heat treatments involved in pelleting and extrusion could inactivate endogenous lipases and prevent triacylglycerol hydrolysis. The increase of FFA in mash feeds during storage was more pronounced in feeds containing crude and refined oils than in feeds containing the by-products (AS and PFAD). This is because these by-products are already rich in FFA (> 61%) and poor in triacylglycerols (< 26%), the substrates of lipases. Thus, in mash feeds containing crude and refined oils, FFA % increased from 11-14% up to 40-47% during the storage for 60 days. As expected, the heat treatments that took place during the pelleting and extrusion of feeds produced a decrease in tocols and an increase in the peroxide value of freshly prepared feeds, but

Abbreviations: ALC, alcohols; ALD, aldehydes; AO, acid oil; AS, soybean acid oil; CS, crude soybean oil; CP, crude palm oil; DAG, diacylglycerols; FA, fatty acids; FAD, fatty acid distillates; FFA, free fatty acids; FUR, furans; HC, hydrocarbons; I, insoluble impurities; KET, ketones; MAG, monoacylglycerols; M, moisture and volatile matter; MIU, sum of M, I and U; *p*-AnV, *p*-anisidine value; PFAD, palm fatty acid distillates; PV, peroxide value; RP, refined palm oil; RS, refined soybean oil; T, tocopherols; T3, tocotrienols; T+T3, sum of tocopherols and tocotrienols; TAG, triacylglycerols; U, unsaponifiable matter.

the oxidative stability of pelleted and extruded feeds during storage did not decrease compared to mash feeds. This could be due to the fact that FFA % did not increase in pelleted and extruded feeds during the storage, which could compensate for the lower levels of tocols found in these feeds throughout the storage.

Thus, under these storage conditions, the formulation of feeds with by-products rich in FFA did not pose a relevant problem of lipid hydrolysis or oxidative stability, while the formulation with crude oils (the most used in feed formulation) could result in a much higher than expected FFA content in mash feeds, which could imply a deterioration in the digestibility and absorption of the fatty acids in young animals.

ARTICLE INFO

Keywords: Acid oil; fatty acid distillate; feed processing; storage temperature; lipid hydrolysis; oxidative stability.

1. Introduction

Fats and oils are energy ingredients and palatability enhancers used in feed formulation (Baião and Lara, 2005). Although crude fats and oils, mainly constituted by triacylglycerols (TAG), are commonly used in monogastric diets, some by-products from edible oil refining present a relevant potential from a nutritional, economic and sustainability point of view, being their use in feeds a way to upcycle and valorize them (Rodriguez-Sanchez et al., 2019a, 2019b, 2021; Jimenez-Moya et al., 2021a, 2021b; Varona et al., 2021a, 2021b). The main by-products of edible oil refining are the acid oils (AO) and the fatty acid distillates (FAD) which come from the free fatty acid (FFA) removal steps. Concretely, AO are obtained from the alkali neutralization step in chemical refining (after the acidification of the soapstocks) and FAD from the deodorization step in physical refining (Bockisch, 1998; Gunstone, 2011; FEDIOL, 2021). Thus, these by-products are very rich in FFA, being the content higher in FAD (82.5%, determined by titration) than in AO (57.0%), thus being in both cases much higher than in crude (usually 0.5—7%, depending on the oil) and refined (usually < 0.3%) oils (Codex Alimentarius, 1999; Gunstone, 2011; Ginting et al., 2020; Varona et al., 2021b). These FFA-rich by-products are considered a good source of energy, essential fatty acids (FA) and lipid soluble vitamins for animal nutrition (Leeson and Summers, 2008; Varona et al., 2021b). However, the incorporation of these by-products in feeds increases their FFA content, which could entail a higher lipid oxidizability and an impairment of FA digestibility and absorption, which consequently would produce a reduction of the dietary energy provided by the feeds. Some studies have reported that the reductions in FA digestibility and absorption were particularly important for saturated FA and depended on the animal species and especially on the age, being larger for young animals (Vilà and

Esteve-Garcia, 1996; Wiseman et al., 1998; Rodriguez-Sanchez et al., 2019a). Complementary, Rodriguez-Sanchez et al. (2019a; 2019b; 2021) in various studies in broilers found that saturated FA contents had a greater influence on FA absorption than FFA contents, and that the FFA utilization improved with the age of the animal. Moreover, these studies reported that in grower-finisher-broilers, depending on the unsaturated/saturated FA ratio in the diet, FFA contents up to 30-56% in feeds do not have a negative effect on FA utilization (Jimenez-Moya et al., 2021a, 2021b; Rodriguez-Sanchez et al., 2021). The decreased absorption of FA in young chickens has been attributed to different factors, an insufficient emulsification process due to less mature gastrointestinal tract, a lower rate of bile secretion and a less efficient turnover of bile acids (Serafin and Nesheim, 1970; Krogdahl, 1985; Noy and Sklan, 1995). In addition, some studies in oils have reported that FFA are more susceptible to oxidation than esterified FA (Choe and Min, 2006). However, at present there are no systematic studies that clearly elucidate the role of FFA in the oxidative stability of other complex matrices beyond fats and emulsions. Furthermore, it is well known that the oxidative stability of a matrix always depends on many factors, that is, the availability of substrates (unsaturated lipids and oxygen) and the balance between antioxidant and prooxidant factors and compounds (Johnson and Decker, 2015).

Back to the FFA, they may also originate by the action of endogenous lipases coming from feed ingredients. In fact, Dierick and Decuypere (2002) reported lipase activity in several feedstuffs stored at room temperature, being this activity higher in non-intact cereals (ground or milled) and lower in feedstuffs that suffer a thermal treatment during their obtention, such as extruded cereals or soybean meal. Thus, an increase in FFA% during storage has been reported in compound feeds that contained ingredients with endogenous lipases (Dierick and Decuypere, 2002). As these lipases can be inactivated by heat treatments (Camire et al., 1990; Dierick and Decuypere, 2002; Lampi et al., 2015), the endogenous lipolysis could be a problem in mash feed but not in pelleted and extruded feeds. For instance, Dierick and Decuypere (2002) reported that FFA increased up to 25 g/100 g of total FA acids after storage for 42 days at room temperature of a mash piglet feed containing among their ingredients milled barley and wheat (as the main sources of endogenous lipases) and soybean oil (as the main fat source). Therefore, the endogenous lipolysis can produce a relevant increase of FFA during the storage of mash feeds formulated with crude oils, which are mainly constituted by TAG (the substrates of the lipases) and have low FFA content. A considerable increase of FFA in this type of feed, as commented above, might be relevant from a nutritional point of view, especially when these feeds are intended for young animals.

The processing and storage of feeds can also affect their oxidative stability. The loss of minor compounds with antioxidant activity (e.g., tocopherols, carotenoids) during feed processing procedures involving heat treatments (pelleting and extrusion) has been previously reported

(Leeson and Summers, 2008; Svihus and Zimonja, 2011; Morin et al. 2021). Also, the storage conditions affect the loss of these antioxidants in feeds, feedstuffs and food systems (Bauernfeind and Desai, 1977; Leeson and Summers, 2008; Seppanen et al., 2010; Morin et al. 2021). In addition, the loss of these compounds has been related with an increase of lipid oxidation in foods (García-Pascual et al., 2003; Player et al., 2006; Lee et al., 2007). Finally, high levels of lipid oxidation in feeds have been associated with harmful effects on animal health and growth performance (Shurson et al., 2015; Overholt et al., 2018) and, therefore, it seems appropriate to control the degree of oxidation in feeds.

To date, in the literature, there are no systematic studies on the effects of processing and storage conditions on lipid hydrolysis and oxidative stability of feeds containing fat by-products rich in FFA. Thus, in this study, the objective was to evaluate the hydrolytic and oxidative stability of feeds prepared with different fat sources, including by-products from vegetable oil refining industry, and under different processing conditions (mash, pelleted and extruded). To do so, the selected fat products were two by-products with a different FFA content: an AO coming from the chemical refining of soybean oil and a FAD coming from the physical refining of palm oil; their corresponding crude oils (soybean and palm oil) because they are the most used in feed formulation; and their corresponding refined oils because they show the lowest levels of FFA, peroxide values and impurities. Thus, six different mash feeds were prepared, and then they were subjected to two processing conditions and two storage temperatures (for 60 days) to compare the evolution of the lipid hydrolysis and oxidative stability (loss of tocopherols (T) and tocotrienols (T3) and accumulation of primary and secondary oxidation compounds).

2. Material and methods

2.1. Fats used to formulate feeds

Crude and refined oils from soybean (unsaturated source) and palm fruit (more saturated source) were used. Also, two by-products rich in FFA were used: the acid oil obtained during the chemical refining of soybean oil, and the palm fatty acid distillate (PFAD) obtained during the physical refining of palm oil. The soybean oils (crude, CS; refined, RS, and acid, AS) were supplied by Bunge Limited International (Amsterdam, The Netherlands) and the palm oils, crude (CP) and refined (RP), and PFAD were supplied by Lipidos Santiga S.A. (Santa Perpètua de Mogoda, Spain).

2.2. Feed production and storage

All feeds were prepared from a basal diet manufactured at Pinsos Molinet S.A. (Prats de Lluçanès, Spain) in mash form. To this basal diet for grower-finisher chickens (Table 1), the six different fat sources were added at a 6% in a mixer at Lindo Pet Global S.A. (Castellar del Vallès,

Spain). Thus, 6 feeds with different fat sources were obtained in mash form. From these feeds, a part was kept as mash, another was pelleted, and another was extruded at Lindo Pet Global S.A.

Table 1
Ingredients of the experimental basal diet for grower-finisher chickens.

Ingredients (%)			
Wheat	44.02	Titanium dioxide	0.50
Soybean meal 47%	27.25	Vitamin and mineral premix2	0.40
Barley	18.58	Sodium chloride	0.35
Experimental fats[1]	6.00	DL-Methionine	0.17
Calcium carbonate	1.39	L-Lysine	0.12
Monocalcium phosphate	1.20	L-Threonine	0.02

[1]CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate and RP, refined palm oil. [2] Provides per kg of feed: vitamin A (from retinol), 10,000 IU; vitamin D3 (from cholecalciferol), 4,800 IU; vitamin E (from α-tocopheryl acetate), 45 mg; vitamin B1, 3 mg; vitamin B2, 9 mg; vitamin B6, 4.5 mg; vitamin B12, 40 µg; vitamin K3, 3 mg; calcium pantothenate, 16.5 mg; nicotinic acid, 51 mg; folic acid, 1.8 mg; biotin, 150 µg; Fe (from $FeSO_4 \cdot 7H_2O$), 54 mg; I (from $Ca(I_2O_3)_2$), 1.2 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 12 mg; Mn (from MnO), 90 mg; Zn (from ZnO), 66 mg; Se (from Na_2SeO_3), 0.18 mg; β-glucanase 150 U; xylanase 270 U.

The pelleted feeds were produced in a LT70-II Twin Screw Extruder (Shandong Light M&E Co., Ltd., Jinan, China) by using 15 kg of the mash feed to which 1.050 kg of water were added to approximately achieve 18% of moisture. To simulate the pelleting conditions, the temperatures of the different segments of the extruder barrel were set at 50, 55, 65 and 90 °C, and after 1 min 10 s the mash feed exited the extruder pelleted at 78.2 °C. To produce the extruded feeds, 25 kg of mash feed were mixed with 2.250 kg of water to approximately achieve a 20% moisture. The extrusion conditions were as follows: a die nozzle of 2 mm of diameter and 15 mm of length was used, pressure was set at 28 bar, the segment temperatures were set at 55, 170, 175, 190 °C and the product residence time was of 50 s. The temperature of the extruded feeds at the extruder exit was 89.4 °C. The pelleted and extruded feeds were cooled at room temperature for 4 h until were packed in plastic bags of 5 kg. All pelleted and extruded feeds were produced in 28 hours.

Immediately after production, mash, pelleted and extruded feeds were transported to our laboratory and homogenized by using a knife mill (Robot Coupe Blixer 3, Dringomix, Vincennes, France). The time that was used in the transport and homogenization of the feed samples was 5.5 hours. Then, the storage experiment was carried out at two different temperatures in the dark: at room temperature (average temperature of 22 °C), and at 35 °C in an oven (Trade Raypa, Terrassa, Spain) and at three different storage times (0, 30 and 60 days). For 0 days, 500 g of each feed were vacuum-packed in 9 multilayer bags (Cryovac BB3255, permeability to O_2, 17 cm^3/m^2 per day per bar at 23 °C and 0% relative humidity, ASTMD-3985, from Sealed Air, Abrera, Spain) and stored at -20 °C until analysis. Then, homogenized mash, pelleted and extruded feeds were stored in the darkness at both storage temperatures in 1 kg zipper bags partially closed. After 30 and 60 days of

storage, 500 g of each feed from each storage temperature were vacuum-packed in 9 bags multilayer bags (Cryovac BB3255) and stored at -20 °C until analysis.

2.3. Determinations in fats

All the fat sources used to prepare the experimental feeds were analysed in duplicate. The parameters determined were the FA composition, the content of T and T3, the lipid class composition (TAG; DAG, diacylglycerols; MAG, monoacylglycerols; and FFA), the peroxide value (PV), *p*-anisidine value (*p*-AnV), moisture and volatile matter (M), insoluble impurities (I) and unsaponifiable matter (U). The methods adapted by Varona et al. (2021c) to the analysis of AO and FAD were applied to the analysis of AS and PFAD as well as to the analysis of crude and refined oils.

2.4. Determinations in experimental feeds

All determinations were carried out in duplicate, except the determination of volatile compound content. The crude fat content and FA composition was only determined at the initial time on mash feeds (0 days feeds). The rest of determinations were carried out in all feeds.

Moisture content

The M content was determined according to RD 2257/1994. First, the empty moisture dishes (78 mm of internal diameter; 20 mm of internal height) and their lids were dried at 103 °C for 30 min in a forced air oven and then weighed after 1 h in desiccator containing an efficient desiccant (silica gel with moisture indicator and phosphorus pentoxide). Then, about 5 g (± 0.0001 g) of sample were weighed into a tared moisture dish. These open dishes with their lids were placed on the forced air oven for an initial drying period of 4 h at 103 °C. After that time, dishes were lidded and cooled for 1 h in a desiccator, and they were weighed. Then, drying periods of 30 min at 103 °C were carried out until the decrease between the weightings after cooling in the desiccator did not exceed 0.1% of moisture (usually two more drying periods were necessary).

Crude fat

The crude fat in feeds was determined by Soxtec using diethyl ether, following the AOAC official method 2003.05 (AOAC, 2019).

Fatty acid composition

Lipids were extracted from 2g of feed (0.01 of sample) by using diethyl ether according to the Soxtec extraction method 2003.05 of the AOAC international (AOAC, 2019). After extraction, the solvent was not completely evaporated to avoid oxidation and more diethyl ether was added to transfer the lipid extract from Soxtec vessels to two glass test tubes with screw cap (approximately half of the lipid extract to each tube). Diethyl ether was evaporated from tubes under N_2 stream at 30 °C and the lipid extract was stored at −20 °C until the analyses. One tube was used to determine

the FA composition and the other to determine the lipid class composition, as described below. For FA determination, the lipid extract was submitted to a double methylation in methanolic medium and FA methyl esters were determined by GC-FID as described in Varona et al. (2021c) and quantified by internal peak area normalization (% area) by expressing the peak area of a given FA as a percentage of the sum of the areas of all the identified FA peaks.

Lipid class composition

Lipids were extracted from feeds by the Soxtec method as described above. The lipid extract was dissolved in 2 mL of tetrahydrofuran (HPLC grade from Scharlau, Sentmenat, Spain) and then a 1:2 dilution was made in order to obtain a lipid concentration of 15 mg/mL approximately. Then, the percentages of TAG, DAG, MAG and FFA were determined by size molecular exclusion chromatography (HPLC-RID) as described in Varona et al. (2021c) and quantified as peak area normalization (% area).

Tocopherol and tocotrienol content

As described by Bou et al. (2004), T and T3 were extracted from feeds after a saponification. Briefly, 1.5 g (± 0.0001 g) of feed was weighed in a tube with screw cap and was homogenized using a Polytron PT 3100 (Kinematica, Lucerne, Switzerland) at 20,000 rpm for 15 s with 10 mL of absolute ethanol containing 0.8% pyrogallol (w/v), 0.008% butylated hydroxytoluene (w/v), and 0.4% anhydrous citric acid (w/v). Then, 10 mL of 2.2 N methanolic KOH was added, the tube was capped and the saponification was carried at 70 °C for 30 min in a water bath. After cooling, 10 mL of petroleum ether 40-60 °C was added to extract the nonsaponifiables (extraction was repeated three times, with a centrifugation step prior to each extraction). After that, the ethereal extract was filtered through a 0.45 µm Teflon filter (VWR™ International, Llinars del Vallés, Spain). The solvent was evaporated under a nitrogen stream at 30 °C. The residue was dissolved in 500 µL of HPLC grade hexane and injected into a HPLC-FLD (Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA) to determine the T and T3 contents according to Varona et al. (2021c). Quantitation was done by means of calibration curves prepared with α-, β-, γ- and δ-T standards (Calbiochem®, Merck, Darmstadt, Germany) applied to the corresponding T and to each respective T3. The results were calculated in mg/ kg of feed dry matter. This determination was performed under attenuated light conditions to avoid possible degradation of tocols.

Peroxide value

First, 50 g of feed (± 0.01 g) was weighed into a 500 mL Erlenmeyer flask; then, 200 mL of chloroform/methanol mixture (2:1, v/v) were added and the flask content was homogenized for 30 min in a stirring plate. After 1 minute, the supernatant was filtered into another 500 mL Erlenmeyer flask through a Whatman N°1 filter paper. The residue from the first Erlenmeyer was re-extracted in the same way with another 100 mL of the solvent mixture. Then, the total extract was distributed

into three 100 mL centrifuge tubes, where 2 mL of saturated sodium chloride plus 15 mL of distilled water were added before the centrifugation at 757 *g* for 10 min. After this, the lower chloroform phase was transferred to a 250 mL Erlenmeyer flask with anhydrous sodium sulphate, allowed to stand for at least 1 h, filtered through a Whatman N°1 filter paper into 500 mL round flask and the solvent was evaporated in a rotary vacuum evaporator at 30 °C. The round flask was placed into a desiccator under a progressive vacuum for a minimum of 2 h and it was weighed to know the amount of lipid extract. The lipid extract was quantitatively transferred to a 50 mL volumetric flask using chloroform and acetic acid so that the final solution was chloroform/acetic acid (1:1.5, v/v). Then, it was divided exactly into two parts of 25 mL that were used to determine, the peroxide value in duplicate by titration as described in Varona et al. (2021c). The extraction and the determination was performed under attenuated light conditions.

Volatile Compound Content

The SPME-GS-MS analysis was used to determine the volatile content in feeds, adjusting the conditions to extract the volatile compounds achieving the maximum extraction yield, but avoiding the oxidation of the sample, which would lead to artifactual formation of hydroperoxides and their consequent degradation into volatile compounds. For this reason, different parameters were tested: sample weight, extraction temperature and extraction time. Thus, 1 g (± 0.0001 g) of sample was weighted into a 10 mL screw-capped CG vial, in which 3 mL of a 1 mg/kg standard solution of 4-methyl-2-pentanol (Sigma-Aldrich, St. Louis, USA) in distilled deionized water were added plus three glass balls. First, the sample conditioning was done for 10 min at 45 °C under agitation. Then, the SPME sampling was performed by exposing the divinylbenzene/carboxen/polymethylsiloxane (DVD/CAR/PDMS) fibre (2 cm length, 50/30 µm thickness) from Supelco (Bellefonte, PA, USA) to the sample headspace for 30 min at 45 °C. After this, the fibre was desorbed for 10 min at 260 °C in the gas chromatograph injection port of the GC-MS system consisting of an Agilent 6890N Network GC system coupled to a quadrupole mass spectrometer Agilent 5975C Inert MSD (Agilent Technologies, Santa Clara, USA), equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland) with agitator. A Supelcowax-10 capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness, Supelco, Bellefonte, PA, USA) was used for the analyte separation. The oven temperature program began at 40 °C (for 10 min); 3 °C/min up to 150 °C (held for 0.1 min); 15 °C/min up to 250 °C (held for 5 min). Helium was used as carrier gas with a constant flow of 1 mL/min. The temperatures of the ion source and the transfer line were 230 and 280 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionization energy. Acquisition was performed in the selected ion monitoring (SIM) mode, by analyzing the Extracted Ion Chromatogram (EIC) of the following specific ions: m/z 44, 55, 56, 57, 58, 70, 81 and 98. Data were acquired and analyzed by an Agilent MSD ChemStation. Relative amounts of volatile compounds were calculated by the internal standard (IS) method.

Concentrations were expressed as micrograms per gram equivalents of 4-methyl-2-pentanol. Finally, the content of volatile compounds was expressed as milligrams per kilogram of feed dry matter.

2.5. Statistics

The effect of the fat source (CS, AS, RS, CP, PFAD, RP) and feed processing (mash, pelleting, extrusion) on the T and T3 content, the lipid class composition, the peroxide value and the volatile compound content of the freshly prepared feeds (0 days of storage) was evaluated by a multifactor ANOVA (n = 18 resulting from 6 fat types x 3 feed processes) where differences between means were assessed by the Tukey´s post-hoc test. Then, to study how the different fat sources (CS, AS, RS, CP, PFAD, RP), feed processing (mash, pelleting, extrusion), storage times (0, 30, 60 days) and storage temperatures (22 and 35 °C) affected the T and T3 content, the lipid class composition, the peroxide value and the volatile compound content of feeds, a multifactor ANOVA including the interactions between two main factors was used (n = 108, resulting from 6 fat types x 3 feed processes x 3 storage times x 2 storage temperatures). When main factors showed a significant effect, the differences between means were assessed by the Tukey´s post-hoc test. In all cases differences were considered significant at $P \leq 0.05$. Data analysis was performed with IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA).

3. Results

3.1. Composition of the fat sources added to feeds

In this study, six fat sources were added to the experimental feeds, three coming from soybean (CS, AS, RS) and the other three from palm fruit (CP, PFAD, RP). In comparison to the fat sources coming from soybean, the three fat sources coming from palm fruit were richer in saturated fatty acids (SFA) such as C16:0, C18:0 and the sum of total SFA (Table 2). In addition, palm sources showed the highest values of oleic acid (C18:1 n-9) and total *cis*-MUFA. Soybean sources were richer in PUFA (C18:2 n-6, C18:3 n-3 and total *cis*-PUFA). *Trans*-C18:1 was higher in PFAD. Regarding the T and T3 content, the soybean sources presented higher T content (especially γ-T and δ-T) and palm sources were richer in T3 (especially CP and RP). Thus, the sum of T was higher in soybean sources and the sum of T3 in palm sources. About the lipid classes, the AS and PFAD showed much higher FFA and lower TAG. DAG were higher in AS, CP and RP. Regarding lipid oxidation parameters, PV were higher in CP and PFAD and *p*-AnV in the by-products (AS and PFAD). As expected, the MIU content (sum of M, I and U) was higher in the by-products and decreased in this order: AS > CS > RS for soybean fat sources and PFAD > CP > RP for palm fruit fat sources.

Table 2

Composition of the different fat sources used to prepare the experimental feeds (n = 2).

Parameter	CS	AS	RS	CP	PFAD	RP
C16:0 (%)	11.3 ± 0.031	14.9 ± 0.25	11.0 ± 0.01	43.1 ± 0.11	46.6 ± 0.16	43.9 ± 0.48
C18:0 (%)	3.3 ± 0.00	3.5 ± 0.01	3.5 ± 0.00	4.3 ± 0.01	6.6 ± 0.03	4.6 ± 0.04
C18:1 n-9 (%)	23.7 ± 0.05	21.1 ± 0.02	25.1 ± 0.01	38.9 ± 0.10	35.0 ± 0.15	38.4 ± 0.37
C18:1 n-7 (%)	1.6 ± 0.01	1.7 ± 0.00	1.6 ± 0.00	0.7 ± 0.00	0.6 ± 0.01	0.7 ± 0.02
C18:2 n-6 (%)	52.2 ± 0.02	51.7 ± 0.18	51.7 ± 0.00	10.4 ± 0.02	8.5 ± 0.05	9.7 ± 0.07
C18:3 n-3 (%)	6.1 ± 0.03	5.3 ± 0.03	5.3 ± 0.00	0.3 ± 0.00	0.3 ± 0.00	0.1 ± 0.00
SFA (%)	16.0 ± 0.02	19.9 ± 0.22	15.8 ± 0.02	49.4 ± 0.12	55.1 ± 0.19	50.6 ± 0.47
cis-MUFA (%)	25.6 ± 0.06	23.1 ± 0.01	27.1 ± 0.01	39.9 ± 0.10	35.9 ± 0.14	39.4 ± 0.40
cis-PUFA (%)	58.3 ± 0.05	57.0 ± 0.21	57.0 ± 0.01	10.7 ± 0.02	8.8 ± 0.06	9.8 ± 0.07
trans-C18:1 (%)	0.03 ± 0.003	0.04 ± 0.000	0.04 ± 0.001	0.03 ± 0.004	0.22 ± 0.006	0.08 ± 0.003
α-T (mg/kg)	304.1 ± 23.19	99.8 ± 10.32	182.9 ± 24.12	162.1 ± 8.19	35.0 ± 2.87	192.0 ± 1.77
β-T (mg/kg)	46.5 ± 3.68	37.8 ± 3.86	23.0 ± 3.29	2.3 ± 0.31	1.1 ± 0.12	3.3 ± 0.12
γ-T (mg/kg)	967.4 ± 32.05	786.9 ± 44.48	660.7 ± 70.75	2.2 ± 0.59	2.2 ± 1.91	4.1 ± 0.34
δ-T (mg/kg)	333.0 ± 23.53	539.8 ± 53.07	140.7 ± 18.31	ND	4.4 ± 4.11	ND
α-T3 (mg/kg)	ND	ND	ND	244.0 ± 12.31	19.1 ± 1.89	189.2 ± 2.36
β-T3 (mg/kg)	ND	ND	ND	34.3 ± 1.83	3.5 ± 0.23	20.6 ± 0.30
γ-T3 (mg/kg)	ND	ND	ND	286.3 ± 18.53	14.9 ± 1.30	203.3 ± 3.74
δ-T3 (mg/kg)	ND	ND	ND	45.2 ± 3.82	15.1 ± 1.01	18.7 ± 0.91
T (mg/kg)	1651.1 ± 82.45	1464.2 ± 111.74	1007.3 ± 116.47	166.6 ± 9.09	42.8 ± 9.01	199.4 ± 1.55
T3 (mg/kg)	ND	ND	ND	609.7 ± 28.86	52.6 ± 3.96	431.9 ± 6.72
T+T (mg/kg)3	1651.1 ± 82.45	1464.2 ± 111.74	1007.3 ± 116.47	776.4 ± 37.94	95.4 ± 12.97	631.3 ± 8.27
TAG (%)	95.1 ± 0.16	25.3 ± 1.79	96.3 ± 0.24	82.2 ± 0.14	4.3 ± 0.51	92.5 ± 0.47
DAG (%)	3.1 ± 0.04	13.5 ± 0.52	3.2 ± 0.35	10.3 ± 0.14	3.2 ± 0.18	7.5 ± 0.47
MAG (%)	ND	Tr	ND	ND	ND	ND
FFA (%)	1.7 ± 0.13	61.2 ± 1.28	0.5 ± 0.11	7.5 ± 0.28	92.5 ± 0.69	ND
PV (meq O_2/kg)	1.0 ± 0.03	1.2 ± 0.00	0.7 ± 0.03	2.9 ± 0.09	3.3 ± 0.16	0.5 ± 0.03
p-AnV	1.1 ± 0.19	10.8 ± 2.18	3.1 ± 0.68	6.5 ± 0.59	54.3 ± 1.44	6.4 ±0.39
M (%)	0.20 ± 0.01	1.43 ± 0.02	ND	0.03 ± 0.00	0.01 ± 0.04	ND
I (%)	1.46 ± 0.06	1.57 ± 0.36	1.27 ± 0.04	0.80 ± 0.03	3.76 ± 0.23	0.59 ± 0.09
U (%)	0.95 ± 0.07	2.34 ± 0.03	0.99 ± 0.16	0.65 ± 0.05	1.34 ± 0.02	0.21 ± 0.03
MIU (%)	2.62 ± 0.12	5.34 ± 0.34	2.26 ±0.20	1.47 ± 0.03	5.11 ± 0.30	0.85 ± 0.02

[1] Mean ± standard deviation. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0 and C24:0); *cis*-MUFA, *cis*-monounsaturated fatty acids (sum of C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9); *cis*-PUFA, *cis*- polyunsaturated fatty acids (sum of C18:2 n-6 and C18:3 n-3); *trans*-C18:1 (sum of positional isomers); T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; PV, peroxide value; *p*-AnV, *p*-anisidine value; M, moisture and volatile matter; I, insoluble impurities; U, unsaponifiable matter; MIU, M+I+U; ND, not detected and Tr, traces. Fatty acid and lipid class composition were expressed as peak area normalization in %; *p*-AnV as arbitrary units; and M, I and U as a percentage on the wet weight.

3.2. Moisture content of the experimental feeds

The effect of the fat source (CS, AS, RS, CP, PFAD and RP) on M content of freshly prepared feeds (0 days of storage) was not significant. The M content was affected by the interaction between the storage temperature and storage time ($P \leq 0.001$), being the decrease of M over the time much greater when the feeds were stored at 35 °C (Fig. 1). Therefore, to compare the evolution of the

different parameters throughout storage, the parameters expressed per unit mass (T, T3 and volatile compound contents) were expressed on dry matter.

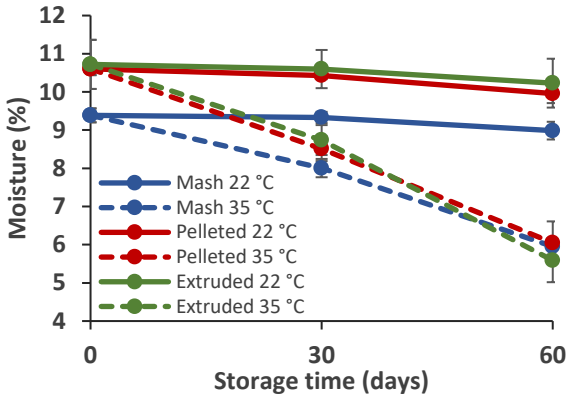


Fig. 1. Evolution of moisture content in feeds (mash, pelleted and extruded, kept at 22 °C and 35 °C) throughout storage time (0, 30 and 60 days) (mean ± SD) (n = 54).

3.3. Fatty acid composition of the mash feeds at zero days of storage

The FA composition of the freshly prepared mash feeds (Table 3) reflected the FA composition of the fat sources added to the feeds (Table 2). Thus, soybean feeds (CS, AS and RS) were richer in C18:2 n-6, C18:3 n-3 and total *cis*-PUFA, while palm feeds (CP, PFAD and RP) had higher C16:0, C18:0, total SFA, C18:1 n-9 and total cis-MUFA. Also, the *trans*-C18:1 were higher in PFAD compared to the rest of fat sources.

Table 3

Fatty acid composition (expressed as peak area normalization in %) in freshly prepared mash feeds (0 days of storage) according to the added fat source (n = 2).

Fatty acids	CS	AS	RS	CP	PFAD	RP
C16:0	12.4 ± 0.231	15.3 ± 0.01	12.0 ± 0.01	39.3 ± 0.02	41.5 ±0.22	39.4 ± 0.13
C18:0	3.4 ± 0.06	3.5 ± 0.03	3.5 ± 0.00	4.2 ± 0.00	6.2 ± 0.02	4.5 ± 0.01
C18:1 n-9	23.1 ± 0.03	20.8 ± 0.00	24.4 ± 0.01	35.8 ± 0.10	33.1 ± 0.04	36.0 ± 0.07
C18:1 n-7	1.5 ± 0.01	1.6 ± 0.02	1.6 ± 0.02	0.8 ± 0.01	0.7 ± 0.00	0.8 ± 0.00
C18:2 n-6	51.8 ± 0.26	51.6 ± 0.08	51.4 ± 0.03	16.5 ± 0.13	14.9 ± 0.20	15.8 ± 0.04
C18:3 n-3	5.8 ± 0.02	5.2 ± 0.01	5.1 ± 0.00	1.0 ± 0.02	1.0 ± 0.03	0.9 ± 0.01
SFA	17.3 ± 0.33	20.4 ± 0.07	17.0 ± 0.01	45.5 ± 0.05	49.6 ± 0.27	46.0 ± 0.12
cis-MUFA	25.0 ± 0.04	22.9 ± 0.02	26.4 ± 0.01	37.0 ± 0.10	34.3 ± 0.04	37.2 ± 0.08
cis-PUFA	57.6 ± 0.29	56.7 ± 0.09	56.5 ± 0.04	17.5 ± 0.15	15.9 ± 0.23	16.7 ± 0.05
trans-C18:1	0.04 ± 0.002	0.04 ± 0.006	0.04 ± 0.006	0.03 ± 0.000	0.19 ± 0.003	0.09 ± 0.009

[1] Mean ± standard deviation. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0; C22:0, C23:0 and C24:0); *cis*-MUFA, *cis*-monounsaturated fatty acids (sum of C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9); *cis*-PUFA, total *cis*-polyunsaturated fatty acids (sum of C18:2 n-6 and C18:3 n-3) and *trans*-C18:1 (sum of positional isomers).

3.4. Lipid composition and oxidation of all the experimental feeds at zero days of storage

The T contents in freshly prepared feeds (Table 4) agreed with the content of these compounds in the added fats (Table 2). Thus, summarizing the total T content was the lowest in PFAD feeds and the highest in CS feeds. The total T3 content was higher in CP and RP feeds than in PFAD and soybean feeds. Thus, total T + T3 was the lowest in PFAD and the highest in CS. In relation to the feed

elaboration process, some T and T3 decreased significantly in pelleted and extruded feeds and, therefore, the total contents of T, T3 and T + T3 were significantly higher in mash feeds (Table 4).

The lipid class composition (TAG, DAG, MAG and FFA) of the freshly prepared feeds (Table 4) is also in accordance with the composition of the added fats. Thus, the TAG% decreased significantly in this order, CS, RS and RP > CP > AS > PFAD and the FFA% decreased in the reverse order. The highest DAG% were found in AS, CP and RP feeds and the highest MAG% in AS feeds. In relation to the feed processing, the TAG% was lower in mash feeds, while the MAG and FFA were higher.

The PV was higher in soybean feeds than in palm feeds. The feed elaboration process did not significantly affect the PV at zero days of storage (Table 4). The fat source added to the feeds did not affect the volatile compound content, while the feed processing did it. Thus, mash feeds showed lower pentanal content and higher totals of hydrocarbons (HC), ketones (KET) and alcohols (ALC) (Table 4 and Table S1).

Table 4

Effect of the fat source and feed processing on the lipid composition and oxidation of the freshly prepared feeds (0 days of storage) [1]

	Fat source								Feed processing				
Parameter	CS	AS	RS	CP	PFAD	RP	SEM[2]	*P*[3]	Mash	Pelleted	Extruded	SEM[2]	*P*[3]
α-T (mg/ kg)	102.7^{b}	100.2^{b}	98.8^{b}	92.9^{b}	77.7^{a}	100.7^{b}	23.332	0.000	107.9^{b}	91.1^{a}	87.5^{a}	1.649	**0.000**
β-T (mg/ kg)	5.8^{b}	3.5^{ab}	4.5^{ab}	3.5^{ab}	2.2^{a}	3.7^{ab}	0.548	0.015	5.4^{b}	3.1^{a}	3.1^{a}	0.388	**0.002**
ϒ-T (mg/ kg)	73.8^{b}	36.7^{ab}	42.9^{ab}	8.0^{a}	5.2^{a}	8.9^{a}	9.696	0.003	46.7	20.7	20.4	6.856	**0.033**
δ-T (mg/ kg)	22.7^{b}	30.5^{b}	10.1^{a}	2.3^{a}	1.9^{a}	2.4^{a}	2.420	0.000	14.2	10.3	10.5	1.711	0.244
α-T3 (mg/ kg)	8.1	7.9	8.9	17.1	8.7	16.3	2.130	0.028	17.1^{b}	8.1^{a}	8.3^{a}	1.506	**0.003**
β-T3 (mg/ kg)	12.1^{bc}	9.8^{ab}	12.5^{bc}	12.2^{bc}	9.0^{a}	13.3^{c}	0.578	0.002	13.8^{b}	10.7^{a}	9.9^{a}	0.408	**0.000**
ϒ-T3 (mg/ kg)	1.1^{a}	1.1^{a}	1.2^{a}	17.9^{b}	2.1^{a}	12.6^{b}	1.126	0.000	7.7	5.3	5.0	0.796	0.072
δ-T3 (mg/ kg)	Tr^{a}	Tr^{a}	Tr^{a}	2.1^{c}	1.2^{b}	0.9^{b}	0.105	0.000	0.8	0.6	0.7	0.074	0.281
T (mg/ kg)	205.0^{d}	170.9^{cd}	156.3^{bcd}	106.8^{ab}	87.0^{a}	115.7^{abc}	12.261	0.000	174.3^{b}	125.2^{a}	121.4^{a}	8.670	**0.003**
T3 (mg/ kg)	21.4^{a}	18.8^{a}	22.6^{a}	49.3^{b}	21.0^{a}	43.1^{b}	2.943	0.000	39.4^{b}	24.7^{a}	24.0^{a}	2.081	**0.001**
T+T3 (mg/ kg)	226.4^{c}	189.7^{bc}	178.9^{bc}	156.1^{ab}	108.1^{a}	158.8^{ab}	11.113	0.000	213.7^{b}	150.0^{a}	145.4^{a}	7.858	**0.000**
TAG (%)	81.3^{d}	30.2^{b}	80.9^{d}	72.1^{c}	18.0^{a}	80.2^{d}	0.660	0.000	57.3^{a}	62.1^{b}	61.9^{b}	0.467	**0.000**
DAG (%)	9.2^{b}	13.7^{d}	9.6^{bc}	13.3^{d}	5.4^{a}	10.9^{c}	0.328	0.000	10.3	10.3	10.4	0.232	0.896
MAG (%)	1.7^{a}	2.9^{b}	1.7^{a}	2.1^{a}	1.5^{a}	1.8^{a}	0.146	0.001	2.4^{b}	1.8^{a}	1.6^{a}	0.103	**0.000**
FFA (%)	7.9^{a}	52.2^{c}	7.8^{a}	12.5^{b}	75.1^{d}	7.1^{a}	0.462	0.000	29.8^{b}	25.6^{a}	25.9^{a}	0.327	**0.000**
PV (meq O_2/kg)	18.8^{b}	12.7^{ab}	14.7^{ab}	7.6^{a}	5.8^{a}	7.9^{a}	2.094	0.010	8.2	13.6	11.9	1.481	0.074
Pentanal (mg/kg)	0.5	0.6	0.3	0.3	0.5	0.3	0.113	0.318	0.1^{a}	0.7^{b}	0.4^{b}	0.080	**0.001**
Hexanal (mg/kg)	2.5	3.0	2.3	1.5	1.6	1.4	0.688	0.556	1.5	3.0	1.6	0.487	0.100
Σ ALD (mg/kg)	3.4	4.3	2.9	2.0	2.4	1.8	0.914	0.453	1.9	4.0	2.4	0.646	0.097
Σ HC (mg/kg)	0.2	0.2	0.3	0.3	0.3	0.3	0.038	0.508	0.5^{b}	0.2^{a}	0.1^{a}	0.027	**0.000**
Σ KET (mg/kg)	0.4	0.7	0.6	0.4	0.4	0.3	0.091	0.057	0.8^{b}	0.3^{a}	0.3^{a}	0.065	**0.000**
Σ ALC (mg/kg)	4.3	4.1	3.3	4.0	4.8	3.8	0.500	0.461	11.1^{b}	0.7^{a}	0.4^{a}	0.354	**0.000**
Σ FUR (mg/kg)	0.3	0.9	0.3	0.4	0.8	0.3	0.218	0.243	0.7	0.5	0.3	0.154	0.149

[1] Pooled means from multifactor ANOVA (n = 18 resulting from 6 fat types x 3 feed processes). [2] SE, standard error of the mean. [3] *P* values in bold are significant ($P \leq 0.05$). [a-d] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; PV, peroxide value; Σ ALD, Σ Aldehydes (sum of pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal and 2,4-nonadienal); Σ HC, Σ Hydrocarbons (sum of hexane and decane); Σ KET, Σ ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one); Σ ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol, 1-octen-3-ol, 2-pentenol and 1-nonanol); Σ FUR, Σ Furans (sum of 2-penthylfuran and 2-furanmethanol); Tr, traces.

3.5. Lipid composition and oxidation of the experimental feeds throughout the storage

Considering the influence of the storage time (Table 5), the total T decreased during storage (0 d > 30 d > 60 d), while the total T3 only decreased at 60 days of storage. In addition, total T and T + T3 contents were higher in feeds kept at 22 °C than in those kept at 35 °C. Regarding TAG%, it was higher at 0 days of storage than at 30 and 60 days, while the FFA% increased over storage time (0 d< 30 d< 60 d). The storage temperature did not affect the lipid class composition. The PV was lower at 30 and 60 days of storage and was not influenced by the storage temperature. The contents of pentanal, HC, KET, ALC and FUR were higher at 0 days of storage. The storage temperature also affected the contents of hexanal, ALD and KET, which were higher when feeds were stored at 22 °C (Table 5).

Several interactions between two of the main factors (fat source x feed processing, fat source x storage time, feed processing x storage time, and feed processing x storage temperature) significantly affected the studied parameters (Tables S2-S6 and S8, supplementary data). The most relevant interactions are presented in Figs. 2, 3, 4 and 5. Fig. 2 and 3 show that mash feeds, in comparison to pelleted and extruded feeds, presented a higher initial content of total T and a steeper decrease in this content from 30 to 60 days of storage (Fig. 2) as well as a higher decrease in TAG% and a higher increase in FFA% during the storage time (Fig. 3). Moreover, the TAG reduction and the FFA increase over the storage time also differed between fat sources added to the feeds, being higher in CS, RS, CP and RP feeds, and lower in AS and PFAD feeds (Fig. 4). In addition, in Fig. 5 it is shown that TAG% was higher and FFA% was lower in pelleted and extruded feeds than in mash feeds, but that the magnitude of these differences depended on the fat source, being the differences larger for CS, RS, CP and RP feeds, and smaller for AS and PFAD feeds.

Table 5

Effect of the fat source, feed processing, storage time and storage temperature on the lipid composition and oxidation of all experimental feeds[1].

	Fat source								Feed processing					Storage time					Storage temperature			
Parameters	CS	AS	RS	CP	PFAD	RP	SEM[2]	*P*[3]	Mash	Pelleted	Extruded	SEM[2]	*P*[3]	Day 0	Day 30	Day 60	SEM[2]	*P*[3]	22°C	35°C	SEM[2]	*P*[3]
α-T (mg/ kg)	93.4^{c}	90.4^{bc}	90.3^{bc}	88.9^{b}	72.3^{a}	90.1^{bc}	1.065	**0.000**	98.8^{b}	82.4^{a}	81.5^{a}	0.753	**0.000**	95.5^{c}	88.6^{b}	78.7^{a}	0.753	**0.000**	88.4	86.8	0.615	0.066
β-T (mg/ kg)	5.3^{e}	3.2^{b}	4.1^{d}	3.4^{bc}	2.1^{a}	3.5^{c}	0.056	**0.000**	5.0^{b}	2.9^{a}	2.9^{a}	0.040	**0.000**	3.9^{c}	3.6^{b}	3.3^{a}	0.040	**0.000**	3.7^{b}	3.5^{a}	0.033	**0.002**
ϒ-T (mg/ kg)	64.4^{d}	30.5^{b}	36.6^{c}	7.4^{a}	4.7^{a}	8.0^{a}	1.108	**0.000**	39.5^{b}	17.8^{a}	18.4^{a}	0.784	**0.000**	29.3^{c}	25.4^{b}	21.1^{a}	0.784	**0.000**	26.6^{b}	24.0^{a}	0.640	**0.006**
δ-T (mg/ kg)	21.6^{c}	27.4^{d}	9.5^{b}	2.3^{a}	1.8^{a}	2.4^{a}	0.216	**0.000**	13.2^{b}	9.5^{a}	9.9^{a}	0.153	**0.000**	11.7^{b}	10.6^{a}	10.3^{a}	0.153	**0.000**	11.0^{b}	10.7^{a}	0.125	**0.049**
α-T3 (mg/ kg)	8.0^{a}	7.4^{a}	8.2^{a}	17.2^{d}	9.2^{b}	15.3^{c}	0.212	**0.000**	16.0^{b}	8.3^{a}	8.3^{a}	0.150	**0.000**	11.2^{b}	11.4^{b}	10.1^{a}	0.150	**0.000**	10.9	10.8	0.122	0.666
β-T3 (mg/ kg)	12.1^{bc}	9.3^{a}	11.9^{b}	12.6^{cd}	9.3^{a}	13.0^{d}	0.163	**0.000**	13.6^{b}	10.4^{a}	10.1^{a}	0.115	**0.000**	11.5^{b}	11.7^{b}	11.0^{a}	0.115	**0.000**	11.4	11.3	0.094	0.278
ϒ-T3 (mg/ kg)	1.1^{a}	1.0^{a}	1.2^{a}	17.5^{d}	2.2^{b}	11.6^{c}	0.110	**0.000**	7.3^{b}	5.1^{a}	4.9^{a}	0.078	**0.000**	6.0^{b}	5.9^{b}	5.4^{a}	0.078	**0.000**	5.8	5.7	0.064	0.307
δ-T3 (mg/ kg)	Tr^{a}	Tr^{a}	Tr^{a}	2.4^{c}	1.2^{b}	1.1^{b}	0.042	**0.000**	0.9^{b}	0.7^{a}	0.7^{a}	0.030	**0.001**	0.7^{a}	0.8^{a}	0.9^{b}	0.030	**0.000**	0.8	0.8	0.024	0.405
T (mg/ kg)	184.8^{e}	151.5^{d}	140.5^{c}	102.1^{b}	80.9^{a}	104.1^{b}	1.955	**0.000**	156.6^{b}	112.7^{a}	112.7^{a}	1.382	**0.000**	140.3^{c}	128.2^{b}	113.4^{a}	1.382	**0.000**	129.7^{b}	124.9^{a}	1.129	**0.004**
T3 (mg/ kg)	21.2^{b}	17.7^{a}	21.3^{b}	49.7^{d}	21.9^{b}	41.1^{c}	0.407	**0.000**	37.8^{b}	24.6^{a}	24.1^{a}	0.288	**0.000**	29.4^{b}	29.7^{b}	27.4^{a}	0.288	**0.000**	29.0	28.7	0.235	0.394
T+T3 (mg/ kg)	205.9^{d}	169.2^{c}	161.7^{c}	151.8^{b}	102.8^{a}	145.2^{b}	2.254	**0.000**	194.4^{b}	137.2^{a}	136.8^{a}	1.594	**0.000**	169.7^{b}	158.0^{a}	140.7^{a}	1.594	**0.000**	158.6^{b}	153.6^{a}	1.302	**0.009**
TAG (%)	72.4^{d}	28.4^{b}	74.2^{d}	65.9^{c}	17.0^{a}	74.0^{d}	1.261	**0.000**	41.8^{a}	62.1^{b}	62.1^{b}	0.892	**0.000**	60.5^{b}	54.0^{a}	51.5^{a}	0.892	**0.000**	56.2	54.5	0.728	0.107
DAG (%)	10.5^{b}	13.7^{d}	10.4^{b}	14.2^{d}	5.7^{a}	12.0^{c}	0.226	**0.000**	12.0^{b}	10.5^{a}	10.7^{a}	0.160	**0.000**	10.3^{a}	11.3^{b}	11.5^{b}	0.160	**0.000**	10.9	11.2	0.131	0.059
MAG (%)	1.9^{bc}	2.7^{d}	1.7^{ab}	2.2^{c}	1.5^{a}	1.8^{ab}	0.072	**0.000**	2.6^{b}	1.7^{a}	1.7^{a}	0.051	**0.000**	2.0	2.0	1.9	0.051	0.169	2.0	1.9	0.041	0.269
FFA (%)	15.1^{ab}	54.1^{c}	13.7^{ab}	17.8^{b}	75.8^{d}	12.3^{a}	1.006	**0.000**	43.5^{b}	25.5^{a}	25.4^{a}	0.711	**0.000**	27.1^{a}	32.5^{b}	34.8^{b}	0.711	**0.000**	30.8	32.1	0.581	0.107
PV (meq O_2/kg)	12.1^{c}	11.3^{c}	12.6^{c}	6.4^{ab}	5.5^{a}	7.5^{b}	0.433	**0.000**	7.5^{a}	10.4^{b}	9.7^{b}	0.306	**0.000**	11.3^{b}	7.7^{a}	8.7^{a}	0.306	**0.000**	9.0	9.5	0.250	0.142
Pentanal (mg/kg)	0.3^{ab}	0.6^{c}	0.3^{b}	0.3^{ab}	0.4^{b}	0.2^{a}	0.035	**0.000**	0.1^{a}	0.5^{c}	0.3^{b}	0.025	**0.000**	0.4^{b}	0.3^{a}	0.3^{a}	0.025	**0.000**	0.4	0.3	0.020	0.097
Hexanal (mg/kg)	1.8^{a}	3.3^{b}	2.1^{a}	1.6^{a}	1.4^{a}	1.1^{a}	0.258	**0.000**	1.7^{a}	2.6^{b}	1.3^{a}	0.182	**0.000**	2.1	1.8	1.8	0.182	0.516	2.1^{b}	1.7^{a}	0.149	**0.031**
Σ ALD (mg/kg)	2.3^{a}	4.4^{b}	2.6^{a}	2.0^{a}	2.0^{a}	1.3^{a}	0.308	**0.000**	2.0^{a}	3.4^{b}	1.9^{a}	0.218	**0.000**	2.8	2.2	2.2	0.218	0.130	2.7^{b}	2.1^{a}	0.178	**0.025**
Σ HC (mg/kg)	0.2^{ab}	0.1^{a}	0.2^{bc}	0.2^{abc}	0.2^{abc}	0.3^{c}	0.020	**0.000**	0.4^{b}	0.2^{a}	0.1^{a}	0.014	**0.000**	0.3^{b}	0.1^{a}	0.2^{b}	0.014	**0.000**	0.2	0.2	0.011	0.369
Σ KET (mg/kg)	0.4^{a}	0.6^{b}	0.4^{ab}	0.3^{a}	0.4^{a}	0.3^{a}	0.038	**0.000**	0.7^{b}	0.3^{a}	0.3^{a}	0.027	**0.000**	0.5^{b}	0.4^{a}	0.4^{ab}	0.027	**0.015**	0.5^{b}	0.4^{a}	0.022	**0.003**
Σ ALC (mg/kg)	4.2	3.2	3.6	3.3	3.9	4.0	0.276	0.076	10.0^{b}	0.7^{a}	0.5^{a}	0.195	**0.000**	4.1^{b}	3.0^{a}	4.0^{b}	0.195	**0.001**	3.8	3.6	0.160	0.517
Σ FUR (mg/kg)	0.3^{ab}	0.5^{b}	0.4^{ab}	0.4^{ab}	0.4^{ab}	0.3^{a}	0.043	**0.034**	0.4^{b}	0.6^{c}	0.2^{a}	0.030	**0.000**	0.5^{b}	0.4^{a}	0.4^{a}	0.030	**0.001**	0.4	0.4	0.025	0.204

[1] Pooled means from multifactor ANOVA (n = 108, resulting from 6 fat sources x 3 feed processing x 3 storage times x 2 storage temperatures). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). [a-e] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. See Table 4 for abbreviations.

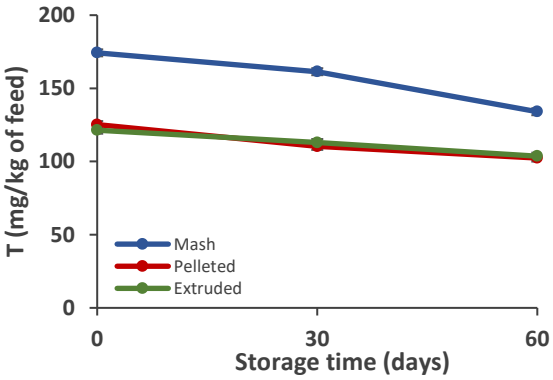
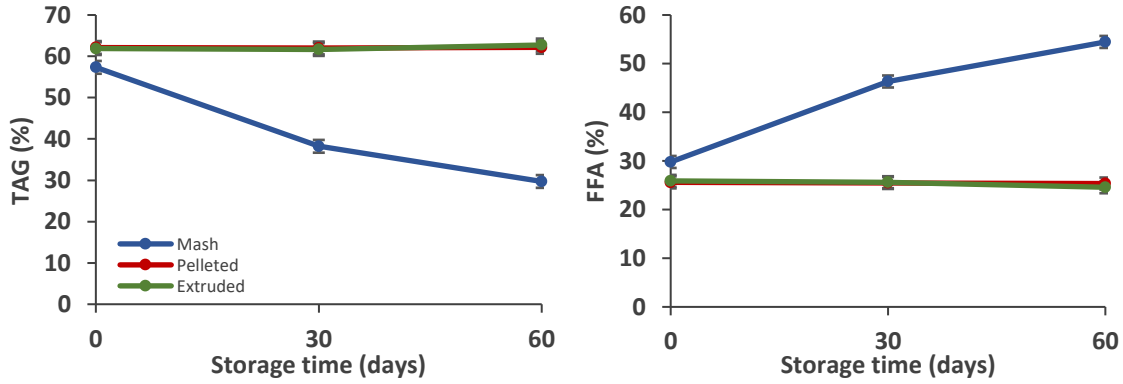
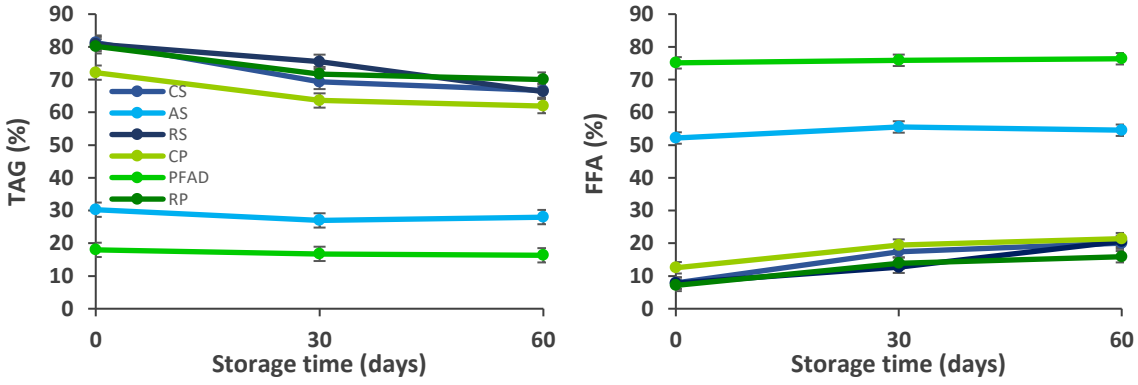


Fig. 2. Effect of the interaction between feed processing (mash, pelleted and extruded feeds) and storage time (0, 30 and 60 days) on the total tocopherol content (pooled means ± standard error from multifactor ANOVA with n = 108, $P \leq 0.000$).



(A) (B)

Fig. 3. Effect of the interaction between feed processing (mash, pelleted and extruded feeds) and storage time (0, 30 and 60 days) on the triacylglycerol (TAG) and free fatty acid (FFA) percentages (pooled means ± standard error from multifactor ANOVA with n = 108): (A) TAG% ($P \leq 0.000$) and (B) FFA% ($P \leq 0.000$).



(A) (B)

Fig. 4. Effect of the interaction between fat source (CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil) and storage time (0, 30 and 60 days) on the triacylglycerol (TAG) and free fatty acid (FFA) percentages (pooled means ± standard error from multifactor ANOVA with n = 108): (A) TAG% ($P = 0.035$) and (B) FFA% ($P = 0.021$).

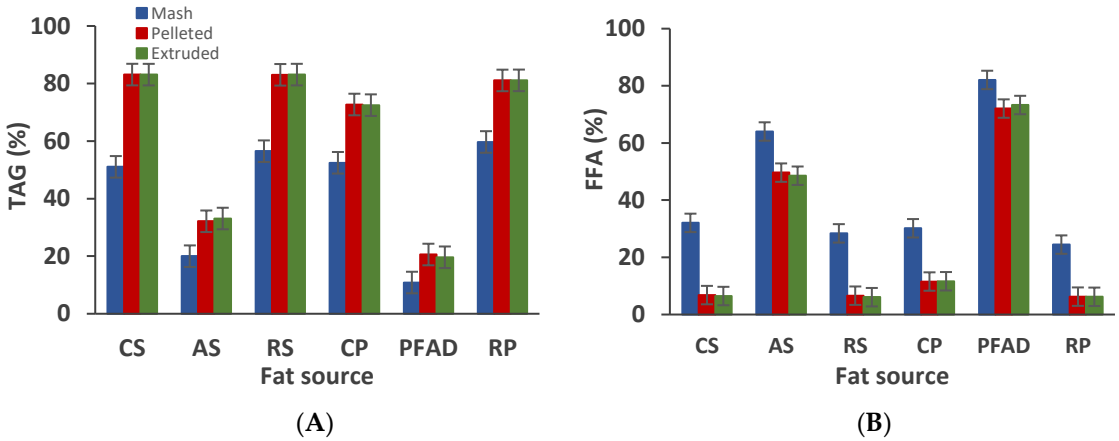


Fig. 5. Effect of the interaction between fat source (CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil) and feed processing (mash, pelleted and extruded feeds) on the triacylglycerol (TAG) and free fatty acid (FFA) percentages (pooled means ± standard error from multifactor ANOVA with n = 108): (A) TAG% ($P \leq 0.000$) and (B) FFA% ($P = 0.001$).

4. Discussion

4.1. Composition of the fat sources added to the feeds

The composition of the fat sources coming from soybean (CS, AS, RS) and from palm fruit (CP, PFAD, RP) was in accordance with the typical FA composition and T and T3 contents of the soybean and palm oils (Codex Alimentarius, 1999; Gunstone, 2011). For instance, palmitic (C16:0) and oleic (C18:1 n-9) acids in the palm fat sources ranged from 43.1 to 46.6% and from 35.0 to 38.9%, respectively, which agrees with the ranges given for palm oils in the Codex Alimentarius (1999), 39.3-47.5% and 36.0-44.0%. In the fat sources coming from soybean, the linoleic (C18:2 n-6) and linolenic (C18:3 n-3) acids ranged from 51.7 to 52.2% and from 5.3 to 6.1%, respectively, which agrees with the ranges given for soybean oils in the Codex Alimentarius (1999), 48.0-59.0% and 4.5-11.0%. Thus, the fat sources coming from palm fruit were richer in SFA and MUFA, while the ones coming from soybean were richer in PUFA. In addition, the highest content of *trans*-C18:1 FA isomers (0.22%) was found in PFAD, which is consistent with the FA isomerization that occurs during the obtention of FAD by deodorization, a process that involves very high temperatures, usually from 180 to 270 °C (Gibon et al., 2007; Vaisali et al., 2015; FEDIOL,2021). These the *trans*-C18:1 values were in agreement with the ranges (from 0.1 to 0.5%) found for other PFAD as reported in Varona et al., (2021b).

About the T and T3, T were more abundant in the soybean fat sources, while T3 in the palm fat sources, agreeing with the results previously reported for soybean and palm oils (Codex Alimentarius, 1999; Gunstone, 2011). The major T in soybean fat sources was, γ-T, followed by δ-T, α-T and β-T, and they were in similar ranges as those reported for these compounds in crude soybean oils (Codex Alimentarius, 1999), respectively, 89-2307 mg γ-T/kg, 154-932 mg δ-T/kg, 9-352 mg α-T/kg and not detected-36 mg β-T /kg. In palm fat sources, the predominant tocols were the T3, being γ-T3 the main T3, followed by α-T3, δ-T3 and β-T3, which agrees with the content ranges reported in crude palm oils (Codex Alimentarius, 1999) for γ-T3 (14-710 mg/kg), α-T3 (4-336 mg/kg) and δ-T3 (not detected-377

mg/kg). The content of T and T3 in the by-products (AS and PFAD) agreed with the contents previously reported by Varona et al. (2021b) for T in soybean acid oils (1180-1464, median = 1322 mg/kg) and in the blends of sunflower and soybean acid oils (524-2242, median = 1035 mg/kg) and for T (2-73 mg/kg, median = 44 mg/kg) and T3 (not detected-339 mg/kg, median = 185 mg/kg) in PFAD samples.

About the differences found in the lipid class composition, the AS and PFAD were those with the lowest TAG% and the highest FFA%, as they are edible oil refining by-products obtained during the FFA removal steps (neutralization in chemical refining or deodorisation in physical refining). The FFA% was higher in PFAD than in AS, which agrees with the fact that FFA content is higher in FAD coming from physical refining than in AO coming from the chemical refining (Nuchi et al., 2009; Varona et al., 2021b). Regarding PV, the results were low for all fat sources ≤ 3.3 meq O_2/kg, which met the recommendations (< 10 meq O_2/kg) for fat sources used in feed production FEDNA guidelines (FEDNA, 2002). In addition, the PV found in AS and PFAD agreed with the low PV previously reported for AO and FAD (Nuchi et al., 2009; Varona et al., 2021a), since the production of these by-products entails heating treatments that decompose the hydroperoxides leading to PV close to O in freshly produced AO and FAD. Thus, the PV in these by-products depends on the formation of lipid hydroperoxides during the storage, which is affected by the oxidizability of the sample and the storage time and conditions (Varona et al., 2021a). PV were lower in refined oils (RS and RP) since hydroperoxides, like other undesirable compounds such as FFA, are efficiently removed during refining (Ghazani and Marangoni, 2013) and their content in refined oils also depends on the oxidizability of the oil and the storage time and conditions. Regarding *p*-AnV, which measures the content of aldehydes, the results were higher in the by-products (AS and PFAD) and agreed with the previously reported results for AO and FAD (Nuchi et al., 2009; Varona et al., 2021a). The PFAD showed the highest *p*-AnV because it is obtained by distillation at low pressures and high temperatures and during this process volatile aldehydes accumulate in the FAD along with many other distillable compounds (Varona et al., 2021a).

As expected, MIU content for soybean and palm fruit fat sources decreased in the order: by-product > crude oil > refined oil. This is because during the refining of crude oils some I are removed, M is reduced and part of the U is lost being some U compounds and I accumulated in AO and FAD (Gunstone, 2011; Varona et al., 2021b).

4.2. Lipid composition and oxidation of feeds at day zero of storage

The fat sources added to feeds greatly influenced their lipid composition because they were added at a 6%, being the crude fat found in these feeds on average a 7.9%. Thus, the FA profile and the T and T3 contents in feeds reflected the contents of these compounds in the fat sources. Compared to soybean feeds, palm feeds were richer in T3 (except PFAD) and in SFA and MUFA, while soybean feeds presented higher T contents (CS) and PUFA. The individual T and T3 contents of feeds also reflected the T and T3 content of the fat sources, being the γ-T and δ-T higher in some soybean feeds and the γ-T3 and δ-T3 in

some palm feeds. In addition, it must be considered that wheat and barley, which respectively represent a 44.02% and a 18.58% of the experimental feeds, are a great source of T and T3 (Granda et al. 2018; Nielsen and Hansen, 2008). Thus, the content of T and T3 in feeds was also affected by the content of these two cereals. This effect was clearly observed for T3, which were only present in palm fat sources but were present in all feeds (Table 4), and specially for α-T3 and β-T3 since barley is an important source of α-T3 and wheat of β-T3. Thus, the high content of T and T3 of these two cereals partially masked the influence of the fat sources on the contents of T and T3 in feeds. Moreover, the vitamin premix supplemented 45 mg of α-T, as α-tocopheryl acetate, which represented the 41.7% of the α-T and the 25.8% of the sum of T found in freshly produced mash feeds.

The lower levels found for some T and T3 in pelleted and extruded feeds in comparison to mash feeds could be related to the heat treatments applied during pelleting and extrusion. Actually, the loss of vitamin E in feeds after heat treatments has been previously reported (Morin et al., 2021). But the reduction observed after pelleting for α-T was 15.6% and after extrusion 18.9%, while the reduction for the sum of T was 28.1% after pelleting and 30.3% after extrusion. This difference in the reduction % for α-T and total T could be due to the different proportion of α-T as α-tocopheryl acetate and also to the different antioxidant activity (stability) of the different structural analogues of the T (Seppanen et al., 2010). It is important to note that the antioxidant activity of the T and T3 analogues depends on the matrix and storage and processing conditions, and that highly controversial results have been reported in the literature (Seppanen et al., 2010). Thus, the decrease observed for the sum of T after pelleting or extrusion is plausible according to the literature review carried out by Morin et al. (2021).

The lipid class composition (% of TAG, DAG, MAG and FFA) of the extracted lipid fractions of the freshly produced feeds (0 days of storage) was mainly influenced by the fat source, since as commented above the fat sources added to the feeds (at a 6%) were the main part of the crude fat of the feeds (7.9%). Thus, the FFA% in feeds decreased in this order PFAD > AS > CP > CS = RS = RP (Table 4) and the TAG% did so in the opposite order. This is in agreement with the content of FFA and TAG found in the fat sources and also with the previously reported results showing that FAD and AO are rich in FFA (Nuchi et al., 2009; Varona et al., 2021b) and crude palm oil can reach up to 12.0% FFA, although the maximum FFA% established by the Codex Alimentarius is a 5% expressed as palmitic acid (Codex Alimentarius, 1999; Ginting et al., 2020). The DAG% in feeds also mainly depended on their content in the added fat sources and was also in agreement with the previously reported results.

The lipid class composition was also affected by the feed processing being FFA and MAG higher in mash feeds and TAG in pelleted and extruded feeds. These results in freshly produced feeds indicated a rapid hydrolysis of TAG in mash feeds due to the presence of lipases coming from the ingredients of the feeds. In fact, the presence of lipases has been reported in cereals such as wheat and barley and also in soybeans (Dierick and Decuypere, 2002; Barros et al., 2010). In addition, Dierick and Decuypere (2002)

reported lipase activity in several feedstuffs stored at room temperature, being this activity higher in non-intact cereals (ground or milled) and lower in feedstuffs that suffer a thermal treatment during their obtention, such as extruded cereals or soybean meal. Thus, lipases in our feeds might come from grinded wheat and barley and to lower extent from soybean meal. As these lipases can be inactivated by heat treatments (Camire et al., 1990; Dierick and Decuypere, 2002; Lampi et al., 2015), this would explain the lower FFA% found in pelleted and extruded feeds.

The PV in recently produced feeds was, in general, higher in soybean feeds than in palm feeds. This agreed with higher oxidizability of soybean oils than palm oils. This primary oxidation could have occurred mainly immediately after feed production (during the necessary short time interval between the production of the feed and the start of the storage study, see material and methods section), because, although the difference was not significant, the PV tended to be higher in pelleted and extruded feeds as the manufacturing processes involved heat treatments. In freshly produced feeds, the fat source had no significant influence on the content of major volatile compounds (Table 4 and Table S1, supplementary data), but, the heat treatments that took place during the pelleting and extrusion seemed to accumulate some ALD (significantly for pentanal, octanal and nonanal) and reduce the HC, KET and ALC (Table 4 and Table S1), being the magnitude of the reduction very large for ALC (mainly for 1-octen-3-ol and 1-hexanol) and hexane (Table S1, supplementary data). A large number of these volatile compounds (ALD, KET, HC, ALC and 2-alkylfurans) derive from secondary oxidation of unsaturated FA (Frankel, 1982; Choe and Min, 2006). During heat treatments, these compounds can be formed by decomposition of lipid hydroperoxides but they can also be lost by volatilization; thus, their accumulation depends on the balance between these two phenomena. The higher content of these volatiles in mash feeds might be related with the ingredients; for instance, the 1-octen-3-ol and the 1-hexanol can come from wheat, barley and soybeans (Boué et al., 2003; Cramer et al., 2005) and 1-octen-3-ol also from linoleic acid autoxidation of the fat sources (Frankel, 1982). If these volatile alcohols mainly came from the ingredients, it is plausible that the lost predominated over the formation during pelleting and extrusion.

4.3. Lipid composition and oxidation of all the experimental feeds throughout the storage

In general, the content of T and T3 in feed decreased over storage time, being the decrease of total T (Fig. 2) and of α-, β- and γ-T, and α-, β- and γ-T3 (Table S5) more accentuated after 30 days in mash than in pellet or extruded feeds, as revealed by the significant interactions. The T decrease throughout storage has been previously reported in foods and feedstuffs (mainly ground cereals) stored around room temperature (Bauernfeind and Desai, 1977; Seppanen et al., 2010); however, there are almost no studies on the stability of T and T3 during storage of compound feeds, and those that exist report controversial results and usually do not report how much of the T content comes naturally from feed ingredients or from the supplementation with α-tocopheryl acetate (Gadient et al., 1998; Kostadinović

et al., 2013). As discussed above, in our case, 41.7% of the α-T and the 25.8% of the sum of T found in freshly produced mash feeds were supplied in form of α-tocopheryl acetate by the premix. This is crucial because the esterification of the phenolic group with the acetate blocks the antioxidant activity of this group and greatly increases the oxidative stability of T in the form of α-tocopheryl acetate compared to the free forms of T and T3. Apart from this, as previously discussed, the heat conditions involved in pelleting and extrusion reduced the contents of T and T3 in freshly produced feeds, but storage at 35 °C significantly reduced the content of β-T, γ-T and total T in mash feeds but not for pelleted or extruded feeds (Table S6). This seems to disagree with the conclusion of the revision of Morin et al. (2021), who reported a higher reduction of T + T3 over storage time when extrusion was carried at 120-140 °C than when it was carried at 110 °C; however, their conclusion was based on a single extrusion study dealing with extruded rice bran stored for a long period (1 year) and clear differences were only observed at/from 150 days of storage (Shin et al., 1997). In our case, we studied storage up to 60 days and the T and T3 content remaining in our feeds after pelleting and extrusion (0 days of storage) was high (T = 125.2 and T3 = 24.7 mg/kg for pelleted feeds; T = 121.4 and T3 = 24.0 for extruded feeds, Table S5). These results indicated that in these storage conditions, in these matrices the stability of T and T3 during the storage time did not decrease more in those feeds that had undergone a more aggressive heat treatment (pelleted and extruded). Thus, as discuss below, this amount of tocols (T + T3), effectively protected feeds against oxidation during storage time for 60 days and, thus, the loss of tocols was moderate during storage. However, a greater decrease of total T in mash feeds than in pelleted and extruded feeds from 30 to 60 days of storage was observed (Fig. 2) and could be explained by the starch gelatinization in pelleted and extruded feeds that might have decreased oxygen penetration (Jimenez et al., 2012).

During storage, the TAG% decreased while DAG% and FFA% increased, which agrees, as commented above, with the lipase activity of some of the feed ingredients, particularly milled wheat and barley (Dierick and Decuypere, 2002). The storage temperature did not significantly affect lipid class composition, which might be due to a low difference in lipase activity between the two storage temperatures assayed (22 and 35 °C). However, the reduction of TAG% and the increase of FFA% throughout the storage time differed between fat sources added to the feeds, being higher in CS, RS, CP and RP feeds, and lower in AS and PFAD feeds (Fig 4). This lower magnitude of the reduction in AS and PFAD feeds might be related to their lower initial TAG%, which are the substrate of lipases. This fact also explained the interaction observed between the fat source and feed processing (Fig 5): even if higher TAG% and lower FFA% were found in pelleted and extruded feeds than in mash feeds which was related to a higher lipase inactivation by heat treatments in pelleting and extrusion, the magnitude of these TAG and FFA differences depended on the fat source, being the differences also larger for CS, RS, CP and RP feeds, and smaller for AS and PFAD feeds. A detailed look at the FFA% results obtained for each feed

type evidenced the influence of the three factors on the feed FFA%: fat source, feed processing and storage time, leading to clear differences that could also agree with the lipase activity in each type of feed during storage (Fig S1). Indeed, the FFA% increased during storage time in a different way depending on the feed processing and on the fat source. No increase in FFA% was observed for pelleted and extruded feeds during the storage for 60 days, regardless of the fat source. Therefore, pelleting and extrusion could be good processes to avoid lipase activity and TAG hydrolysis in feeds up to 60 days as was also observed in extruded rice bran (Shin et al., 1977). On the contrary, in mash feeds a large increase in FFA% was already observed after 30 days (similar at both storage temperatures) (Fig. 3), particularly for the fat sources that had more TAG (CS, RS, CP and RP) (Fig S1). For instance, after 30 days at 22 °C, FFA% had increased from 10.8% to 35.7% in CS feeds and from 14.4% to 32.2% in CP feeds. In the case of the mash feeds that included fat by-products (AS and PFAD), this increase was lower (from 55.8% to 64.6% for AS and from 78.4% to 83.3% for PFAD) because these two fat sources had a much lower TAG%, particularly PFAD. Increases of FFA% during storage of milled cereals and compound feeds have been previously reported (Shin et al., 1997; Dierick and Decuypere, 2002). The increases of FFA% observed in our study during storage of mash feeds could be relevant from a nutritional point of view, particularly when fat sources rich in TAG are added to the feeds (crude or refined oils). In fact, it is highly remarkable that at 60 days, the FFA% reached by CS mash feeds was similar to that in recently produced AS feeds. In this sense, previous studies have reported that increasing FFA contents led to a significant reduction in FA digestibility and absorption and, thus, in the dietary energy value of fat sources. These reductions were particularly important for saturated FA and depended on the animal species and especially on the age, being larger for young animals (Vilà and Esteve-Garcia, 1996; Wiseman et al., 1998; Rodriguez-Sanchez et al., 2019a). Complementary, in various studies in broilers, Rodriguez-Sanchez et al. (2019a; 2019b; 2021) found that saturated FA contents had a greater influence on FA absorption than FFA contents, and that the FFA utilization improved with the age of the animal. Therefore, in grower-finisher-broilers (22 to 37 days old) FFA contents up to 35% in feeds did not have a negative effect on FA absorption using saturated (palm fats) and unsaturated (soybean oils) diets (Rodriguez-Sanchez et al., 2021). Subsequently, it has been observed in grower-finisher broilers (23 to 35 days old) that if the FFA in the diet came from an unsaturated fat source such as AS, FA utilization was not affected up to 56.3% of FFA (unsaturated/saturated FA ratio in the diet = 3.88; Jimenez-Moya et al., 2021a), while if FFA came from a saturated fat source such as PFAD it was up to 30.0% of FFA (unsaturated/saturated FA ratio in the diet = 2.61; Jimenez-Moya et al., 2021b). Thus, the hydrolysis observed during storage of mash feeds formulated with crude and refined oils could be important for the mash feeds intended for young animals. The decreased absorption of FA in young chickens has been attributed to different factors, an insufficient emulsification process due to less mature gastrointestinal tract, a lower rate of bile secretion

and a less efficient turnover of bile acids (Serafin and Nesheim, 1970; Krogdahl, 1985; Noy and Sklan, 1995).

Regarding primary oxidation in feeds stored for 30 and 60 days, PV kept being higher in pelleted and extruded feeds compared to mash feeds (becoming significant), and it also kept being higher in feeds containing soybean sources compared to those containing palm sources (Table 5), even if the PV decreased at 30 days. These effects were observed regardless of the storage temperature (22 or 35 °C; no significant interactions with temperature; Table S2). The PV decrease at 30 days was observed especially in soybean feeds (Table S4), and especially in pelleted and extruded feeds as they had higher initial PV (Table S5). This might be due to a predominance of the decomposition of lipid hydroperoxides over their formation. Overall, a low formation of lipid hydroperoxides during storage was encountered and may be related with a high oxidative stability of all the feeds that conducted to a moderate reduction of T + T3 throughout storage time as explained above. In fact, in different foods stored between 25-50 °C (García-Pascual et al., 2003; Player et al., 2006; Lee et al., 2007) the T reduction was accompanied by a proportional increase of PV. For instance, Lee et al. (2007) in a refined soybean oil stored at 25 °C in the dark did not observe a significant increase of the PV at 90 days with a 16.6% reduction of total T; while at 120 days, when the total T were reduced a 20.9%, the increase of PV was notable.

Regarding the evolution of the content of volatile compounds during storage, even if a slight decrease in various ALD and ALC compounds was observed, the levels of the total volatile compounds were similar to the levels observed in freshly produced feeds, which agrees with a high oxidative stability of all feeds during storage. However, some slight but significant differences during storage were observed for some volatiles depending on the fat source and on the feed processing. For instance, AS feeds showed higher contents of pentanal, hexanal, Σ ALD, Σ KET and Σ FUR (mainly 2-pentylfuran, Tables 5 and S7 in supplementary data), which might be related to a slightly higher oxidation of the feeds formulated using this by-product in comparison with CS and RS feeds. On the contrary, the feeds formulated with PFAD did not show these differences in volatile compounds with CP and RP feeds at any storage time, which might indicate that the inclusion of the by-product (PFAD) did not affect the oxidative stability of feeds formulated with palm fat sources (Table 5). Regarding the influence of the feed processing on the content of volatile compounds, the differences observed for stored feeds (Table 5) were similar to the trend observed for freshly produced feeds (Table 4), except for the Σ FUR. Thus, the content of aldehydes was significantly higher in pelleted feeds, and the Σ HC, Σ KET and Σ ALC were significantly lower for pelleted and extruded feeds. In stored feeds, the Σ FUR (mainly 2-pentylfuran, Tables 5 and S7 in supplementary data) was higher for pelleted feeds. It is important to note that ALD were mainly accumulated in pelleted feeds during the pelleting process, and not during subsequent storage; while the Σ FUR (mainly 2-pentylfuran) slightly increased during storage of pelleted feeds (see the interaction between feed processing x storage time factors in Table S5, supplementary data). Thus,

this is the only result that could indicate that pelleted feeds were slightly more oxidized during storage than mash and extruded feeds, which would agree with the fact that 2-pentylfuran is produced by autooxidation of linoleic acid (Frankel, 1981). However, the levels of several volatile compounds were slightly higher in freshly produced feeds (Tables 5 and S7 in supplementary data). As commented above, these compounds during heat treatments and also during storage can be formed by decomposition of lipid hydroperoxides and also lost by volatilization, and their accumulation depends on the balance between these two phenomena. In addition, the content of several volatile compounds was higher in feeds stored at 22 °C than at 35 °C (Tables 5 and S7, in supplementary data), which could be due to a low formation rate of these compounds and a higher loss of them by volatilization at 35 °C, which in turn is compatible with a high oxidative stability of all feeds during the storage for 60 days under the conditions assayed.

5. Conclusions

The effect of processing and storage conditions on lipid hydrolysis and oxidative stability of feeds containing various fats and fat by-products was studied. As expected, a great influence of the added fat source on lipid composition of freshly produced feeds was confirmed. Regarding the lipid hydrolysis, it was observed that FFA increased very quickly in mash feeds, but not in pelleted and extruded feeds. Thus, it is plausible that heat treatments involved in pelleting and extrusion inactivated endogenous lipases and prevented the hydrolysis of the TAG. The increase of FFA in mash feeds during the storage was more pronounced in feeds containing crude and refined oils than in feeds containing the by-products (AS and PFAD). This is explained by the fact that these by-products are already rich in FFA (> 61%) and poor in TAG (< 26%), the substrates of lipases. In the case of mash feeds containing crude and refined oils, the FFA % increased from 11-14% up to 40-47% during the storage for 60 days, which could be relevant from a nutritional point of view, especially for feeds intended for young animals. The different storage temperatures (22 and 35 °C) did not significantly affect lipid hydrolysis. Although FFA content clearly increased in mash feeds during storage, this had little influence on oxidative stability of feeds under the storage conditions assayed. As expected, the heat treatments that took place during the pelleting and extrusion of feeds produced a decrease in tocols and an increase in the PV of freshly prepared feeds, but the oxidative stability of pelleted and extruded feeds during storage did not decrease compared to mash feeds. This could be due to the fact that FFA % did not increase in pelleted and extruded feeds during the storage, which could compensate for the lower levels of tocols found in these feeds. On the contrary, the levels of tocols were higher in mash feeds along the storage, but in this case also the FFA % increased. Also, aldehydes were mainly accumulated during the pelleting process, not during the subsequent storage, while the Σ FUR (mainly 2-pentylfuran) slightly increased during storage of pelleted feeds. Thus, this is the only result that could indicate that pelleted feeds had a slightly lower oxidative stability than mash and extruded feeds. However, in general, the evolution of the loss in

T, PV and content of volatile compounds during the storage indicated a high oxidative stability of all feeds.

Thus, under the storage conditions assayed, the formulation of feeds with by-products rich in FFA (AS and PFAD) did not pose a relevant problem of lipid hydrolysis or oxidative stability, whereas the formulation with crude oils (the most used in feed formulation) could result in a much higher than expected FFA content in stored mash feeds.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version

CRediT authorship contribution statement

Elisa Varona and Vitor Andre Vidal: Analytical work at the laboratory; Elisa Varona, Alba Tres, Stefania Vichi and Francesc Guardiola: Data curation; Alba Tres, Magdalena Rafecas, David Solà-Oriol and Francesc Guardiola: Conceptualization; Elisa Varona, Alba Tres, Stefania Vichi and Francesc Guardiola: Formal analysis; Magdalena Rafecas and Francesc Guardiola: Funding acquisition; Elisa Varona, Alba Tres, Stefania Vichi and Francesc Guardiola: Methodology; Magdalena Rafecas and Francesc Guardiola: Project administration; David Solà-Oriol and Francesc Guardiola: Resources; Elisa Varona, Alba Tres and Stefania Vichi: Software; Alba Tres, Magdalena Rafecas, Stefania Vichi, David Solà-Oriol and Francesc Guardiola: Supervision; Elisa Varona and Francesc Guardiola: Writing – original draft-review & editing; Vitor Andre Vidal, Magdalena Rafecas, Stefania Vichi, David Solà-Oriol and Alba Tres: Writing – review & editing.

Declaration of Competing Interest

Declarations of interest: none.

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References

AOAC (Association of Official Analytical Chemists), 2019. Official Method of Analysis 2003.05, Crude fat in feeds, cereal grains, and forages. Randall/Soxtec/diethyl ether extraction-submersion method, 21st ed. AOAC, Rockville, MD.

Baião, N.C., Lara, L.J.C., 2005. Oil and fat in broiler nutrition. Rev. Bras. Ciência Avícola 7, 129–141. https://doi.org/10.1590/s1516-635x2005000300001.

Barros, M., Fleuri, L.F., Macedo, G.A., 2010. Seed lipases: Sources, applications and properties - A review. Brazilian J. Chem. Eng. 27, 15–29. https://doi.org/10.1590/S0104-66322010000100002.

Bauernfeind, J.C., Desai, I.D., 1977. The tocopherol content of food and influencing factors. Crit. Rev. Food Sci. Nutr. 8, 337–382. https://doi.org/ 10.1080/10408397709527226.

Bockisch, M., 1988. Fats and Oils Handbook, first ed. Academic Press and AOCS Press, Illinois, USA.

Bou, R., Guardiola, F., Tres, A., Barroeta, A.C., Codony, R., 2004. Effect of dietary fish oil, α-tocopheryl acetate, and zinc supplementation on the composition and consumer acceptability of chicken meat. Poult. Sci. 83, 282–292. https://doi.org/10.1093/ps/83.2.282.

Boué, S.M., Shih, B.Y., Carter-Wientjes, C.H., Cleveland, T.E., 2003. Identification of volatile compounds in soybean at various developmental stages using solid phase microextraction. J. Agric. Food Chem. 51, 4873–4876. https://doi.org/10.1021/jf030051q.

Camire, M.E., Camire, A., Krumhar, K., 2009. Critical reviews in food science and nutrition chemical and nutritional changes in foods during extrusion chemical and nutritional changes in foods during extrusion. Food Sci. Nutr. 29, 35–57. https://doi.org/10.1080/10408399009527513.

Choe, E., Min, D.B., 2006. Mechanisms and factors for edible oil oxidation. Compr. Rev. Food Sci. Food Saf. 5, 169–186.

Codex Alimentarius. Standard for Named Vegetable Oils (CXS 210-1999). In Codex Alimentarius, International Food Standards; Codex Alimentarius: Rome, Italy, 1999; Adopted in 1999. Revised in 2001, 2003, 2009, 2017, 2019. Amended in 2005, 2011, 2013, 2015, 2019.

Cramer, A.C.J., Mattinson, D.S., Fellman, J.K., Baik, B.K., 2005. Analysis of volatile compounds from various types of barley cultivars. J. Agric. Food Chem. 53, 7526–7531. https://doi.org/10.1021/jf0506939.

Dierick, N.A., Decuypere, J.A., 2002. Endogenous lipolysis in feedstuffs and compound feeds for pigs: Effects of storage time and conditions and lipase and/or emulsifier addition. Anim. Feed Sci. Technol. 102, 53–70. https://doi.org/10.1016/S0377-8401(02)00224-9.

FEDIOL, 2021. The European Vegetable Oil and Protein Meal Industry Association - Refining of Edible Oils. http://www.fediol.eu/web/refining/1011306087/list1187970096/f1.html (accessed 27 August 2021).

FEDNA, 2002. Fundación Española para el Desarrollo de la Nutrición Animal. Normas FEDNA de Control de Calidad de Ingredientes para Piensos- Especificación Técnica de Materia Prima, Grasas

y Aceites. http://www.fundacionfedna.org/sites/default/files/8_Grasas_y_aceites.pdf (accessed on 15 April 2021).

Frankel, E.N., Neff, W.E., Selke, E., 1981. Analysis of autoxidized fats by gas chromatography-mass spectrometry: VII. volatile thermal decomposition products of pure hydroperoxides from autoxidized and photosensitized oxidized methyl oleate, linoleate and linolenate. Lipids 16, 279–285.

Frankel, E.N., 1982. Volatile lipid oxidation products. Prog. Lipid Res. 22, 1-33. https://doi.org/0163-7827/82/010001-33516.50/0.

Gadient, M., Fenster, R., 1994. Stability of ascorbic acid and other vitamins in extruded fish feeds. Aquaculture 124, 207–211. https://doi.org/10.1016/0044-8486(94)90379-4.

García-Pascual, P., Mateos, M., Carbonell, V., Salazar, D.M., 2003. Influence of storage conditions on the quality of shelled and roasted almonds. Biosyst. Eng. 84, 201–209. https://doi.org/10.1016/S1537-5110(02)00262-3.

Ghazani, S.M., Marangoni, A.G., 2013. Minor components in canola oil and effects of refining on these constituents: A review. J. Am. Oil Chem. Soc. 90, 923–932. https://doi.org/10.1007/s11746-013-2254-8.

Gibon, V., De Greyt, W., Kellens, M., 2007. Palm oil refining. Eur. J. Lipid Sci. Technol. 109, 315–335. https://doi.org/10.1002/ejlt.200600307.

Ginting, R., Wanli, Fauzi Malik, A., 2020. Crude palm oil product quality control using seven tools (case study: XYZ Company). IOP Conf. Ser. Mater. Sci. Eng. 851, 012046. https://doi.org/10.1088/1757-899X/851/1/012046.

Granda, L., Rosero, A., Benešová, K., Pluháčková, H., Neuwirthová, J., Cerkal, R., 2018. Content of selected vitamins and antioxidants in colored and nonpigmented varieties of quinoa, barley, and wheat grains. J. Food Sci. 83, 2439–2447. https://doi.org/10.1111/1750-3841.14334.

Gunstone, F.D., 2011. Vegetable Oils in Food Technology: Composition, Properties and Uses, second ed. Blackwell, Oxford, UK. https://doi.org/10.1002/9781444339925.ch3.

IUPAC (International Union of Pure and Applied Chemistry), 1991. Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography (method 2508). In Standard Methods for the Analysis of Oils, Fats and Derivatives, seventh ed. IUPAC, Blackwell Scientific Publications: Oxford, UK.

Jiménez, A., Fabra, M.J., Talens, P., Chiralt, A., 2012. Edible and biodegradable starch films: a review. Food Bioprocess Technol. 5, 2058–2076. https://doi.org/10.1007/s11947-012-0835-4.

Jimenez-Moya, B., Barroeta, A.C., Tres, A., Soler, M.D., Sala, R., 2021a. Replacement of palm oil with soybean acid oil in broiler chicken diet: fat digestibility and lipid class content along the intestinal tract. Animals 11, 2586. https://doi.org/10.3390/ani11041035.

Jimenez-Moya, B., Barroeta, A.C., Tres, A., Soler, M.D., Sala, R., 2021b. Soybean oil replacement by palm fatty acid distillate in broiler chicken diets: Fat digestibility and lipid-class content along the intestinal tract. Animals 11, 1035. https://doi.org/10.3390/ani11041035.

Johnson, D.R., Decker, E.A., 2015. The role of oxygen in lipid oxidation reactions: a review. Annu. Rev. Food Sci. Technol. 6, 171–190. https://doi.org/10.1146/annurev-food-022814-015532.

Kostadinović, L., Teodosin, S., Spasevski, N., Đuragić, O., Banjac, V., Vukmirović, Đ., Sredanović, S., 2014. Effect of pelleting and expanding processes on stability of vitamin A in animal feeds. J. Process. Energy Agric. 18, 44–46.

Krogdahl, A., 1985. Digestion and absorption of lipids in poultry. J. Nutr. 115, 675–685. https://doi.org/10.1093/jn/115.5.675.

Lampi, A.M., Damerau, A., Li, J., Moisio, T., Partanen, R., Forssell, P., Piironen, V., 2015. Changes in lipids and volatile compounds of oat flours and extrudates during processing and storage. J. Cereal Sci. 62, 102–109. https://doi.org/10.1016/j.jcs.2014.12.011.

Lee, J., Lee, Y., Choe, E., 2007. Temperature dependence of the autoxidation and antioxidants of soybean, sunflower, and olive oil. Eur. Food Res. Technol. 226, 239–246. https://doi.org/10.1007/s00217-006-0532-5.

Leeson, S., Summers, J.D., 2008. Commercial Poultry Nutrition, third. ed. Nottingham, UK.

Morin, P., Gorman, A., Lambrakis, L., 2021. A literature review on vitamin retention during the extrusion of dry pet food. Anim. Feed Sci. Technol. 277, 114975. https://doi.org/10.1016/j.anifeedsci.2021.114975.

Nielsen, M.M., Hansen, Å., 2008. Rapid high-performance liquid chromatography determination of tocopherols and tocotrienols in cereals. Cereal Chem. 85, 248–251. https://doi.org/10.1094/CCHEM-85-2-0248.

Noy, Y., Sklan, D., 1995. Digestion and absorption in the young chick. Poult. Sci. 74, 366–373. https://doi.org/10.3382/ps.0740366.

Nuchi, C., Guardiola, F., Bou, R., Bondloli, P., Bella, L.D., Codony, R., 2009. Assessment of the levels of degradation in fat co-and byproducts for feed uses and their relationships with some lipid composition parameters. J. Agric. Food Chem. 57, 1952–1959. https://doi.org/10.1021/jf803369h.

Overholt, M.F., Dilger, A.C., Boler, D.D., Kerr, B.J., 2018. Influence of feeding thermally peroxidized soybean oil on growth performance, digestibility, and gut integrity in finishing pigs. J. Anim. Sci. 96, 2789–2803. https://doi.org/10.1093/jas/sky091.

Player, M.E., Kim, H.J., Lee, H.O., Min, D.B., 2006. Stability of α-, γ-, or δ-tocopherol during soybean oil oxidation. J. Food Sci. 71. https://doi.org/10.1111/j.1750-3841.2006.00153.x.

Rodriguez-Sanchez, R., Tres, A., Sala, R., Garcés-Narro, C., Guardiola, F., Gasa, J., Barroeta, A.C., 2019a. Effects of dietary free fatty-acid content and saturation degree on lipid-class composition and fatty-acid digestibility along the gastrointestinal tract in broiler starter chickens. Poult. Sci. 98, 4929–4941. https://doi.org/10.3382/ps/pez253.

Rodriguez-Sanchez, R., Tres, A., Sala, R., Guardiola, F., Barroeta, A.C., 2019b. Evolution of lipid classes and fatty acid digestibility along the gastrointestinal tract of broiler chickens fed different fat sources at different ages. Poult. Sci. 98, 1341–1353. https://doi.org/10.3382/ps/pey458.

Rodriguez-Sanchez, R., Tres, A., Sala, R., Soler, M.D., Guardiola, F., Barroeta, A.C., 2021. Effects of free-fatty-acid content and saturation degree of the dietary oil sources on lipid-class content and fatty-acid digestibility along the gastrointestinal tract in broilers from 22 to 37 days of age. Poult. Sci. 100, 101261. https://doi.org/10.1016/j.psj.2021.101261.

Seppanen, C.M., Song, Q., Saari Csallany, A., 2010. The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. J. Am. Oil Chem. Soc. 87, 469–481. https://doi.org/10.1007/s11746-009-1526-9.

Serafin, J.A., Nesheim, M.C., 1970. Influence of dietary heat-labile factors in soybean meal upon bile acid pools and turnover in the chick. J. Nutr. 100, 786–796. https://doi.org/10.1093/jn/100.7.786.

Shin, T.S., Godber, J.S., Martin, D.E., Wells, J.H., 1997. Hydrolytic stability and changes in E vitamers and oryzanol of extruded rice bran during storage. J. Food Sci. 62, 704–728. https://doi.org/10.1111/j.1365-2621.1997.tb15440.x.

Shurson, G.C., Kerr, B.J., Hanson, A.R., 2015. Evaluating the quality of feed fats and oils and their effects on pig growth performance. J. Anim. Sci. Biotechnol. 6, 1-11. https://doi.org/10.1186/s40104-015-0005-4.

Svihus, B., Zimonja, O., 2011. Chemical alterations with nutritional consequences due to pelleting animal feeds: A review. Anim. Prod. Sci. 51, 590–596. https://doi.org/10.1071/AN11004.

Vaisali, C., Charanyaa, S., Belur, P.D., Regupathi, I., 2015. Refining of edible oils: A critical appraisal of current and potential technologies. Int. J. Food Sci. Technol. 50, 13–23. https://doi.org/10.1111/ijfs.12657.

Varona, E., Tres, A., Rafecas, M., Vichi, S., Sala, R., Guardiola, F., 2021a. Oxidative quality of acid oils and fatty acid distillates used in animal feeding. Animals 11, 2559. https://doi.org/10.3390/ani11092559.

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F., 2021b. Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding. Animals 11, 196. https://doi.org/10.3390/ani11010196.

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F., 2021c. Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding. MethodsX. 8, 101334. https://doi.org/10.1016/j.mex.2021.101334.

Vilà, B., Esteve-Garcia, E., 1996. Studies on acid oils and fatty acids for chickens. II. Effect of free fatty acid content and degree of saturation of free fatty acids and neutral fat on fatty acid digestibility. Br. Poult. Sci. 37, 119–130. https://doi.org/10.1080/00071669608417842.

Wiseman, J., Powles, J., Salvador, F., 1998. Comparison between pigs and poultry in the prediction of the dietary energy value of fats. Anim. Feed Sci. Technol. 71, 1–9. https://doi.org/10.1016/S0377-8401(97)00142-9.

5.1.5. Use of soybean acid oil and palm fatty acid distillate in broiler diets: effects on lipid composition and oxidative stability of meat/ Uso de aceite ácido de soja y ácido graso destilado de palma en dietas para pollos: efectos en la composición lipídica y estabilidad oxidativa de la carne

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F., *Poult. Sci*. En preparación.

Los aceites ácidos (AA) y los ácidos grasos destilados (AGD) son subproductos que provienen del refinado químico y físico de grasas y aceites comestibles, respectivamente. A pesar de su composición variable, son ingredientes energéticos potenciales en la alimentación animal porque son subproductos con alto contenido de ácidos grasos libres (AGL). Como es un hecho conocido que la composición lipídica de la carne refleja la composición lipídica de los alimentos, la formulación de la dieta es una herramienta relevante para controlar la calidad de la carne, en términos de composición, especialmente considerando los ácidos grasos (AG) y el perfil de tocoferoles (T) y de tocotrienoles (T3), pero también los parámetros de oxidación también pueden influir en la calidad.

El objetivo principal de este estudio fue evaluar cómo el uso de subproductos grasos (AA obtenido del refinado de aceite de soja (AS), AGD del aceite de palma (PFAD)) en la alimentación de pollos de engorde en comparación con su correspondiente crudo (CS, aceite crudo de soja); CP, aceite crudo de palma) y aceites refinados (RS, aceite refinado de soja; RP, aceite refinado de palma) pueden afectar la composición y estabilidad oxidativa de la carne. Un total de 72 pollos de engorde hembras de 1 a 37 días se distribuyeron aleatoriamente en 36 jaulas experimentales correspondientes a los seis tratamientos dietéticos (CS, AS, RS, CP, PFAD y RP). Luego, una vez tomadas las muestras, las muestras de carne fueron sometidas a diferentes tratamientos tecnológicos: fresco (F), cocido (C) y cocido y almacenamiento refrigerado (CR) para estudiar sus efectos en la calidad de la carne. Así, se realizó un diseño factorial 6 x 3, con los 6 tipos de fuentes grasas (CS, AS, RS, CP, PFAD y RP), y 3 procesos tecnológicos (F, C y CR) dando un total de 108 muestras recogidas. La carne de los pollos de engorde alimentados con los subproductos no mostró diferencias significativas en cuanto a composición y oxidación cuando se compararon con los correspondientes aceites crudos o refinados. De acuerdo con estos resultados se concluye un posible aprovechamiento de estos subproductos si mantienen una calidad adecuada, por lo que no afectan la calidad lipídica de la carne. Además, el proceso tecnológico afectó claramente los parámetros de oxidación de la carne, especialmente en las carnes cocidas y cocidas almacenadas en refrigeración que presentaron los valores más altos de estos parámetros.

Use of soybean acid oil and palm fatty acid distillate in broiler diets: effects on lipid composition and oxidative stability of meat

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ABSTRACT Acid oils (**AO**) and fatty acid distillates (**FAD**) are by-products obtained from the chemical and physical refining of edible fats and oils, respectively. Despite their variable composition they are potential energetic ingredients in animal feeding because they are high-free fatty acid (**FFA**) by-products. As it is a well-known fact that the lipid composition of meat reflects the lipid composition of feeds, diet´s formulation is a relevant tool to control the quality of meat, in terms of composition, especially considering fatty acid (**FA**) and tocopherol (**T**) and tocotrienol (**T3**) profiles, and of oxidative stability. The main objective of this study was to assess how the use of fat by-products (AO obtained from soybean oil refining (**AS**), FAD from palm oil (**PFAD**) refining) in broiler feed compared to their corresponding crude (**CS**, crude soybean oil; **CP**, crude palm oil) and refined oils (**RS**, refined soybean oil; **RP**, refined palm oil) may affect the composition and oxidative stability of meat. The nutritional quality of the AS and PFAD used in this study was slightly better than average, compared to the by-products present on the market, being their content of non-energy compounds (MIU value) only slightly above 5%. A total of 144 female broiler chickens from 1 to 37 days were randomly distributed in 36 experimental cages corresponding to the six dietary treatments (CS, AS, RS, CP, PFAD and RP). Then, once samples were taken, meat samples were subjected to different technological treatments: fresh (**F**), cooked (**C)** and cooked and refrigerated storage (**CR**) to study their effects on meat oxidative quality. In general, differences in FA and T and T3 composition and on primary oxidation were more related to the botanical origin of the fats used, while meat from broilers fed the by-products did not show significant differences about composition and oxidation when they were compared to the corresponding crude or refined oils. Cooking and refrigeration of cooked meat increased secondary oxidation regardless of the use of the by-products. According to these results it is concluded that, at least when the nutritional quality of the by-products is acceptable (slightly better than the average

market level), it is possible the use of these by-products in broiler feeding without modifying the composition and oxidizability to broiler meat as sustainable and cheaper alternative to the use of crude oils.

Key words: acid oil, fatty acid distillate, fat by-product, oxidative stability, meat quality

INTRODUCTION

Feeding acid oils (**AO**) and fatty acid distillates (**FAD**) might be a sustainable and cheaper alternative to common fats used in animal feeds. They are by-products from the refining of edible vegetable oils and fats, mainly characterized by their high free fatty acid (**FFA**) content and their contents of naturally bioactive compounds such as tocopherols (**T**), tocotrienols (**T3**), and sterols, among others (Dumont and Narine, 2007; Varona et al., 2021a). Therefore, they can be considered valuable and more economic sources of energy, essential fatty acids (**FA**) and fat-soluble vitamins for feeds (Varona et al., 2021a). Nevertheless, their nutritive and oxidative quality differ from that of crude and refined oils as AO and FAD usually show high contents of moisture and volatile matter (**M**), insoluble impurities (**I**) and unsaponifiable matter (**U**) (known as MIU value) and oxidation compounds (Varona et al., 2021a,b). Even if their profile of saturated fatty acids (**SFA**), monounsaturated fatty acids (**MUFA**) and polyunsaturated fatty acids (**PUFA**) is, in general terms, quite similar to that of the crude oil from which they are produced, they present a much higher FFA content (Nuchi et al., 2009; Varona et al., 2021a). Even if high FFA contents have been related with lower fat absorption rates, the magnitude of this effect depends on the degree of saturation of the fat and the age of the animal (Wiseman and Salvador, 1991; Rodriguez-Sanchez et al., 2019a,b; Jimenez-Moya et al., 2021a,b). In this regard, the saturation degree may impact higher the fat utilization than the FFA content, being both effects more relevant in young animals (Rodriguez-Sanchez et al., 2019a; Jimenez-Moya et al., 2021b,c). All this may influence their digestibility and energy value and thus, the animal growth performance (Ravindran et al., 2016; Lindblom et al., 2019; Wealleans et al., 2021).

It is well known that the diet composition influences the composition, quality and stability of meat. On the one hand, tissue FA composition is significantly influenced by the FA profile of the diet, reflecting the composition of fat source added to feeds (Pinchasov and Nir, 1992; Bou et al., 2009; Ayed et al., 2015). On the other hand, the extent of oxidation in meat and its oxidative stability are related to the balance between unsaturated fatty acids (**UFA**) (substrates), pro-oxidants (some inorganic elements or enzymes) and antioxidant content, being the more UFA and the lower antioxidant content in the diet, the higher the formation of oxidation products in meat (Wood et al., 2004; Bou et al., 2009). Also, some secondary oxidation products from fats added to feeds are reported to be partly absorbed in the intestines and accumulated in the liver (Kanazawa and Ashida, 1998). Regarding primary oxidation compounds, those supplied by the diet seem to be decomposed before

being absorbed (Kanazawa and Ashida, 1998). However, in many cases, the occurrence of primary oxidation compounds in the diet might also entail a reduction of available antioxidants that, in turn, might imply a reduction of T in the tissues and therefore an increase in their oxidability (Sheehy et al., 1994; Bou et al., 2006a; Tres et al., 2013). Overall, the increase in meat oxidation leads to impairments of its sensorial quality (off-odor and off-flavor), nutritional value and shelf-life (Bou et al., 2009; Zhang et al., 2011).

Since the composition and oxidation status of AO and FAD differs from that of crude oils (Varona et al., 2021a,b), attention should be paid to the assessment of their effects on meat composition so that its nutritive quality and oxidative stability are adequate from a human nutrition approach. Nevertheless, scarce studies have focused on the use of these particular by-products in broiler feeding and on their repercussion on meat quality. Our hypothesis is that since the composition of AO and FAD differs from that of crude oils, their effects on meat FA and T composition and on oxidative stability might also differ. Moreover, it could be possible that this influence was different depending on the type of by-product because the composition of AO and FAD is influenced both by the type of refining and by the composition of the crude oil (Varona et al., 2021a,b). On the one hand, AO are obtained from chemical refining which is usually applied to unsaturated oils and fats such as crude soybean oil (**CS**). In this refining, FFA are neutralized with an alkali, removed in form of soap-stocks by centrifugation, and acidulated to obtain the AO. But together with FFA, this process also washes out some acylglycerols, M, T, polymeric compounds and gums (if not previously removed) that end up in the AO (Varona et al., 2021a,b). On the other hand, FAD are by-products from physical refining which is commonly applied to refine saturated fats and oils such as crude palm oil (**CP**). In this case, FAD are obtained from the distillation step in which FFA are removed, but concomitantly with them, other compounds such as low-molecular weight secondary oxidation compounds, T and sterols might also be distilled out from the refined oil and end up in the FAD (Varona et al., 2021a,b). In turn, as the triacylglycerols (**TAG**) are hardly distillable, the FFA content of FAD (87.2 to 93.6%) is usually higher than that of AO (31.7 to 65.3%) (Varona et al., 2021a).

Given the above facts, the aim of this study is to evaluate the effects of using AO and FAD as feed fats for broilers on the composition and oxidative stability of chicken meat, comparing their effects with those of crude and refined oils. Also, the effects on fresh meat (**F**) were compared with those obtained after subjecting meats to oxidative stress procedures: cooking (**C**) and cooking and refrigerated storage (**CR**).

MATERIALS AND METHODS

Experimental design

Soybean AO (**AS**) and palm FAD (**PFAD**) were the selected AO and FAD because they are highly available by-products. They were both compared with their corresponding crude oils (CS and CP, respectively), because crude oil is the type of feed fat that is commonly used in broiler feeding, being CS one of the mostly used. The corresponding refined oils (refined soybean oil, **RS** and refined palm oil, **RP**) were also included in the design as control fats because during refining some compounds are washed out from crude oils and accumulated in the by-products so that refined oils can meet the standards for human consumption. The three different soybean oils (CS, AS and RS) were provided by Bunge International Limited (Amsterdam, The Netherlands); the three different palm oils (CP, PFAD and RP) were provided by Lipidos Santiga S.A. (Santa Perpètua de Mogoda, Spain). The following parameters in the experimental fats were analyzed in duplicate as described in Varona et al. (2021c): M, I, U, the FA composition, the T and T3 contents, the lipid class composition (TAG, diacylglycerols (**DAG**); monoacylglycerols (**MAG**) and FFA), the peroxide value (**PV**) and the *p*-anisidine value (***p*-AnV**).

The experimental feeds were produced from a basal diet formulated following the FEDNA (2008) nutritional requirements for broilers (Table 1) and manufactured at Pinsos Molinet S.A. (Prats de Lluçanès, Spain). This basal diet was supplemented with 6% (w/w) of the different fat sources in a mixer at room temperature at Lindo Pet Global S.A. (Castellar del Vallès, Spain).

Table 1. Ingredients (%) of the experimental basal diet.

	Starter diet (from 0 to 21 days)	Grower-finisher diet (to 37 days)
Wheat	54.49	44.02
Soybean meal 47%	35.40	27.25
Barley	-	18.58
Experimental fats[1]	6.00	6.00
Calcium carbonate	1.44	1.39
Monocalcium phosphate	0.99	1.20
Titanium dioxide	0.50	0.50
Vitamin and mineral premix[2]	0.40	0.40
Sodium chloride	0.40	0.35
DL-Methionine	0.23	0.17
L-Lysine	0.15	0.12
L-Threonine	-	0.02

[1] CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate and RP, refined palm oil. [2] Provides per kg of feed: vitamin A (from retinol), 10,000 IU; vitamin D_3 (from cholecalciferol), 4,800 IU; vitamin E (from α-tocopheryl acetate), 45 mg; vitamin B_1, 3 mg; vitamin B_2, 9 mg; vitamin B_6, 4.5 mg; vitamin B_{12}, 40 µg; vitamin K_3, 3 mg; calcium pantothenate, 16.5 mg; nicotinic acid, 51 mg; folic acid, 1.8 mg; biotin, 150 µg; Fe (from $FeSO_4 \cdot 7H_2O$), 54 mg; I (from $Ca(I_2O_3)_2$), 1.2 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 12 mg; Mn (from MnO), 90 mg; Zn (from ZnO), 66 mg; Se (from Na_2SeO_3), 0.18 mg; β-glucanase 150 U; xylanase 270 U.

Thus, six different feeds were obtained in mash form, corresponding to the addition of CS, AS, RS, CP, PFAD and RP. Feed samples were taken immediately after production, ground and 55 g aliquots

were vacuum-packed in high-barrier multilayer bags (Cryovac BB3255, permeability to O_2, 17 cm^3/m^2 per day per bar at 23 °C and 0% relative humidity, ASTMD-3985, from Sealed Air Spain, Abrera, Spain) and stored at -20 °C until analysis. The following determinations were conducted in the experimental feeds in duplicate as described in "(E. Varona, unpublished data)": M, FA composition ,T and T3 content, PV and volatile compounds (**VC**).

Meat sampling

A total of 144 female broiler chickens (Ross 308) were housed and fed at the animal experimental facilities of the Universitat Autònoma de Barcelona (Bellaterra, Spain) under standard conditions of temperature, humidity and ventilation. This experimental procedure received the prior approval from the Animal Protocol Review Committee of this University (number code: 3938) and agreed with the European Union guidelines for the care and use of animals in research (2010/63/EU). Birds were randomly distributed into 36 experimental cages (4 animals per cage) corresponding to the six dietary treatments containing the experimental fats at 6% w/w (CS, AS, RS, CP, PFAD and RP), with 6 replicates each one (each experimental unit was a cage). In all cases, feed and water were provided ad libitum. Broilers were slaughtered when they reached 37 days of age according to commercial procedures. Then, legs with skin from two animals from each cage were hand-deboned, pooled and immediately ground. Meat was divided into three types: F, C and CR. For F meats, sample aliquots of approximately 20 g were vacuum-packed (up to 76 cm Hg) in high-barrier multilayer bags (Cryovac BB3055; permeability to O_2, 17 cm^3/m^2 per day per bar at 23 °C and 0% or 100% relative humidity, ASTMD-3985 from Sealed Air Spain, Abrera, Spain) and stored at -20 °C until analysis. For C and CR meats, aliquots of 20 g were packed in Cryovac HT3000 bags (permeability to O_2, 10 and 19 cm^3/m^2 per day at 23 °C and 0% and 80% relative humidity respectively, ASTMD-3985 from Sealed Air Spain, Abrera, Spain) and then cooked by means of a 1000-LC autoclave (programed to reach up to 82 °C in 20 min, then it was kept at 82 °C for 35 min and last the temperature was decreased to 40 °C in 15 min; reached internal temperature was 83 °C). Once this cooking process was completed, the samples were divided into two groups, a first group (C meat) was directly vacuum-packed in Cryovac BB3055 bags and stored at -20 °C until they were analyzed, and the second group (CR meat) was maintained in refrigeration for 17 days (average temperature of 4 °C) and then vacuum-packed in a BB3055 bags and stored at -20 °C until they were analyzed. All determinations in meat samples were carried out in duplicate, except the determination of VC contents (where once repeatability was demonstrated, only one replicate was performed for each sample).

Meat Fatty Acid Composition

The FA composition of F meat was determined according to Bou et al. (2001), where 1 g of chicken meat plus 20 mL of chloroform: methanol (2:1, v/v) were used in the lipid extraction. The FA

methyl esters were obtained by a double methylation procedure, determined by GC-FID and identified as described by Varona et al. (2021c). Results were expressed as internal peak area normalization (%). All the reagents used for the lipid extraction were of analytical grade purchased from Scharlau (Sentmenat, Spain).

Meat Tocopherol and Tocotrienol Content

The T and T3 content was determined after saponifying 2 g of chicken meat with methanolic KOH and extraction of the unsaponifiable fraction with petroleum ether (boiling point 40-60 °C) according to Bou et al. (2004). The analysis of the different tocol analogues was done by HPLC-FLD according to Varona et al. (2021c) using a Phenomenex Luna Silica (2) column (4.6 mm I.D. x 150 mm, 3 µm diameter of the particles, pore size: 100 Å; Phenomenex, Torrance, CA) and the identification and quantitation were done by means of calibration curves built with α-, β-, γ-, δ-T standards purchased from Calbiochem® (Merck, Darmstadt, Germany). The content of α-, β-, γ- and δ-T3 was calculated by applying the calibration curve obtained for the corresponding T analogue. All reagents and antioxidants used were of ACS grade, and were obtained from Scharlau (Sentmenat, Spain) or Panreac (Castellar del Vallés, Spain), except for butylated hydroxytoluene (BHT) and pyrogallol that were obtained from Sigma-Aldrich (St. Louis, USA). Solvents used as mobile phase in the HPLC separation (1,4-dioxane and n-hexane 99%) were of HPLC grade and were obtained from Scharlau (Sentmenat, Spain).

Meat Lipid Hydroperoxide Content and Susceptibility to Oxidation

The content of lipid hydroperoxides (**LHP**) of meat and its susceptibility to oxidation were evaluated by means of the ferrous-oxidation xylenol-orange (**FOX**) method, carried out as described by Grau et al. (2000a) but weighing 2 g of meat sample in 15 mL of methanol. An aliquot of 190 or 140 µl of the previous extract for F meat or C and CR meat, respectively, was added to a glass cuvette containing the FOX reagents and was incubated in the dark for 96 h (at that point, absorbance was steady). Absorbance was measured at 560 nm (Shimadzu UV-3600 (Kyoto, Japan)) at 30 min (as a measure of the LHP present in meat samples from the beginning, named LHP content) and at 96 h (as a measure of the amount of LHP formed depending on sample´s susceptibility to oxidation – named LHP final value) (Grau et al., 2000a; Aleman et al., 2010). The LHP were quantified by means of a calibration curve built between increasing concentrations of cumene hydroperoxide standard (**CHP**, Sigma-Aldrich , St. Louis, MO, USA) and the corresponding absorbances measured at 30 min, and expressed as mmol of CHP eq/kg of meat. The methanol (HPLC grade) and the other reagents (ACS grade) were purchased from Scharlau (Sentmenat, Spain).

Meat TBA (2-Thiobarbituric Acid) Value

The thiobarbituric acid (**TBA**) value was measured after an extraction with trichloroacetic acid, reaction with TBA at 70 °C for 30 min, measurement of the absorbance spectrum between 400 and

650 nm, the calculation of the 3rd derivative of the spectrum at 521.5 nm (peak height) and the quantification by means of a calibration curve built with increasing concentrations of malondialdehide (**MDA**) obtained by hydrolyzing tetraethoxypropane and the peak height of the derivative of the spectrum at 521.5 nm (Sigma-Aldrich, St Louis, MO, USA) (Grau et al., 2000b). Results were expressed as µg of MDA/kg of meat. Reagents were of ACS grade (Scharlau, Sentmenat, Spain).

Meat Volatile Compounds (VC)

The content of VC in meat was determined by SPME-GC-MS. First, 1 g of meat was weighed into a 10 mL screw-capped GC vial, in which 2 mL of a 1 mg/kg standard solution of 4-methyl-2-pentanol was added plus 0.5 mL of 2000 mg/kg EDTA solution and 0.5 mL of 200 mg/kg propyl gallate (**PG**) solution (all the solutions were prepared every day of analysis in distilled deionized water) as antioxidants to protect the sample during the analytical process. The mixture was homogenized twice for 30 s using a T18 Ultra-Turrax (IKA®, Staufen, Germany) covering the upper part of the vial with Parafilm® and keeping it in ice during the homogenization. Before capping the vials with caps having a silicone/PTFE septum, three glass balls were added. Then, samples were injected in the GC-MS (Agilent 6890N Network GC system coupled to a quadrupole mass spectrometer Agilent 5975C Inert MSD (Agilent Technologies, Santa Clara, USA)), equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland) with agitator. Sample conditioning was done for 10 min at 45 °C under agitation. After this, a divinylbenzene/carboxen/polymethylsiloxane (**DVD/CAR/PDMS**) fibre 50/30 µm, 2 cm long from Supelco (Bellefonte, PA, USA) was exposed to the sample headspace during 30 min at 45 °C, and then desorbed for 10 min at the GC injector port at 260 °C. A Supelcowax-10 capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness, Supelco, Bellefonte, PA, USA) was used for the analytes separation. The oven temperature program began at 40 °C (held for 10 min); 3 °C/min up to 150 °C (held for 0.1 min); 15 °C/min up to 250 °C (held for 5 min). Helium was used as carrier gas with a constant flow of 1 mL/min. The temperatures of the ion source and the transfer line were 230 and 280 °C, respectively. Acquisition was performed in the selected ion monitoring (**SIM**) mode, by analyzing the Extracted Ion Chromatogram (**EIC**) of the following specific ions: m/z 44, 55, 56, 57, 58, 70, 81 and 98. The ionization energy was 70 eV. Data were acquired and analyzed by an Agilent MSD ChemStation. The reagents, PG and EDTA were purchased from Scharlau (Sentmenat, Spain) and the standard 4-methyl-2-pentanol from Sigma-Aldrich (St. Louis, MO, USA). Relative amounts of VC were calculated by the internal standard (IS) method. Finally, VC results were expressed as milligrams per kilogram as equivalents of 4-methyl-2-pentanol .

Statistical Analysis

For F meats (n=36, resulting from 6 dietary treatments x 6 replicates per treatment), one-way ANOVA was performed to evaluate whether the type of added fat influenced meat FA composition

and tocol content. Then, to study how the different dietary treatments (CS, RS, AS, CP, RP, PFAD) and the technological treatment (F, C and CR) affected the T and T3 content, the LHP content, the LHP final value, the TBA value and the VC of broiler meat, a multifactorial ANOVA including interactions between the two main factors was used (n = 108, resulting from 6 dietary treatments x 6 replicates per treatment x 3 types of meat (F, C, CR)). Significantly different means of main effects were evaluated by using Tukey´s post-hoc tests (the results were considered significant at $P \leq 0.05$). Data analysis was performed with IBM SPSS Statistics (v 23, IBM, Armonk, NY, USA).

Principal component analysis (**PCA**) was conducted on T and T3 content, LHP content, LHP final value, TBA values and VC composition of F, C and CR meats to explore the natural distribution and grouping of samples, to detect outlying samples, and to investigate correlations among variables. Data matrix used in this study consisted of 108 rows (samples) x 24 columns corresponding to the 24 variables (all variables were mean-centered and scaled to unit variance). The software used for PCA calculation was SIMCA v13.0 (Umetrics AB, Umea, Sweden).

RESULTS AND DISCUSSION

Chemical Characterization of Fat Sources

The analyzed composition and oxidation status of the fat sources included in the experimental feeds is shown in Table 2. As expected, comparing soybean and palm sources, the later showed higher SFA and *cis*-MUFA, and lower *cis*-PUFA. Comparing the FA composition of crude oils, refined oils and the by-products, no high differences in magnitude were observed, although slight lower C18:1 n-9 was found in both by-products compared to crude and refined oils, as well as lower C18:2 n-6 in PFAD and lower C18:3 n-3 in AS with respect to their crude and refined counterparts. Also, slightly higher trans FA were observed in PFAD compared with CP or RP, although the amount was quite low (0.22%).

The T and T3 contents agreed with the typical composition given for soybean and palm sources according to Codex Alimentarius (1999) being the T contents higher in soybean sources, and the T3 higher in palm sources (especially γ-T3 followed by α-T3), leading to a higher global sum of T+T3 in soybean (Table 2). Within soybean oils, the highest T content was observed in CS, closely followed by AS (lower than CS by 11.32%) and RS (lower than CS by 39.43%), being in all cases γ-T the major T. Within palm sources, the highest T was found in RP closely followed by CP (lower than RP by 16.54%) while PFAD presented a much lower content (78.54% less compared to RP).

The M, I, U, FFA, and *p*-AnV values encountered for the by-products were within the range of values encountered for similar AO and FAD: while the M content of AS and the *p*-AnV of PFAD were similar to the median values reported for other AS (M median=1.08%) and PFAD (*p*-AnV median =54.7) from the market, the U value of AS and the I value of PFAD were below the medians encountered for similar AS (U median=4.38%) and PFAD (I median=4.86%) and the FFA of AS was above (FFA median

=53.2%) (Varona et al., 2021a,b). This comparison with other AS and PFAD samples from the market, indicates that the quality of the experimental AS and PFAD used in this study was superior than that usually encountered within these by-products. In fact, their MIU value was only slightly above 5% (5.34% for AS; 5.11% for PFAD) which is the maximum MIU recommended by some associations for the development of animal feeding strategies, while that of many AS and PFAD in the market is above this recommendation (Varona et al., 2021a).

Table 2. Chemical composition of the different fat sources used in the animal diets (n = 2) [1].

	CS	AS	RS	CP	PFAD	RP
M (g/100 g)	0.20 ± 0.01	1.43 ± 0.02	ND	0.03 ± 0.00	0.01 ± 0.04	ND
I (g/100 g)	1.46 ± 0.06	1.57 ± 0.36	1.27 ± 0.04	0.80 ± 0.03	3.76 ± 0.23	0.59 ± 0.09
U (g/100 g)	0.95 ± 0.07	2.34 ± 0.03	0.99 ± 0.16	0.65 ± 0.05	1.34 ± 0.02	0.21 ± 0.03
C16:0 (%)	11.3 ± 0.03	14.9 ± 0.25	11.0 ± 0.01	43.1 ± 0.11	46.6 ± 0.16	43.9 ± 0.48
C18:0 (%)	3.3 ± 0.00	3.5 ± 0.01	3.5 ± 0.00	4.3 ± 0.01	6.6 ± 0.03	4.6 ± 0.04
C18:1 n-9 (%)	23.7 ± 0.05	21.1 ± 0.02	25.1 ± 0.01	38.9 ± 0.10	35.0 ± 0.15	38.4 ± 0.37
C18:2 n-6 (%)	52.2 ± 0.02	51.7 ± 0.18	51.7 ± 0.00	10.4 ± 0.02	8.5 ± 0.05	9.7 ± 0.07
C18:3 n-3(%)	6.1 ± 0.03	5.3 ± 0.03	5.3 ± 0.00	0.3 ± 0.00	0.3 ± 0.00	0.1 ± 0.00
SFA (%)	16.0 ± 0.02	19.9 ± 0.22	15.8 ± 0.02	49.4 ± 0.12	55.1 ± 0.19	50.6 ± 0.47
cis-MUFA (%)	25.6 ± 0.06	23.1 ± 0.01	27.1 ± 0.01	39.9 ± 0.10	35.9 ± 0.14	39.4 ± 0.40
trans-C18:1 (%)	0.03 ± 0.003	0.04 ± 0.000	0.04 ± 0.001	0.03 ± 0.004	0.22 ± 0.006	0.08 ± 0.003
cis-PUFA (%)	58.3 ± 0.05	57.0 ± 0.21	57.0 ± 0.01	10.7 ± 0.02	8.8 ± 0.06	9.8 ± 0.07
TAG (%)	95.1 ± 0.16	25.3 ± 1.79	96.3 ± 0.24	82.2 ± 0.14	4.3 ± 0.51	92.5 ± 0.47
DAG (%)	3.1 ± 0.04	13.5 ± 0.52	3.2 ± 0.35	10.3 ± 0.14	3.2 ± 0.18	7.5 ± 0.47
MAG (%)	ND	ND	ND	ND	ND	ND
FFA (%)	1.7 ± 0.13	61.2 ± 1.28	0.5 ± 0.11	7.5 ± 0.28	92.5 ± 0.69	0.0 ± 0.00
α-T (mg/kg)	304.1 ± 23.19	99.8 ± 10.32	182.9 ± 24.12	162.1 ± 8.19	35.0 ± 2.87	192.0 ± 1.77
γ-T (mg/kg)	967.4 ± 32.05	786.9 ± 44.48	660.7 ± 70.75	2.2 ± 0.59	2.2 ± 1.91	4.1 ± 0.34
δ-T (mg/kg)	333.0 ± 23.53	539.8 ± 53.07	140.7 ± 18.31	ND	4.4 ± 4.11	ND
α-T3 (mg/kg)	ND	ND	ND	244.0 ± 12.31	19.1 ± 1.89	189.2 ± 2.36
γ-T3 (mg/kg)	ND	ND	ND	286.3 ± 18.53	14.9 ± 1.30	203.3 ± 3.74
T (mg/kg)	1651.1 ± 82.45	1464.2 ± 111.74	1007.3 ± 116.47	166.6 ± 9.09	42.8 ± 9.01	199.4 ± 1.55
T3 (mg/kg)	ND	ND	ND	609.7 ± 28.86	52.6 ± 3.96	431.9 ± 6.72
T+T3 (mg/kg)	1651.1 ± 82.45	1464.2 ± 111.74	1007.3 ± 116.47	776.4 ± 37.94	95.4 ± 12.97	631.3 ± 8.27
PV (meq O_2/kg)	1.0 ± 0.03	1.2 ± 0.00	0.7 ± 0.03	2.9 ± 0.09	3.3 ± 0.16	0.5 ± 0.03
p-AnV	1.1 ± 0.19	10.8 ± 2.18	3.1 ± 0.68	6.5 ± 0.59	54.3 ± 1.44	6.4 ± 0.39

[1] Mean ± standard deviation. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; M, moisture and volatile matter; I, insoluble impurities (expressed as wet weight); U, unsaponifiable matter (expressed as wet weight); SFA, saturated fatty acids (sum of C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0 and C24:0); *cis*-MUFA, *cis*-monounsaturated fatty acids (sum of C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9); *cis*-PUFA, *cis*-polyunsaturated fatty (sum of C18:2 n-6 and C18:3 n-3); trans-C18:1, includes a sum of positional isomers; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T+T3, sum of tocopherols and tocotrienols; PV, Peroxide Value; *p*-AnV, *p*-anisidine value; ND, not detected.

Comparing the composition of by-products with that of crude and refined oils, the I amount in PFAD was higher than that of AS and other experimental fats (Table 2). In both by-products (AO and FAD) the FFA contents were higher compared to their corresponding crude and refined oils, being indeed higher in PFAD than in AS (Table 2). This was explained by the fact that PFAD was obtained by

distillation, a procedure that washed out less TAG from the crude oil than the soap-stock removal in chemical refining to obtain AS (Varona et al., 2021a). This also explained why AS showed higher M, as in PFAD production it would be evaporated (Varona et al., 2021a), and why PFAD showed higher *p*-AnV, which is a measure of secondary oxidation aldehydes, much of which are volatile and thus, distilled during the deodorization step in physical refining and accumulated in PFAD (Varona et al., 2021b). However, PV were quite low in all six fat sources (Table 2), because on the one hand, the edible oil refining process efficiently removes hydroperoxides from crude oils leading to refined seed oils with PV<10 meq O_2/kg, and on the other hand, the high temperatures reached during the by-product obtention cause the decomposition of hydroperoxides leading to low PV in AO and FAD (Varona et al., 2021b).

Influence of Diet on Fresh Meat Composition and Oxidation

Feed and meat FA and tocol composition is shown in Table 3. In general, feed FA and T+T3 composition reflected the differences found in the added fats, and in turn, this influenced the values found in meat. For instance, C18:2 n-6, C18:3 n-3 and total *cis*-PUFA showed higher contents in feeds containing soybean sources (CS, AS and RS) while C16:0, C18:0, total SFA, C18:1 n-9 and total *cis*-MUFA were higher in feeds containing palm sources (CP, PFAD and RP) (Table 3). This entailed that F meat coming from broilers fed soybean sources was richer in PUFA while meat coming from broilers fed palm sources was richer in SFA (such as C16:0) and MUFA (Table 3). In addition, long chain PUFA such as arachidonic acid (**AA**, C20:4 n-6), eicosapentaenoic (**EPA,** C20:5 n-3) and docosahexaenoic (**DHA,** C22:6 n-3) acids that were not supplied by feeds, were also significantly higher in all the soybean meats than in the palm meats because they were synthetized from the linoleic (C18:2 n-6) or linolenic (C18:3 n-3) acids that were supplied in a higher proportion by the soybean feeds (Nyquist et al., 2013; Alagawany et al., 2019).

Table 3. Comparison of fatty acid, tocopherol and tocotrienol composition and oxidation parameters between mash feeds and fresh meat.

	Feeds (n=2)[1]						Fresh meat (n=6)[2]						
	CS	AS	RS	CP	PFAD	RP	CS	AS	RS	CP	PFAD	RP	*P*[3]
C16:0 (%)	12.4 ± 0.23	15.3 ± 0.01	12.0 ± 0.01	39.3 ± 0.02	41.5 ±0.22	39.4 ± 0.13	19.3 ± 0.41^{a}	20.8 ± 0.22^{b}	18.5 ± 0.59^{a}	28.2 ± 0.55^{cd}	27.3 ± 1.03^{c}	29.1 ± 0.51^{d}	**0.000**
C18:0 (%)	3.4 ± 0.06	3.5 ± 0.03	3.5 ± 0.00	4.2 ± 0.00	6.2 ± 0.02	4.5 ± 0.01	5.9 ± 0.48	6.0 ± 0.36	6.0 ± 0.19	5.6 ± 0.26	5.9 ± 0.42	5.5 ± 0.36	**0.048**
C18:1 n-9 (%)	23.1 ± 0.03	20.8 ± 0.00	24.4 ± 0.01	35.8 ± 0.10	33.1 ± 0.04	36.0 ± 0.07	30.3 ± 0.83^{b}	29.0 ± 0.84^{a}	30.3 ± 1.06^{b}	43.0 ± 0.37^{c}	42.9 ± 0.91^{c}	42.4 ± 0.47^{c}	**0.000**
C18:1 n-7 (%)	1.5 ± 0.01	1.6 ± 0.02	1.6 ± 0.02	0.8 ± 0.01	0.7 ± 0.00	0.8 ± 0.00	1.7 ± 0.10^{a}	1.8 ± 0.05^{ab}	1.8 ± 0.18^{ab}	2.0 ± 0.20^{b}	2.0 ± 0.08^{b}	1.8 ± 0.10^{ab}	**0.003**
C18:2 n-6 (%)	51.8 ± 0.26	51.6 ± 0.08	51.4 ± 0.03	16.5 ± 0.13	14.9 ± 0.20	15.8 ± 0.04	32.9 ± 1.10^{b}	32.6 ± 0.87^{b}	33.8 ± 1.35^{b}	12.7 ± 0.78^{a}	12.6 ± 0.67^{a}	12.4 ± 0.38^{a}	**0.000**
C18:3 n-3 (%)	5.8 ± 0.02	5.2 ± 0.01	5.1 ± 0.00	1.0 ± 0.02	1.0 ± 0.03	0.9 ± 0.01	3.4 ± 0.11^{c}	3.0 ± 0.09^{b}	3.0 ± 0.11^{b}	0.8 ± 0.05^{a}	0.8 ± 0.05^{a}	0.7 ± 0.01^{a}	**0.000**
C20:4 n-6 (%)	-	-	-	-	-	-	1.01 ± 0.10^{b}	1.08 ± 0.10^{b}	1.05 ± 0.07^{b}	0.66 ± 0.16^{a}	0.71 ± 0.10^{a}	0.61 ± 0.05^{a}	**0.000**
C20:5 n-3 (%)	-	-	-	-	-	-	0.07 ± 0.00^{d}	0.07 ± 0.00^{d}	0.06 ± 0.01^{c}	0.03 ± 0.01^{a}	0.04 ± 0.00^{b}	0.03 ± 0.00^{a}	**0.000**
C22:6 n-3 (%)	-	-	-	-	-	-	0.12 ± 0.00^{b}	0.10 ± 0.02^{b}	0.11 ± 0.03^{b}	0.06 ± 0.02^{a}	0.06 ± 0.01^{a}	0.05 ± 0.02^{a}	**0.000**
SFA (%)	17.3 ± 0.33	20.4 ± 0.07	17.0 ± 0.01	45.5 ± 0.05	49.6 ± 0.27	46.0 ± 0.12	26.1 ± 0.50^{a}	27.7 ± 0.54^{b}	25.4 ± 0.68^{a}	34.9 ± 0.73^{cd}	34.4 ± 1.39^{c}	35.8 ± 0.63^{d}	**0.000**
cis-MUFA(%)	25.0 ± 0.04	22.9 ± 0.02	26.4 ± 0.01	37.0 ± 0.10	34.3 ± 0.04	37.2 ± 0.08	36.7 ± 1.27^{a}	35.8 ± 1.00^{a}	36.9 ± 1.27^{a}	51.1 ± 0.23^{b}	51.6 ± 1.39^{b}	50.5 ± 0.63^{b}	**0.000**
cis-PUFA (%)	57.6 ± 0.29	56.7 ± 0.09	56.5 ± 0.04	17.5 ± 0.15	15.9 ± 0.23	16.7 ± 0.05	37.1 ± 1.27^{b}	36.5 ± 0.92^{b}	37.7 ± 1.52^{b}	13.9 ± 0.84^{a}	13.9 ± 0.74^{a}	13.6 ± 0.40^{a}	**0.000**
α-T (mg/kg)	124.4 ± 14.59	110.7 ± 0.39	112.8 ± 3.21	108.7 ± 1.05	94.5 ± 4.68	105.6 ± 1.78	25.2 ± 3.98	21.5 ± 4.22	24.8 ± 7.12	20.1 ± 8.12	24.3 ± 5.31	29.3 ± 5.33	0.138
β-T (mg/kg)	7.1 ± 0.07	7.0 ± 0.10	5.8 ± 0.25	4.3 ± 0.21	4.1 ± 0.19	4.2 ± 0.03	0.4 ± 0.12^{b}	0.4 ± 0.06^{b}	0.3 ± 0.16^{b}	Tr^{a}	Tr^{a}	Tr^{a}	**0.000**
Υ-T (mg/kg)	102.5 ± 0.42	87.3 ± 0.50	60.1 ± 2.00	10.2 ± 0.12	10.0 ± 0.62	10.3 ± 0.14	6.2 ± 1.81^{c}	3.9 ± 0.78^{b}	4.0 ± 1.60^{b}	0.4 ± 0.17^{a}	0.4 ± 0.17^{a}	0.4 ± 0.07^{a}	**0.000**
δ-T (mg/kg)	23.4 ± 1.75	42.9 ± 1.60	11.1 ± 0.66	2.6 ± 0.30	2.7 ± 0.28	2.5 ± 0.22	0.4 ± 0.16^{bc}	0.5 ± 0.13^{c}	0.3 ± 0.09^{b}	ND	ND	ND	**0.000**
α-T3 (mg/kg)	11.9 ± 0.28	10.8 ± 0.66	11.7 ± 0.21	30.5 ± 0.15	12.5 ± 0.03	24.9 ± 0.19	0.5 ± 0.12^{a}	0.5 ± 0.14^{a}	0.4 ± 0.16^{a}	1.7 ± 0.64^{b}	0.3 ± 0.13^{a}	1.5 ± 0.47^{b}	**0.000**
β-T3 (mg/kg)	14.5 ± 1.19	13.9 ± 0.07	14.8 ± 0.54	13.8 ± 0.23	11.7 ± 0.04	14.2 ± 0.88	0.4 ± 0.11	0.3 ± 0.07	0.3 ± 0.18	0.3 ± 0.08	0.2 ± 0.09	0.3 ± 0.08	0.166
Υ-T3 (mg/kg)	1.5 ± 0.12	1.3 ± 0.20	1.5 ± 0.05	23.6 ± 0.71	2.7 ± 0.10	15.7 ± 0.60	0.2 ± 0.10	0.2 ± 0.08	0.2 ± 0.07	0.2 ± 0.09	0.2 ± 0.23	0.1 ± 0.05	0.940
δ-T3 (mg/kg)	ND	ND	ND	2.3 ± 0.24	1.7 ± 0.23	0.8 ± 0.06	ND	ND	ND	ND	ND	ND	-
T (mg/kg)	257.4 ± 12.34	247.9 ± 0.80	189.8 ± 6.11	125.8 ± 0.84	111.2 ± 5.76	122.5 ± 1.72	32.2 ± 5.99	26.2 ± 4.82	29.4 ± 8.81	20.4 ± 8.26	24.7 ± 5.47	29.8 ± 5.38	0.057
T3 (mg/kg)	27.9 ± 1.35	26.0 ± 0.79	28.1 ± 0.38	70.1 ± 1.34	28.6 ± 0.32	55.6 ± 1.35	1.1 ± 0.27^{ab}	1.0 ± 0.28^{a}	1.0 ± 0.40^{a}	2.2 ± 0.77^{c}	0.7 ± 0.35^{a}	1.9 ± 0.58^{bc}	**0.000**
T+T3 (mg/kg)	285.3 ± 10.98	274.0 ± 1.59	217.9 ± 6.49	195.9 ± 0.50	139.8 ± 6.09	178.2 ± 0.37	33.3 ± 6.22	27.2 ± 5.00	30.3 ± 9.20	22.6 ± 8.94	25.4 ± 5.62	31.7 ± 5.90	0.105
PV (meq O_2/kg fat)	8.2 ± 0.16	14.1 ± 0.78	13.3 ± 0.69	4.7 ± 0.31	3.6 ± 0.68	5.6 ± 0.13	NA	NA	NA	NA	NA	NA	
LHP content (mmol of CHP/kg)	NA	NA	NA	NA	NA	NA	0.12 ± 0.010^{b}	0.10 ± 0.010^{ab}	0.10 ± 0.006^{ab}	0.10 ± 0.011^{a}	0.10 ± 0.013^{ab}	0.10 ± 0.012^{a}	**0.014**
LHP final value (mmol of CHP/kg)	NA	NA	NA	NA	NA	NA	1.44 ± 0.292^{bc}	1.61 ± 0.067^{c}	1.44 ± 0.408^{bc}	0.93 ± 0.363^{ab}	1.18 ± 0.297^{b}	0.43 ± 0.277^{a}	**0.000**
TBA (µg MDA/kg)	NA	NA	NA	NA	NA	NA	42.0 ± 16.53	41.9 ± 16.66	51.7 ±39.30	26.9 ± 4.09	34.5 ± 16.68	21.4 ± 11.76	0.153
Pentanal (mg/kg)	0.211	0.096	0.153	0.040	0.032	0.108	Tr^{ab}	Tr^{ab}	0.0040 ± 0.00^{b}	Tr^{a}	Tr^{a}	ND^{a}	**0.011**
Hexanal (mg/kg)	2.292	1.058	3.498	0.404	0.095	1.789	0.045 ± 0.03^{ab}	0.025 ± 0.02^{a}	0.095 ± 0.09^{b}	0.008 ± 0.00^{a}	0.006 ± 0.00^{a}	0.007 ± 0.00^{a}	**0.001**
2-Pentylfuran (mg/kg)	0.411	1.003	0.365	0.400	1.933	0.248	0.009 ± 0.01	0.009 ± 0.00	0.013 ± 0.01	0.001 ± 0.01	0.008 ± 0.00	0.008 ± 0.00	0.426
Σ ALD (mg/kg)	2.955	1.363	4.061	0.642	0.301	2.108	0.055 ± 0.03^{ab}	0.033 ± 0.02^{a}	0.106 ± 0.09^{b}	0.015 ± 0.00^{a}	0.014 ± 0.01^{a}	0.013 ± 0.00^{a}	**0.002**
Σ HC (mg/kg)	0.418	0.289	0.524	0.524	0.515	0.502	0.046 ± 0.02	0.040 ± 0.02	0.053 ± 0.03	0.035 ± 0.01	0.034 ± 0.01	0.035 ± 0.01	0.231

Σ KET (mg/kg)	0.926	1.118	1.133	0.735	0.662	0.406	0.008 ± 0.00	0.007 ± 0.00	0.010 ± 0.00	0.006 ± 0.00	0.016 ± 0.03	0.027 ± 0.05	0.661
Σ ALC (mg/kg)	11.520	9.852	9.469	11.566	13.299	10.816	0.023 ± 0.01^{ab}	0.025 ± 0.01^{ab}	0.066 ± 0.06^{b}	0.009 ± 0.01^{a}	0.007 ± 0.00^{a}	0.007 ± 0.00^{a}	**0.002**

[1] Mean ± standard deviation (n = 2, analytical replicates per feed; statistical analysis for feeds was not carried out as the replicates were analytical replicates). [2] Mean ± standard deviation (n = 6 experimental replicates per treatment). [3] *P* values in bold are significant ($P \leq 0.05$). [a-d] For meat results, means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; SFA, saturated fatty acids; *cis*-MUFA, *cis*-monounsaturated fatty acids; *cis*-PUFA, *cis*-polyunsaturated fatty acids; T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; PV, peroxide value; LHP content, Content of lipid hydroperoxides measured by the xylenol orange method after 0.5 h of incubation, measures the LHP content of the sample ; LHP final value, amount of lipid hydroperoxides measured by the xylenol orange method after 96 h of incubation, measures the content of LHP formed after 96 h of incubation and estimates the oxidative stability of the sample; TBA, 2-thiobarbituric acid; MDA, malondialdehyde; Σ ALD, Σ Aldehydes (sum of pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal and 2,4-nonadienal); Σ HC, Σ Hydrocarbons (sum of hexane and decane); Σ KET, Σ Ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one) and Σ ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol and 1-octen-3-ol). Considering mash feeds, SFA (sum of C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0; C22:0, C23:0 and C24:0), *cis*-MUFA (sum of C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9); *cis*-PUFA (sum of C18:2 n-6 and C18:3 n-3). Regarding fresh meat, SFA (sum of C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C23:0 and C24:0), *cis*-MUFA (sum of C14:1, C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7, C20:1 n-9, C22:1 n-9), *cis*-PUFA (sum of C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C18:3 n-3, C20:3 n-3, C20:5 n-3 and C22:6 n-3). NA, not analyzed; ND, not detected; Tr, traces.

Regarding the differences in the feed FA composition between fat types (crude, refined or by-products), their variation within the three soybean feeds or within the three palm feeds was minimal. Only slightly higher C16:0 and slightly lower C18:1 n-9 in AS and PFAD feeds compared to CS and CP feeds were encountered, as well as slightly lower C18:3 n-3 in AS and RS feeds compared with CS feeds, and C18:2 n-6 in PFAD compared to CP. Therefore, the differences in meat FA composition between animals fed the different palm sources were also minimal. Within soybean meats, higher C16:0 and lower C18:1 n-9 in AS meats, higher C18:3 n-3 in CS meats and higher EPA in CS and AS meats was found. Regarding meats from animals fed palm sources, no differences between CP, PFAD and RP were observed except for a slight higher EPA and a slight lower C16:0 and total SFA that were found in PFAD even if for SFA the tendency in feeds was the contrary.

About the content of tocols in feed, it was remarkable that the T3 were not detected in soybean fats but they were present in feeds; therefore, they might have been supplied by cereals present in the feed such as barley and wheat (Falk et al., 2004). Many previous studies consistently agree that the composition of barley grain contains all eight tocol isomers, being α-T3 the most predominant, while wheat grains only contain α- and β- forms, being β-T3 the main isomer found (Nielsen and Hansen, 2008; Granda et al., 2018).

The differences observed in T and T3 between the three palm fats were reflected in feeds (Tables 2 and 3); thus, PFAD had the lowest T and T3 contents. In case of soybean feeds, AS and CS presented similar total T and T3 contents that were higher than those in RS feeds. In all cases, α-T was the major T, followed by γ-T in the case of soybean feeds and by α-T3 in palm feeds. These tocol profiles were the result of the tocol profiles of the added fat (in which γ-T was the main tocol in soybean fats and γ-T3 and α-T3 in palm fats) "(E. Varona, unpublished data)", of the cereals (supplying T and various T3 as explained above), and of the vitamin premix added to feeds (Table 1). This premix contained α-tocopheryl acetate as vitamin E source. It is an acetylated form of α-T to increase its stability in feeds blocking its antioxidant properties until pancreatic esterases release the free form which is absorbed in the small intestine, ensuring its absorption and accumulation in the meat, contributing to its oxidative stability (Schneider, 2005; Bou et al., 2009; Pogorzelska-Nowicka et al. 2018).

In F meat, α-T was the highest tocol both in soybean and palm meats, and the most efficiently transferred from feed to meat even if other tocols (such as γ-T) were also quite abundant in feeds. Although all tocols seem to be similarly absorbed through the intestines (Reboul et al., 2006), α-T is transferred by the hepatic α-T transfer protein to the circulation preferentially to other tocols such as γ-T or α-T3 (Schneider, 2005). In spite of this, PFAD meats still reflected the lower α-T3 contents observed in feeds compared to the other palm meats, and CS meats the higher γ-T contents compared to other soybean meats. Soybean and palm meats presented quite similar α-T contents, except for RP that tended to show slightly higher amounts, even if it was not the fat or feed with the highest α-T

content.

In general, feed oxidation was low, being the PV values lower in palm than in soybean feeds, as well as the hexanal, total ALD and KET, although within palm feeds, hexanal was lower in PFAD than in CP and RP, and within soybean feeds it was lower in AS than in CS and RS. On the contrary, 2-pentylfuran was higher in AS and PFAD feeds with respect to the feeds containing the corresponding crude or refined fats.

In F meats, primary oxidation (LHP content) and secondary oxidation (TBA values and VC content) parameters were in general low. Considering the meat primary oxidation (LHP contents) even if some significant differences were observed for the LHP content, they were of low magnitude. About the susceptibility to oxidation, the LHP final values reached were also low and similar to those previously reported for similar meats (Bou et al., 2006a), being the values in soybean meats slightly higher than those of palm meats, in agreement with their higher total PUFA content that are the substrates of lipid peroxidation. However, no significant differences between dietary treatments were observed for the meat TBA values or for some VC content, neither between by-products and crude and refined fats, nor between soybean and palm fats. TBA value evaluates the formation of MDA, which is an aldehyde mainly released from the oxidation of FA with more than two double bonds. Even if this type of PUFA in soybean meats were higher than those in palm meats, their % was quite low (about 4%, being 3% linolenic acid). In agreement with this, no significant differences were found in the contents of 2,4-heptadienal and 2-pentylfuran (Table S1, Supplementary material) that are mainly formed from linolenic acid, while the two major VC analyzed (pentanal and hexanal), even if their amount was still low, tended to be higher in soybean than in palm meats, especially in CS and RS, as they are mainly formed from the oxidation of linoleic acid (Snyder et al., 1985) that was quite abundant in these meats (Table S1, Supplementary material). Moreover, the tocol amount also explained this general low oxidation in F meat. The content of α-T in meat was between 20.1 and 29.3 mg/kg, not being significantly different between meats from different dietary treatments. Differences in F meats were only observed for minor tocols such as β, γ- and δ-T that were higher in soybean meats, and for α-T3 that was higher in palm meats, while the T and T+T3 contents in F meats was similar between dietary treatments. The αT amounts were similar to those obtained by other studies on broiler meat from animals fed feeds supplemented for a similar feeding period with 150 mg/kg of α-tocopheryl acetate (Bou et al., 2006a), which considering also the rest of feed ingredients, would have supplied the animals a similar tocol amount to our study. Besides, the low TBA values found in F meat were similar between that study (Bou et al., 2006a), amounts that led to TBA reductions when compared to non-supplemented feeds. In fact, the total T+T3 amount present in feeds was above 150 mg/kg, an amount that has been suggested as a general recommendation to supplement feeds (as α-tocopheryl acetate) to obtain an adequate oxidative stability in raw broiler meat from animals fed vegetable fats

such as those in the present study (Bou et al., 2006a, 2009). Moreover, it needs to be taken into account that F meat had not been subjected to any oxidative stress procedure further than grinding and packing (that were done in less than 12 hours after slaughter) (Grau et al., 2001; Bou et al., 2009). Therefore, with regards to primary and secondary oxidation of F meat, it seems that the differences in the composition between by-products and crude and refined oils from soybean and palm origin were not relevant, as they were counteracted by a the T amount supplied by feeds.

Meat Tocol Composition, Oxidative Status and Susceptibility to Oxidation after the Technological Treatments

The effects of the dietary and technological treatments on meat tocol composition and oxidative status have been visualized by PCA (Figure 1) and assessed by multifactor ANOVA (Table 4). The PCA is multivariate data analysis used to explore the natural distribution and grouping of samples as well as to assess the relationships between variables. The cumulative variance explained by the two firsts components was 62.10%.

In general terms, the PCA score plots developed on the tocol composition and oxidation parameters of F, C and CR meats, revealed that meat samples clustered in two main groups which agreed with the fat origin (soybean vs palm fat sources, Figure 1a) while no clear clusters were observed for the type of fat products used (CS, AS, RS, CP, PFAD and CP, Figure 1a). This separation between soybean and palm meats was mainly related to PC2. By comparing the score and loading plots (Figure 1a and 1c) it was revealed that the separation of botanical origins was mostly driven by meat tocol composition, as well as by its LHP content and the LHP final value. As commented above, meat samples that came from broilers fed soybean sources agreed with a higher contribution of LHP parameters and T contents (especially due to the minor tocols such as γ-T, β-T and δ-T), while those coming from broilers on palm sources were more related with α-T3 (Figure 1c).

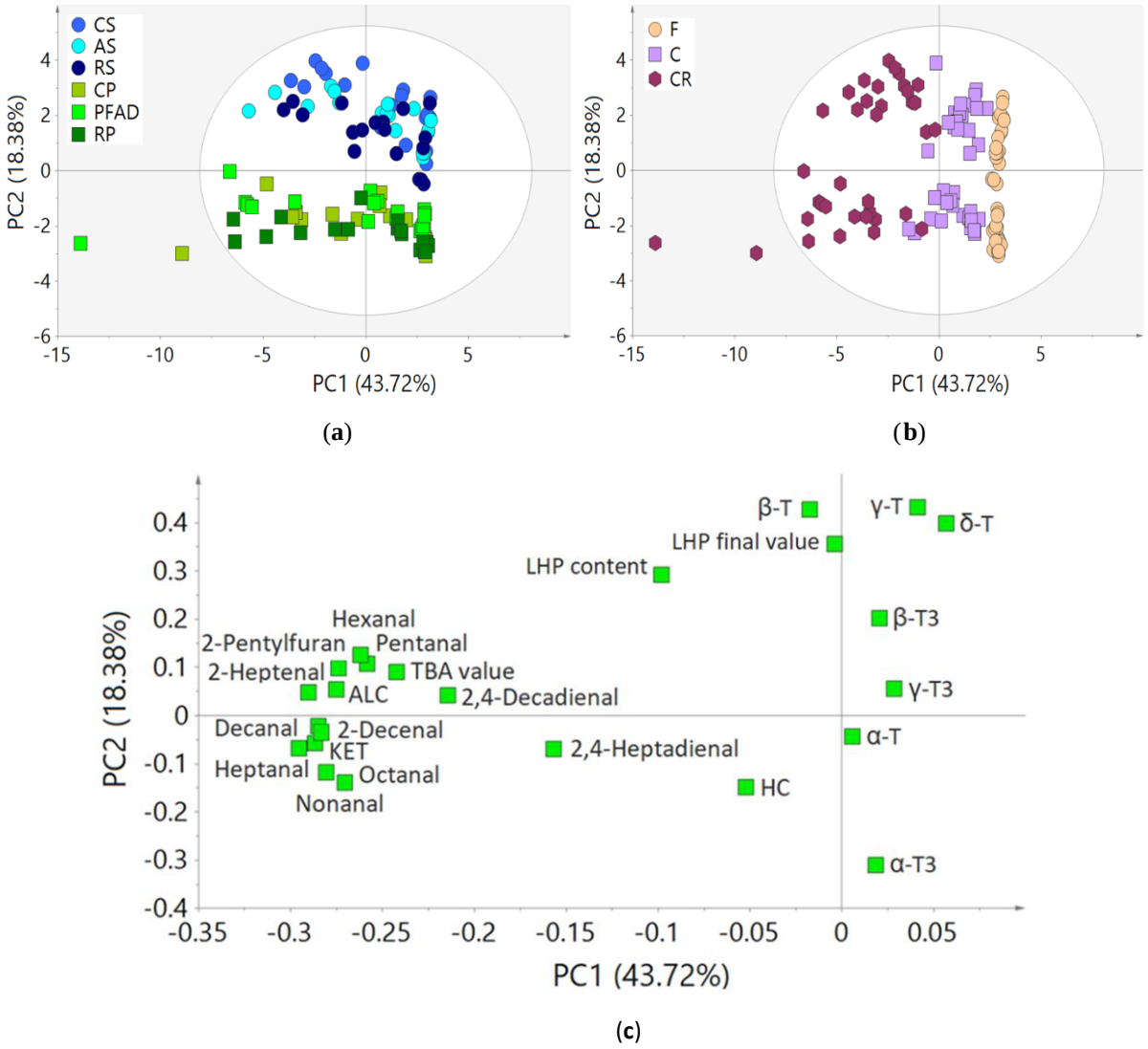

Fig. 1. Principal component analysis on the tocol composition and oxidation parameters (24 variables, mean centered and scaled to unit variance) of fresh, cooked and cooked and refrigerated meat samples (n=108). (**a**) Score plot colored according to the dietary treatment; (**b**) score plot colored according to the technological treatment; (c) loading plot. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; F, fresh meat; C, cooked meat; CR, cooked and refrigerated meat; LHP content, Content of lipid hydroperoxides measured by the xylenol orange method after 0.5 h of incubation, measures the LHP content of the sample ; LHP final value, amount of lipid hydroperoxides measured by the xylenol orange method after 96 h of incubation, measures the content of LHP formed after 96 h of incubation and estimates the oxidative stability of the sample; TBA, 2-thiobarbituric acid; HC, Σ Hydrocarbons (sum of hexane and decane); KET, Σ Ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one) and ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol and 1-octen-3-ol).

Thus, the botanical origin of the fat (soybean or palm sources) might have a higher influence on meat composition and oxidative stability than the type of fat (crude, refined or by-product). Nevertheless, some slight differences were observed between fat types after cooking and refrigeration, leading to a tendency of separation within the three types of CR soybean meats (Figure 1a), as well as to a significant interaction for the initial LHP content between the dietary treatment and the technological treatment revealed by multifactor ANOVA (Figure 2). The initial LHP content (after 0.5 h of incubation) increased significantly in the order F< C< CR which is consistent with the fact that the heat treatment during the cooking process leads to an increase in the formation of hydroperoxides,

which continue increasing with further refrigeration of cooked meat (Table 4), but this LHP increase was larger in the case of soybean sources, especially in CS, (Figure 2a) which was in agreement with their higher PUFA (n-6 and n-3) content that might have been oxidized leading to higher LHP content (Table 4).

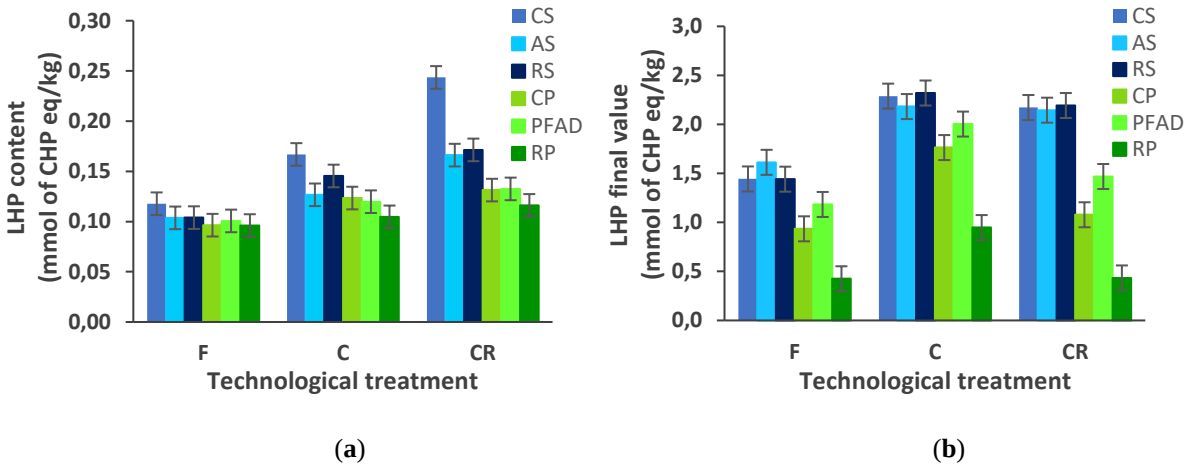

Fig. 2 Effect of the interaction between fat source (CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil) and technological treatment (F, fresh; C, cooked and CR, cooked and refrigerated) on the (**a**) LHP content (P = 0.002) and (**b**) LHP final value (P = 0.042) expressed both as mmol of cumene hydroperoxide (CHP) equivalents/kg (pooled means ± standard error of the pooled means from multifactor ANOVA, n = 108).

The susceptibility to oxidation (LHP final value) followed a different trend in F, C and CR meats depending on the type of fat, leading to an interaction between dietary treatment and technological treatment (Figure 2b): first, it increased as a result of cooking (from F to C), but this increase was lower for RP meat which agreed with their numerically lowest PUFA and highest T+T3. The increase of the LHP final values from F to C meats agreed with the effects of cooking on meat structure and composition, such as disruption of cell membranes leading to release of oxygen and iron from heme compounds or inactivation of antioxidant enzymes, which might have created a more prooxidant status on C meat compared to F meat (Min and Ahn, 2005; Amaral et al., 2018; Domínguez et al., 2019). This more prooxidant status might have been reflected in the methanolic extract of C meats used in the FOX method, leading to higher LHP final values when it was incubated with the FOX reagents. However, no further increases of the susceptibility to oxidation (LHP final value) were observed in CR meats; and even palm CR meats tended to present lower LHP final values. This indicated that the effects of cooking on the composition of prooxidants and antioxidants of the methanolic extract obtained from meats were more relevant than the changes in the composition of this extract caused by refrigerated storage.

Table 4. Effect of the dietary and technological treatments on the tocopherol and tocotrienol composition, lipid hydroperoxide contents, TBA value and volatile compound contents of cooked, refrigerated and cooked and refrigerated meat samples.

	Dietary treatment[1]								Technological treatment[2]					Dietary treatment × Technological treatment
	CS	AS	RS	CP	PFAD	RP	SE[3]	*P*[4]	F	C	CR	SE[3]	*P*[4]	*P*[5]
α-T (mg/kg)	24.8^{a}	20.5^{a}	24.2^{a}	20.6^{a}	22.4^{a}	31.3^{b}	1.090[2]	**0.000**	24.2	24.0	23.7	0.771	0.915	0.839
Υ-T (mg/kg)	6.1^{c}	3.9^{b}	3.8^{b}	0.4^{a}	0.6^{a}	0.5^{a}	0.211	**0.000**	2.5	2.5	2.6	0.149	0.968	0.960
α-T3 (mg/kg)	0.4^{a}	0.4^{a}	0.4^{a}	1.5^{b}	0.5^{a}	1.4^{b}	0.073	**0.000**	0.8^{b}	0.8^{ab}	0.7^{a}	0.052	0.029	0.413
Υ-T3 (mg/kg)	0.2^{ab}	0.2^{ab}	0.2^{ab}	0.2^{b}	0.1^{a}	0.2^{ab}	0.023	**0.011**	0.2	0.2	0.2	0.016	0.912	0.609
T (mg/kg)	31.6^{c}	25.4^{ab}	28.5^{bc}	21.1^{a}	23.1^{a}	31.9^{c}	1.214	**0.000**	27.1	27.0	26.7	0.858	0.950	0.877
T3 (mg/kg)	1.0^{a}	0.8^{a}	0.9^{a}	2.1^{b}	0.7^{a}	1.9^{b}	0.090	**0.000**	1.3^{b}	1.3^{b}	1.1^{a}	0.063	**0.032**	0.691
T+T3 (mg/kg)	32.6^{c}	26.2^{ab}	29.4^{bc}	23.2^{a}	23.8^{a}	33.8^{c}	1.262	**0.000**	28.4	28.3	27.8	0.892	0.887	0.886
LHP content (mmol of CHP/kg)	0.18^{c}	0.13^{ab}	0.14^{b}	0.12^{ab}	0.12^{ab}	0.11^{a}	0.006	**0.000**	0.10^{a}	0.13^{b}	0.16^{c}	0.005	**0.000**	**0.002**
LHP final value (mmol of CHP/kg)	1.97^{c}	1.98^{c}	1.99^{c}	1.26^{b}	1.55^{b}	0.60^{a}	0.074	**0.000**	1.17^{a}	1.92^{c}	1.58^{b}	0.052	**0.000**	**0.042**
TBA value (µg MDA/kg)	896.8	974.4	1204.7	1076.1	1083.7	897.2	135.01	0.547	36.4^{a}	528.3^{b}	2501.7^{c}	95.47	**0.000**	0.488
Pentanal (mg/kg)	0.622	0.668	0.727	0.675	0.626	0.613	0.069	0.852	Tr^{a}	0.687^{b}	1.277^{c}	0.049	**0.000**	0.265
Hexanal (mg/kg)	10.425	11.669	12.465	11.127	10.493	9.883	0.987	0.477	0.031^{a}	11.030^{b}	21.970^{c}	0.698	**0.000**	0.679
2-Pentylfuran (mg/kg)	0.188	0.173	0.153	0.180	0.215	0.202	0.024	0.518	0.009^{a}	0.098^{b}	0.448^{c}	0.017	**0.000**	0.623
Σ ALD (mg/kg)	11.337	12.730	13.526	12.633	12.210	11.390	1.093	0.703	0.039^{a}	12.028^{b}	24.846^{c}	0.773	**0.000**	0.630
Σ HC (mg/kg)	0.068^{abc}	0.049^{ab}	0.043^{a}	0.110^{cd}	0.092^{bcd}	0.126^{d}	0.012	**0.000**	0.040^{a}	0.128^{c}	0.076^{b}	0.008	**0.000**	**0.002**
Σ KET (mg/kg)	0.230^{a}	0.279^{a}	0.254^{a}	0.447^{ab}	0.557^{b}	0.339^{ab}	0.056	**0.000**	0.012^{a}	0.265^{b}	0.775^{c}	0.040	**0.000**	**0.000**
Σ ALC (mg/kg)	1.761	2.295	1.806	1.846	2291.3	1.672	0.260	0.356	0.023^{a}	1.569^{b}	4.244^{c}	0.184	**0.000**	0.922

[1] Values are pooled means from multifactor ANOVA (n = 18 per group, resulting from 6 experimental replicates x 3 technological treatments). [2] Values are pooled means from multifactor ANOVA (n = 36 per group, resulting from 6 experimental replicates x 6 dietary treatments). [3] SE, standard error of the pooled mean. [4] *P* values of the main effects in bold are significant ($P \leq 0.05$). [5] *P* values of the interaction between the dietary and the technological treatment in bold are significant ($P \leq 0.05$) [a-d] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; F, fresh meat; C, cooked meat; CR, cooked and refrigerated meat; T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; LHP content, Content of lipid hydroperoxides measured by the xylenol orange method after 0.5 h of incubation, measures the LHP content of the sample ; LHP final value, amount of lipid hydroperoxides measured by the xylenol orange method after 96 h of incubation, measures the content of LHP formed after 96 h of incubation and estimates the oxidative stability of the sample; TBA, 2-thiobarbituric acid; MDA, malondialdehyde; Σ ALD, Σ Aldehydes (sum of pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal and 2,4-nonadienal); Σ HC, Σ Hydrocarbons (sum of hexane and decane); Σ KET, Σ Ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one) and Σ ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol and 1-octen-3-ol).

Regarding secondary oxidation, by comparing the PCA score and loading plots, it was revealed that the TBA value and most VC had a high contribution on the separation of CR meats from C meats and from F meats, while T and T3 had almost no contribution (Figure 1c). This again agreed with the ANOVA results (Table 4), which revealed that cooking significantly increased TBA values and most VC while it did not modify T or T3, and further refrigeration of cooked meat only significantly decreased total T3 (Table 4). The major VC (hexanal and pentanal) were plotted in the PCA close to TBA value showing a tendency to correlate positively. Both TBA values and ALD increased from F to C and to CR being the highest increase from C to CR. In fact, almost all the individual ALD and 2-pentylfurane showed this increase from F to CR (Table S2, Supplementary material). Indeed, the TBA values reached in CR meats were above the thresholds suggested by Bou et al. (2001) for the acceptability of meats and the detection of rancid flavor. Thus, even if the T content of raw meat was enough to avoid differences in oxidability between the type of fat added to diets, lipid oxidation took place when thermal treatments were applied leading to higher LHP content in C meats and in CS meats, to a higher susceptibility to oxidation after cooking and to higher secondary oxidation both after cooking and after the refrigerated storage of cooked meat. Nevertheless, this did not led to a reduction of T contents in C or CR meats compared to F meats (only a non-significant tendency) although significant reductions in meat α-T during cooking have been previosuly reported in other studies using vegetable oils and leading to similar TBA values (Bou et al., 2006b; Grau et al., 2001). Moreover, in general these effects were similar for the different types of fat by-products added to feeds.

CONCLUSIONS

This study on the AO and FAD effects on meat composition and oxidative stability when used as feed ingredients is a step forward to for the valorization of these by-products from the oil refining industry, complementing the findings on the evaluation of their energy value (Varona et al., 2021a), oxidation status (Varona et al., 2021b), and their digestibility and absorption (Rodriguez-Sanchez et al., 2019a,b, 2021; Jimenez-Moya et al., 2021a,b). Overall, the results of this study have shown an influence of the added fat composition in feed and meat composition in terms of FA profile and T and T3. The FA composition, T and T3 contents and primary oxidation parameters of meat were more dependent on the botanical type of fat added (soybean or palm) than on the fact that refining by-products rich in FFA (AO and FAD) were used instead of crude or refined oils. This was related to the fact that the FA composition of the by-products was quite similar to that of the crude and refined oils. Regarding their T and T3 contents, even if they were lower than that of crude and refined oils, the final differences in feeds were diminished by the presence of other T and T3 from other ingredients and the premix.

Cooking led to an increase in primary and secondary oxidation, and further refrigeration of cooked meat increased the secondary oxidation compounds even more. However, again these increases were more dependent on the botanical origin of the oils rather on the fact that they were by-products. Thus it seems that the higher FFA content of both by-products, nor the higher *p*-AnV or I in PFAD compared to crude and refined oils did not led to relevant changes in the composition or oxidative stability of meat. However, it needs to be taken into account that the nutritional quality of the by-products used in this study was rather high compared to other by-products in the market.

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REFERENCES

1. Alagawany, M., S. S. Elnesr, M. R. Farag, M. E. Abd El-Hack, A. F. Khafaga, A. E. Taha, R. Tiwari, M. Iqbal Yatoo, P. Bhatt, S. K. Khurana, K. Dhama. 2019. Omega-3 and omega-6 fatty acids in poultry nutrition: Effect on production performance and health. Animals 9:573. doi:10.3390/ani9080573

2. Aleman, M., C.D. Nuchi, R. Bou, A. Tres, J. Polo, F. Guardiola, R. Codony. 2010. Effectiveness of antioxidants in preventing oxidation of palm oil enriched with heme iron: A model for iron fortification in baked products. Eur. J. Lipid Sci. Technol. 112:761–769. doi:10.1002/ejlt.200900220

3. Amaral, A. B., M. V. Da Solva, and S. C. S. Lannes. 2018. Lipid oxidation in meat: Mechanisms and protective factors - a review. Food Sci. Technol. 38:1–15. doi:10.1590/fst.32518

4. Ayed, H. B., H. Attia, and M. Ennouri. 2015. Effect of oil supplemented diet on growth performance and meat quality of broiler chickens. Adv. Tech. Biol. Med. 4:1–5. doi:10.4172/2379-1764.1000156

5. Bou, R., F. Guardiola, A. Grau, S. Grimpa, A. Manich, A. C. Barroeta, R. Codony. 2001. Influence of dietary fat source, α-tocopherol, and ascorbic acid supplementation on sensory quality of dark shicken meat. Poult. Sci. 80:800–807. doi:10.1093/ps/80.6.800

6. Bou, R., F. Guardiola, A. Tres, A. C. Barroeta, R. Codony. 2004. Effect of dietary fish oil, α-tocopheryl acetate, and zinc supplementation on the composition and consumer acceptability of chicken meat. Poult. Sci. 83:282–292. doi:10.1093/ps/83.2.282

7. Bou, R., S. Grimpa, M. D. Baucells, R. Codony, and F. Guardiola. 2006a. Dose and duration effect of α-tocopheryl acetate supplementation on chicken meat fatty acid composition, tocopherol content, and oxidative status. J. Agric. Food Chem. 54:5020–5026. doi:10.1021/jf060535x

8. Bou, R., S. Grimpa, F. Guardiola, A.C. Barroeta, R. Codony. 2006b. Effects of various fat sources, α-tocopheryl acetate, and ascorbic acid supplements on fatty acid composition and α-tocopherol content in raw and vacuum-packed, cooked dark chicken meat. Poult. Sci. 85, 1472–1481. doi:10.1093/ps/85.8.1472

9. Bou, R., R. Codony, A. Tres, E. A. Decker, and F. Guardiola. 2009. Dietary strategies to improve nutritional value, oxidative stability, and sensory properties of poultry products. Crit. Rev. Food Sci. Nutr. 49:800–822. doi:10.1080/10408390902911108

10. Codex Alimentarius. Standard for named vegetable oils (CXS 210-1999). In Codex Alimentarius, International Food Standards; Codex Alimentarius: Rome, Italy, 1999; Adopted in 1999. Revised in 2001, 2003, 2009, 2017, 2019. Amended in 2005, 2011, 2013, 2015, 2019.

11. Domínguez, R., M. Pateiro, M. Gagaoua, F.J. Barba, W. Zhang, J. M. Lorenzo. 2019. A comprehensive review on lipid oxidation in meat and meat products. Antioxidants 8:429. doi:10.3390/antiox8100429

12. Dumont, M. J. and S. S. Narine. 2007. Soapstock and deodorizer distillates from north american vegetable oils: review on their characterization, extraction and utilization. Food Res. Int. 40:957–974. https://doi.org/10.1016/j.foodres.2007.06.006

13. Falk, J., A. Krahnstöver, T. A. W. Van Der Kooij, M. Schlensog, and K. Krupinska. 2004. Tocopherol and tocotrienol accumulation during development of caryopses from barley (Hordeum vulgare L.). Phytochemistry 65:2977–2985. doi:10.1016/j.phytochem.2004.08.047

14. FEDNA. 2008. Fundación Española para el Desarrollo de la Nutrición Animal. Necesidades Nutricionales para Avicultura: Pollos de Carne y Aves de Puesta; FEDNA: Madrid, Spain, 2008; ISBN 9788409065295. Available at http://www.fundacionfedna.org/sites/default/files/NORMAS_FEDNA_AVES_2018v.pdf.

15. Granda, L., A. Rosero, K. Benešová, H. Pluháčková, J. Neuwirthová, R. Cerkal. 2018. Content of selected vitamins and antioxidants in colored and nonpigmented varieties of quinoa, barley, and wheat grains. J. Food Sci. 83:2439–2447. doi: 10.1111/1750-3841.14334

16. Grau, A., R. Codony, M. Rafecas, A. C. Barroeta, F. Guardiola. 2000a. Lipid hydroperoxide determination in dark chicken meat through a ferrous oxidation-xylenol orange method. J. Agric. Food Chem. 48:4136–4143. https://doi.org/10.1021/jf991054z

17. Grau, A., F. Guardiola, J. Boatella, A. Barroeta, R. Codony. 2000b. Measurement of 2-thiobarbituric acid values in dark chicken meat through derivative spectrophotometry: Influence of various parameters. J. Agric. Food Chem. 48:1155–1159. doi:10.1021/jf990518q

18. Grau, A., F. Guardiola, S. Grimpa, A. C. Barroeta, and R. Codony. 2001. Oxidative stability of dark chicken meat through frozen storage: Influence of dietary fat and α-tocopherol and ascorbic acid supplementation. Poult. Sci. 80:1630–1642. doi:10.1093/ps/80.11.1630

19. Jimenez-Moya, B., D. Martin, C. Soler-Rivas, A. C. Barroeta, A. Tres, R. Sala. 2021a. Acid versus crude oils for broiler chicken diets: In vitro lipid digestion and bioaccessibility. Anim. Feed Sci. Technol. 276:114926. doi:10.1016/j.anifeedsci.2021.114926

20. Jimenez-Moya, B., A. C. Barroeta, A. Tres, M.D. Soler, and R. Sala. 2021b. Soybean oil replacement by palm fatty acid distillate in broiler chicken diets: Fat digestibility and lipid-class content along the intestinal tract. Animals 11:1035. doi:10.3390/ani11041035

21. Jimenez-Moya, B., A. C. Barroeta, A. Tres, M. D. Soler, and R. Sala. 2021c. Replacement of palm oil with soybean acid oil in broiler chicken diet: fat digestibility and lipid class content along the intestinal tract. Animals 11:2586. doi:10.3390/ani11092586

22. Kanazawa, K. and H. Ashida. 1998. Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body. Biochim. Biophys. Acta 1393:349–361. doi:10.1016/S0005-2760(98)00089-7

23. Lindblom, S. C., N. K. Gabler, E. A. Bobeck, and B. J. Kerr. 2019. Oil source and peroxidation status interactively affect growth performance and oxidative status in broilers from 4 to 25 d of age. Poult. Sci. 98:1749–1761. doi:10.3382/ps/pey547

24. Nielsen, M. M., and Å. Hansen. 2008. Rapid high-performance liquid chromatography determination of tocopherols and tocotrienols in cereals. Cereal Chem. 85:248–251.

25. Nuchi, C., Guardiola, F., Bou, R., Bondloli, P., Bella, L.D., Codony, R. 2009. Assessment of the levels of degradation in fat co-and byproducts for feed uses and their relationships with some lipid composition parameters. J. Agric. Food Chem. 57:1952–1959. doi:10.1021/jf803369h

26. Nyquist, N. F., R. Rødbotten, M. Thomassen, and A. Haug. 2013. Chicken meat nutritional value when feeding red palm oil, palm oil or rendered animal fat in combinations with linseed oil, rapeseed oil and two levels of selenium. Lipids Health Dis. 12:69. doi:10.1186/1476-511X-12-69

27. Pinchasov, Y., and I. Nir. 1992. Effect of dietary polyunsaturated fatty acid concentration on performance, fat deposition, and carcass fatty acid composition in broiler chickens. Poult. Sci. 71:1504–1512. doi:10.3382/ps.0711504

28. Pogorzelska-Nowicka, E., A. G. Atanasov, J. Horbańczuk, and A. Wierzbicka. 2018. Bioactive compounds in functional meat products. Molecules 23:307. doi:10.3390/molecules23020307

29. Ravindran, V., P. Tancharoenrat, F. Zaefarian, and G. Ravindran, G. 2016. Fats in poultry nutrition: Digestive physiology and factors influencing their utilisation. Anim. Feed Sci. Technol. 213:1–21. doi:10.1016/j.anifeedsci.2016.01.012

30. Reboul, E., A. Klein, F. Bietrix, B. Gleize, C. Malezet-Desmoulins, M. Schneider, A. Margotat, L. Lagrost, X. Collet, P. Borel. 2006. Scavenger receptor class B type I (SR-BI) is involved in vitamin E transport across the enterocyte. J. Biol. Chem. 281:4739–4745. doi:10.1074/jbc.M509042200

31. Rodriguez-Sanchez, R., A. Tres, R. Sala, C. Garcés-Narro, F. Guardiola, J. Gasa, A. C. Barroeta. 2019a. Effects of dietary free fatty-acid content and saturation degree on lipid-class composition and fatty-acid digestibility along the gastrointestinal tract in broiler starter chickens. Poult. Sci. 98:4929–4941. doi:10.3382/ps/pez253

32. Rodriguez-Sanchez, R., A. Tres, R. Sala, F. Guardiola, and A. C. Barroeta. 2019b. Evolution of lipid classes and fatty acid digestibility along the gastrointestinal tract of broiler chickens fed different fat sources at different ages. Poult. Sci. 98:1341–1353. doi:10.3382/ps/pey458

33. Rodriguez-Sanchez, R., A. Tres, R. Sala, M. D. Soler, F. Guardiola, and A. C. Barroeta. 2021. Effects of free-fatty-acid content and saturation degree of the dietary oil sources on lipid-class content and fatty-acid digestibility along the gastrointestinal tract in broilers from 22 to 37 days of age. Poult. Sci. 100:101261. doi:10.1016/j.psj.2021.101261

34. Sheehy, P. J. A., P. A. Morrissey, and A. Flynn. 1994. Consumption of thermally-oxidized sunflower oil by chicks reduces α-tocopherol status and increases susceptibility of tissues to lipid oxidation. Br. J. Nutr. 71:53–65. doi:10.1079/bjn19940110

35. Schneider, C. 2005. Chemistry and biology of vitamin E. Mol. Nutr. Food Res. 49:7–30. doi:10.1002/mnfr.200400049

36. Snyder, J.M., E.N. Frankel, and E. Selke. 1985. Capillary gas chromatographic analyses of headspace volatiles from vegetable oils. J. Am. Oil Chem. Soc. 62:1675–1679. doi:10.1007/BF02541664

37. Tres, A., R. Bou, F. Guardiola, C. D. Nuchi, N. Magrinyá, R. Codony. 2013. Use of recovered frying oils in chicken and rabbit feeds: Effect on the fatty acid and tocol composition and on the oxidation levels of meat, liver and plasma. Animal 7:505–517. doi:10.1017/S1751731112001607

38. Varona, E., A. Tres, M. Rafecas, S. Vichi, A. C. Barroeta, F. Guardiola. 2021a. Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding. Animals 11:196. doi:10.3390/ani11010196

39. Varona, E., A. Tres, M. Rafecas, S. Vichi, R. Sala, F. Guardiola. 2021b. Oxidative quality of acid oils and fatty acid distillates used in animal feeding. Animals 11:2559. doi:10.3390/ani11092559

40. Varona, E., A. Tres, M. Rafecas, S. Vichi, A. C. Barroeta, F. Guardiola. 2021c. Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding. MethodsX 8:101334. doi:10.1016/j.mex.2021.101334

41. Wiseman, J., and F. Salvador. 1991. The influence of free fatty acid content and degree of saturation on the apparent metabolizable energy value of fats fed to broilers. Poult. Sci. 70:573–582. doi:10.3382/ps.0700573

42. Wealleans, A. L., K. Bierinckx, and M. Benedetto. 2021. Fats and oils in pig nutrition : Factors affecting digestion and utilization. Anim. Feed Sci. Technol. 277:114950. doi:10.1016/j.anifeedsci.2021.114950

43. Wood, J. D., R. I. Richardson, G.R. Nute, A. V. Fisher, M.M. Campo, E. Kasapidou, P. R. Sheard, M. Enser, 2004. Effects of fatty acids on meat quality: a review. Meat Sci. 66:21–32. doi:10.1016/S0309-1740(03)00022-6

44. Zhang, W., S. Xiao, E. J. Lee, and D. U. Ahn. 2011. Consumption of oxidized oil increases oxidative stress in broilers and affects the quality of breast meat. J. Agric. Food Chem. 59:969–974. doi:10.1021/jf102918z

5.2. Discusión global de los resultados

Debido al creciente interés por el uso de subproductos en la formulación de piensos, tanto por su rendimiento a nivel productivo, como debido a los precios competitivos que presentan respecto a las grasas convencionales, conseguir un incremento en su utilización asegurando su eficiencia alimentaria constituye una alternativa sostenible y atractiva desde el punto de vista económico. Por ello, uno de los objetivos de esta Tesis es la caracterización de subproductos de la industria de la refinación de grasas y aceites comestibles, concretamente los AA y AGD, para poder controlar su calidad y usarlos de manera eficaz como ingredientes en alimentación animal. Además, en esta Tesis se aporta información sobre su inclusión en los piensos, así como, resultados sobre la composición y estabilidad oxidativa de la carne de pollos alimentados con ellos. Así, se consigue una evaluación profunda de estos subproductos en varios aspectos de su utilización. A continuación, se presentan y discuten los resultados más relevantes de esta tesis.

5.2.1. Adaptación de los métodos analíticos para los aceites ácidos y ácidos grasos destilados y caracterización de estos subproductos usados en alimentación animal

La parte inicial de esta Tesis se centra en la caracterización de los AA y AGD, matrices grasas muy particulares y de gran heterogeneidad, de forma que se conozca de una forma más exhaustiva cuáles son los factores que influyen en la variabilidad de su composición, para que puedan ser utilizados de una forma eficaz y eficiente en la formulación de dietas animales. Para ello, es preciso el control de calidad de dichas fuentes grasas, determinando los parámetros que influyen directamente en su valor energético y nutricional. Actualmente es escasa la información sobre la calidad y variabilidad de los AA y AGD, y los pocos datos disponibles demuestran que no están correctamente estandarizados [13,72]. Esto hace que resulte difícil establecer niveles aceptables tanto de compuestos de degradación como de compuestos con nulo o muy bajo valor energético que actúan como diluyentes de la energía en alimentación animal. En esta Tesis se llevó a cabo una caracterización de AA y AGD recogidos del mercado español mediante la determinación de parámetros relacionados con su valor energético y nutricional, y con su grado de oxidación y estabilidad oxidativa. Se determinaron diversos parámetros que afectan al valor energético de los AA y AGD, entre ellos, los que estiman el contenido en diluyentes energéticos (MIU y contenido de diversos productos de oxidación) y los que afectan a la digestibilidad y absorción de los AG y así a su energía dietética (el contenido de AGL y la relación I/S), además

de otros parámetros de composición (perfil de AG y contenido en T y T3) relacionados con el valor nutricional de los AA y AGD y con su estabilidad oxidativa (Tabla 2).

Tabla 2. Principales relaciones entre los parámetros determinados en aceites ácidos y ácidos grasos destilados y sus características más relevantes[1].

	Parámetro	Valor energético	Valor nutricional	Grado de oxidación	Estabilidad Oxidativa
MIU	M (g/100 g)	✓			
	I (g/100 g)	✓			
	U (g/100 g)	✓	✓		
Composición en ácidos grasos, tocoferoles y tocotrienoles	Composición en AG (%)	✓	✓		✓
	Relaciones I/S y AGI/AGS	✓	✓		✓
	T y T3 (mg/kg)		✓		✓
Clases lipídicas	POL (%)	✓	✓	✓	
	TAG, DAG, MAG (%)	✓			
	AGL (%)	✓			✓
Hidrolisis de los TAG	AC (g/100 g)	✓			✓
Compuestos de oxidación	PV (meq O_2/kg)	✓	✓	✓	✓
	p-AnV	✓	✓	✓	
Estabilidad oxidativa	IT (h)				✓

[1] Sólo se especifican las relaciones más relevantes, por ejemplo, los polímeros que se forman mediante un elevado número de enlaces éter o carbono-carbono se pueden encontrar en la materia insaponificable y desde este punto de vista la cantidad de materia insaponificable podría estar relacionada en determinadas situaciones, con en el grado de oxidación. No obstante, para que esto sea significativo el grado de polimerización debe ser muy elevado y no se considera una relación relevante desde el punto de vista práctico puesto que estas grasas tan oxidadas ya no se deben usar como ingredientes para piensos. Por tanto, la tabla sólo recoge las principales relaciones entre los parámetros determinados en estos subproductos y sus características más relevantes.

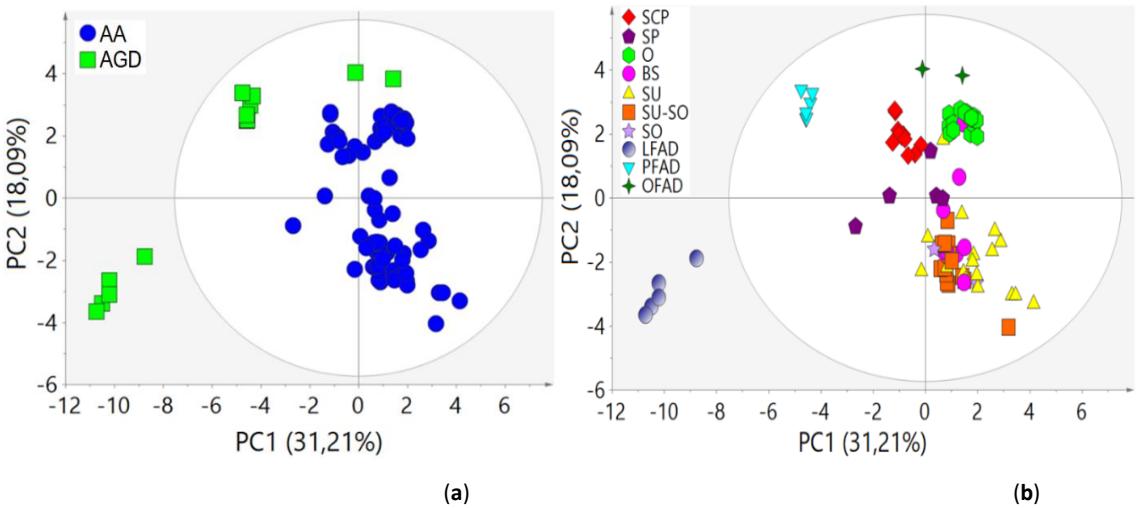
Abreviaciones: M, humedad y materia volátil; I, impurezas insolubles; U, materia insaponificable; MIU, suma de humedad y materia volátil, impurezas insolubles y materia insaponificable; AG, ácidos grasos (expresados según normalización interna de áreas (%)); relación I/S, relación ácidos grasos insaturados/ácidos grasos saturados (esta relación se utiliza para predecir la energía y se calcula incluyendo los AG de 12 carbonos o menos como AG insaturados independientemente de su grado de insaturación, así como considerando los ácidos grasos *trans* como saturados); relación AGI/AGS en este caso todos los AG insaturados se incluyen en el numerador y todos los AG saturados en el denominador, esta relación se utiliza para predecir la susceptibilidad a la oxidación de la fracción lipídica; T, suma de α-, β-, γ- y δ-tocoferoles; T3, suma de α-, β-, γ- y δ-tocotrienoles; POL, compuestos poliméricos; TAG, triacilgliceroles; DAG, diacilgliceroles; MAG, monoacilgliceroles; AGL, ácidos grasos libres (determinados por cromatografía de exclusión molecular); AC, grado de acidez (determinación de los AGL mediante volumetría y expresado como g de ácido oleico/100 g para todas las muestras excepto para los AGD obtenidos a partir de grasas laúricas en las que se expresa como g de ácido laúrico/100 g y para los PFAD, expresados en g de ácido palmítico/100 g); PV, índice de peróxidos; *p*-AnV, índice de *p*-anisidina; IT, período de inducción medido mediante el instrumento Rancimat.

Los pocos datos disponibles sobre la composición y estabilidad de los AA y los AGD muestran que su composición es bastante distinta a la de los aceites crudos. Esto implica que, en muchos casos, los métodos de análisis usualmente aplicados en aceites crudos no sean aplicables a los AA y AGD, matrices de gran heterogeneidad. Por ello, tal y como se ha explicado en la metodología, para poder caracterizar los AA y los AGD, se adaptaron los protocolos de análisis a partir de los métodos recomendados para grasas y aceites crudas o refinadas. Las modificaciones llevadas a cabo en cada uno se justifican en la publicación que se recoge en la sección 5.1.1 [146] y en el Anexo 8.1 correspondiente a la información suplementaria de dicha publicación, se describen detalladamente los métodos de análisis optimizados. El tipo de dificultades encontradas, así como las modificaciones propuestas variaron según los parámetros; sin embargo, las determinaciones de la composición en AG, del contenido en T y T3, así como del U no presentaron ningún problema a la hora de utilizar los métodos descritos en la bibliografía. En cambio, tres de los métodos oficiales tuvieron que ser adaptados para los AA, como fue el caso del contenido en I, el PV y el AC en los que fue necesario disminuir el peso de muestra inicial para poder llevar a cabo el análisis. En el primero, el exceso de I hizo imposible la filtración, mientras que los otros dos parámetros vieron dificultada la valoración volumétrica ya que algunas muestras muy oscuras no permitían observar correctamente el punto final. También, los AA causaron problemas en el desarrollo de otros métodos como el *p*-AnV, donde en algunos casos la turbidez de las muestras impidió la lectura de la absorbancia en el espectrofotómetro, lo que se solventó con una filtración previa. Además, el exceso de compuestos volátiles en algunos AA provocó la ebullición violenta de las muestras y salpicaduras en el interior de la estufa de vacío por lo que fue necesario mantener las muestras durante una noche en el desecador con un incremento progresivo del vacío hasta los 10 mm Hg para asegurar que se eliminaba parte de la humedad y compuestos volátiles. Y, por último, para algunos AA no se obtuvieron los valores del IT (período de inducción, *induction time*) medido con el instrumento Rancimat ya que no se detectó de forma clara el salto repentino de conductividad. En el caso del grupo de subproductos procedentes de grasas laúricas obtenidos mediante refinación física, presentaron problemas en dos parámetros de la caracterización. Debido a la presencia de AG de cadena corta y media (C6:0-C12:0), no fue posible la separación de las diferentes clases lipídicas por exclusión molecular ya que abarcaban un rango demasiado amplio de pesos moleculares que hizo que se encontrasen superpuestas algunas de las diferentes clases lipídicas y, por tanto, se descartase el uso de este método para subproductos con esta composición. A su vez, este tipo de AG también son volátiles lo que condujo a una sobreestimación del valor de humedad y compuestos volátiles calculada por el método de estufa

de vacío, si la determinación se llevaba a peso constante como en el resto de las muestras. Por ello, se hizo una comprobación con el método Karl Fischer y se concluyó que un único período de desecación de 1 h en estufa de vacío (es decir sin llevar la determinación a peso constante, varias desecaciones de 1h) era suficiente para que la sobreestimación no fuese significativa.

El desarrollo de estos métodos de análisis es una parte esencial de esta Tesis para lograr el objetivo de caracterización de los AA y AGD. Tras la adaptación de los métodos y su aplicación, los resultados obtenidos en la presente Tesis Doctoral a partir del estudio de 92 muestras de AA y AGD representativas del mercado español indicaron la influencia de dos factores principales en la calidad final de estos subproductos grasos (sección 5.1.2 [147] y sección 5.1.3 [148]): el proceso de refinación aplicado para la obtención del subproducto y la composición del aceite crudo del que proceden. Además, en algunos parámetros como el PV, podrían también afectar las condiciones y tiempo de almacenamiento del subproducto, aunque estos dos aspectos no fueron objeto de estudio en esta Tesis. Se observó que la composición difirió entre los AA procedentes de refinación química y los AGD procedentes de refinación física. Esto es debido en parte a que, de forma habitual, la refinación física emplea condiciones más drásticas de temperatura y será preferida en aceites y grasas saturados con menor tendencia a la oxidación frente a los aceites más insaturados, los cuales son generalmente refinados por el proceso químico. Por ello, los resultados obtenidos a nivel de composición de los AA y AGD, reflejaron la composición de AG, T y T3 de sus correspondientes aceites crudos, obteniéndose en los AGD un mayor contenido de T3 (mediana= 89.3 mg/kg, AGD, frente 21.8 mg/kg, AA) y AGS (mediana= 53.8%, AGD, frente a un 18.5%, AA), y por tanto una menor relación de I/S (mediana= 1.4, AGD, frente a 4.4, AA), mientras que los AA presentaron contenidos más altos de T (mediana= 813.1 mg/kg, AA, frente 65.6 mg/kg, AGD) y AGI (mediana= 37.3%, AA, frente a un 8.9%, AGD), a la vez que una mayor relación I/S (sección 5.1.2).

En las Figuras 6 y 7 se muestran los resultados en global de las secciones 5.1.2 y 5.1.3. Se trata de un análisis de componentes principales (PCA) donde se han incluido tanto variables referentes al valor nutricional como a la oxidación determinadas en los AA y AGD. El PCA refleja la clara influencia de la procedencia: las muestras se agrupan en varios grupos que coinciden con similar origen botánico y/o proceso de refinación. En el gráfico de variables (Figura 7) se observa cómo los AG más saturados y la variable T3 contribuyen en mayor medida a la separación de los AGD, mientras que T, AGI, y por tanto la relación I/S contribuyeron a la separación de los AA. De hecho, esta influencia se percibe incluso para algunos AG individuales: por ejemplo, los AG de cadena corta y media contribuyen a la separación de las grasas láuricas (LFAD), mientras que el C16:0 lo hace a los PFAD.



(**a**) (**b**)

Figura 6. Análisis de Componentes Principales (PCA), gráficos de muestras. Incluye parámetros de composición y calidad (29 variables, media centrada y escalada a la unidad de varianza) de los aceites ácidos (AA, n = 79) y ácidos grasos destilados (AGD, n = 13). (**a**) Gráfico de muestras coloreado según el proceso de refinación y (**b**) Gráfico de muestras coloreado según el origen botánico (ver Tabla 1 para abreviaciones).

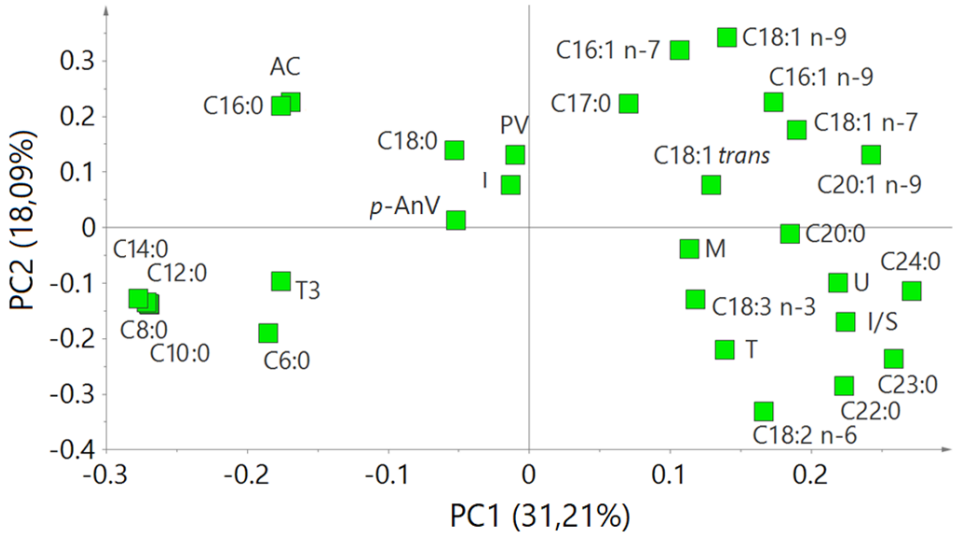


Figura 7. Análisis de Componentes Principales (PCA), gráfico de variables. Incluye 29 variables (media centrada y escalada a la unidad de varianza) correspondientes a los parámetros analizados en la caracterización de los aceites ácidos de refinación química (AA, n = 79) y ácidos grasos destilados (AGD, n = 13). Abreviaciones: M, contenido en humedad y materia volátil; I, contenido en impurezas insolubles; U, contenido en materia insaponificable; relación I/S, relación ácidos grasos insaturados/ácidos grasos saturados; T, suma de α-, β-, γ- y δ-tocoferoles; T3, suma de α-, β-, γ- y δ-tocotrienoles; AC, grado de acidez; PV, índice de peróxidos; *p*-AnV, índice de *p*-anisidina.

Se destaca también la contribución de las variables del *p*-AnV y la AC en la separación de los AGD, debido a que en el proceso de refinación físico la destilación correspondiente a la etapa de desodorización favorece la acumulación de compuestos volátiles en el subproducto, entre los que se encuentran los AGL y los aldehídos de oxidación (evaluados con el *p*-AnV). Además, las altas temperaturas del proceso físico también favorecen la menor retención de agua

en los AGD; en cambio se puede acumular más en los AA ya que se debe añadir agua en determinadas fases del proceso de obtención de los AA a partir de las pastas jabonosas. No obstante, con una optimización del proceso de desecación de los AA se podría reducir considerablemente el contenido de agua de los AA comercializados. Considerando el PV, esta variable básicamente no contribuyó en la varianza explicada, especialmente en el PC1, y en el caso de I muy ligeramente.

Considerando el efecto de la refinación (AA vs AGD), los datos obtenidos en las secciones 5.1.2 y 5.1.3 (mostrados en conjunto en las Figuras 6 y 7) demostraron que el proceso de eliminación de los AGL es determinante, ya que favorece la acumulación de diferentes compuestos en los subproductos. En los AGD obtenidos por destilación en la refinación física, tanto los AGL como el *p*-AnV (medición del contenido de compuesto aldehídicos) alcanzaron valores superiores (medianas de AGL= 90,0% y *p*-AnV= 36,4 respectivamente) frente a los AA (medianas de AGL= 52,9% y *p*-AnV= 16,4 respectivamente), debido a que las condiciones del proceso favorecen la destilación de estos componentes más volátiles. Esto explica los altos niveles de AGL en los AGD, mientras que los compuestos glicerídicos (TAG, DAG y MAG) y los POL son menos destilables y, por lo tanto, su presencia en los AGD sería menor o incluso nula (medianas POL, TAG, DAG Y MAG= ND, 5,2%, 4,0% y 0,0% en AGD, frente a 2,5%, 24,6%, 16,9% y 4,2% en AA). Con respecto al *p*-AnV, hay que tener en cuenta que este índice determina el contenido de aldehídos (especialmente los 2-alquenales y 2,4-alcadienales que proceden principalmente de los AGPI) y que los AA poseen un perfil de AG más insaturado, por lo que se esperarían mayores *p*-AnV en los AA; sin embargo, sucedió lo contrario ya que estos aldehídos son compuestos de bajo peso molecular lo que hace que destilen en la desodorización acumulándose en los AGD [148], hecho que también describió Nuchi et al [13]. Además, las altas temperaturas y bajas presiones empleadas en el proceso de desodorización explican también el menor contenido de agua en los AGD (mediana de M= 0.07 g/100g) frente a los AA (mediana de M= 0.97 g/100g) [147]. Por otro lado, como ya se ha indicado los resultados mostraron cómo la etapa de neutralización de la refinación química favorecía la acumulación del resto de clases lipídicas (TAG, DAG, MAG y POL) en los AA en comparación con los AGD, ya que estos componentes (poco volátiles) son arrastrados al separar las pastas jabonosas del aceite neutralizado. Por otra parte, los T y T3 también son arrastrados diluidos en las pastas jabonosas y fueron más elevados en los AA debido a que estos subproductos proceden de aceites crudos mucho más ricos en T (medianas de T en AA= 813,1 mg/kg y en AGD= 65,6 mg/kg) [147]. Se observa una ligera contribución de la I en la separación de los AGD, los cuales presentaron los valores más altos de medianas (2,85 g/100g en AGD frente a 1,57 g/100 g en los AA), aunque el

valor máximo se encontró en los AA. De forma general, los subproductos tienden a acumular I, las cuales podrían proceder de una contaminación durante el proceso, o también podrían ser I contenidas en el propio aceite crudo y acumuladas tanto en los AA como en los AGD.

Respecto al origen botánico del aceite crudo a partir del cual se obtiene el subproducto, varios parámetros de composición se vieron influenciados, entre ellos, como ya se ha indicado anteriormente el perfil de AG, T y T3, así como la U, ya que entre sus componentes se encuentran precisamente los T y T3 [147]. Por ejemplo, en el caso de los AA, aquellos que además de aceites de semillas contenían palma (SP) y manteca de cacao (SCP) en su composición inicial fueron los que presentaron mayores contenidos en AGS (medianas de SCP= 47,2% y SP= 27,3%), mientras que todos los que procedían de la refinación de aceites de semillas (BS, SU, SU-SO y SO) reflejaron un bajo contenido en este tipo de AG (medianas de SO= 18,1%, SU= 17,1%, SU-SO= 17,6% y BS= 18,2%). En los AGD, las grasas laúricas (LFAD), ricas en AGS de cadena corta y media, fueron las que obtuvieron los mayores contenidos seguidas de la palma (87,1% y 53,4% respectivamente). Por otro lado, en ambos tipos de subproductos, las muestras procedentes de la refinación de aceite de oliva y de orujo de oliva (O y OFAD), seguidas de aquellas que contenían palma en su composición (SP, SCP y PFAD), presentaron los contenidos más altos de AGMI como era de esperar (medianas de O= 67,4% y OFAD= 74,3%). Y finalmente, los AGPI en el caso de los AA mostraron los contenidos más altos para los aceites de semillas, (medianas de SU-SO= 56,7%, SO= 54,6%, SU= 47,5% y BS= 46,6%) lo que coincide con el mayor grado de insaturación (relación AGPI/AGS) de estas fuentes y en el caso de los AGD los contenidos más elevados de AGPI fueron los procedentes de la oliva y orujo de oliva (mediana de OFAD= 11,2%). En cuanto al contenido de T y T3, a rasgos generales, los AA procedentes de la refinación de aceites de semillas reflejaron los contenidos más elevados de la suma de ambos (medianas de SU= 1.699,2 mg/kg seguida de SO= 1.340,2 mg/kg y BS= 1.239,7 mg/kg), mientras que para los AGD no hubo diferencias entre agrupaciones según el origen [147]. Considerando la U, en los AGD destaca su mayor contenido en las muestras de oliva y orujo de oliva, lo que podría explicarse por el hecho que estas grasas suelen presentar escualeno, un hidrocarburo que se ve acumulado en la U. De hecho, también las muestras obtenidas a partir de la refinación química de la oliva y el orujo de oliva presentaron los valores de U más altos junto al grupo de mezcla de semillas (BS) y girasol (SU).

Por otro lado, como se detalla en las secciones 5.1.2 y 5.1.3 se observó una gran variabilidad de composición [147] y oxidación secundaria [148] dentro de un mismo grupo de muestras procedentes de aceites con un mismo origen botánico debido a diferentes aspectos. Esta variabilidad con relación a algunos parámetros (como la composición en AG, el contenido

en T y T3 o la U) se puede explicar por la variabilidad que presentan los aceites crudos de partida [147]. Además, otra posible explicación de dicha variabilidad se debe a que en muchas ocasiones estos subproductos se encuentran como mezclas debido a que algunas refinerías que refinan diversos tipos de aceites y grasas recogen estos subproductos en un mismo tanque, lo cual hace difícil controlar qué proporción de la mezcla final procede de cada aceite o grasa refinado, teniendo en cuenta a su vez, que la materia prima a refinar no presenta siempre la misma acidez y que las pastas jabonosas son extremadamente viscosas y difícilmente homogeneizables en el tanque de recogida.

No obstante, dicha variabilidad también puede deberse a otros factores, por ejemplo, en las muestras de AA procedentes de aceite de girasol se encontraron dos muestras con valores anómalos tanto de T [147] como de *p*-AnV [148]. Estos valores extremos que podrían deberse a la adición de deodestilados, que como se explicó anteriormente en la sección 2.2.1, son subproductos obtenidos en la etapa de desodorización de la refinación química por lo que son muy ricos en tocoferoles y tocotrienoles, compuestos de oxidación volátiles y otros componentes del U; sin embargo, no están incluidos en el Catálogo Europeo de ingredientes para piensos ya que suelen acumular grandes cantidades de contaminantes. También se observó una gran variabilidad en el IT evaluado mediante Rancimat, parámetro que refleja la estabilidad oxidativa de las muestras. Este período de inducción depende del balance entre sustratos de oxidación, antioxidantes y prooxidantes de las muestras. Por ello, se correlacionó de forma negativa con el contenido de AGS [148] y, además, la alta variabilidad observada en el IT se explicaría por el contenido también variable de antioxidantes naturales (T y T3) así como, por una posible adición de antioxidantes sintéticos a los AA que prolongasen el IT de éstos hasta valores muy elevados. De hecho, algunas empresas declararon la adicción de antioxidantes sintéticos como el BHT a los AA, los cuales en algunos casos sí coincidieron con aquellos que presentaron los valores más elevados de IT, sin embargo, en la mayoría de las muestras con valores más altos de IT o en las que no fue posible obtener un valor para esta determinación, no se especificó la adicción de antioxidantes sintéticos.

Finalmente, el PV dio valores próximos a cero y poco variables en casi todas las muestras de AA y AGD, lo que indicó que dichas muestras estaban recién producidas, ya que los peróxidos son altamente inestables a las altas temperaturas aplicadas durante el proceso de obtención de estos subproductos [148]. Esto está de acuerdo con lo reportado por Nuchi et al. [13]. Una vez que los AA Y AGD son producidos, el PV comenzaría a verse incrementando durante el almacenamiento, siendo la tasa y magnitud de este incremento dependiente de la oxidabilidad de la muestra y de las condiciones de almacenamiento.

En base a los resultados obtenidos en la caracterización se compararon estos valores con los límites de control de calidad establecidos en el Catálogo Europeo de ingredientes para piensos [12] y la guía a nivel estatal de Normas FEDNA [71]. En el caso de la M, el Catálogo Europeo indica que es obligatorio declarar su contenido si supera 1 g/100 g, conteniendo valores superiores a este límite 38 de las muestras analizadas en este estudio. Por otro lado, para las I, FEDNA tiene establecido un valor máximo de 0,15 g/100 g, límite que cumplió únicamente una muestra de las caracterizadas en esta Tesis. Respecto al parámetro MIU, FEDNA recomienda valores inferiores a 5 g/100g para ambos tipos de subproductos, encontrándose dentro de este límite 23 de las muestras estudiadas. Considerando, estos tres parámetros a la vez, sólo 1 muestra del total estuvo por debajo de los límites indicados. En cuanto a los parámetros de oxidación, únicamente en FEDNA se incluye un límite de 10 meq O_2/kg para el PV de los AA y AGD, el cual no fue superado por ninguna de las muestras, debido a que en los subproductos recién producidos el valor del índice de peróxidos debe ser próximo a 0 y a que estos subproductos en el campo de la elaboración de piensos tienen una rotación relativamente rápida y normalmente no pasa mucho tiempo desde su producción hasta su utilización para la fabricación de piensos. No obstante, para realizar una evaluación global del estado oxidativo de estos subproductos, además de un parámetro de oxidación primaria (PV), se incluyeron determinaciones de oxidación más avanzada como el *p*-AnV que mide el contenido de aldehídos y el porcentaje de compuestos poliméricos (POL). En ninguno de estos dos casos hay límites o recomendaciones establecidas para las grasas utilizadas en alimentación animal, ni en las guías ni en las regulaciones establecidas. Además, como se ha comentado, el contenido de estos compuestos de oxidación (aldehídos y compuestos poliméricos) en estos subproductos proviene en gran parte del aceite crudo que se refina, influyendo así su grado de oxidación. Además, como su contenido también depende del proceso de refinación aplicado, no son buenos indicadores del grado de oxidación que ha sufrido el subproducto desde su obtención. Por tanto, para interpretar estos resultados y establecer posibles recomendaciones hay que tener en cuenta todos estos aspectos. En este sentido, y simplificando desde un punto de vista práctico, es más interesante la determinación del % de compuestos poliméricos, puesto que un % elevado de estos compuestos (como entre el 2-7 % que se encuentran en más del 50% de las muestras de AA) implicará una reducción significativa del valor energético del subproducto.

Por último, a partir de los parámetros analizados de I/S, AGL y MIU, se calculó la energía metabolizable aparente (EMA, para pollos) y la energía digestible (ED, para cerdos) de las muestras de AA y AGD recogidas, haciendo uso de la ecuación de Wiseman et al. [30] tanto para animales jóvenes como adultos ya que es una de las más aplicadas por el sector ganadero.

Además, el cálculo se hizo también aplicando una corrección de esta ecuación con el valor de MIU de acuerdo con lo sugerido por Wealleans et al. [94] (Tabla S2, Anexo 8.2 [147]). Principalmente se observó el claro efecto del origen botánico, siendo las fuentes más insaturadas (con mayor relación I/S) aquellas con mayores valores energéticos debido al hecho bien reconocido de la mayor facilidad de formación de micelas favoreciendo la digestión y absorción de los AGI frente a los AGS. También, como se ha explicado anteriormente, los principales constituyentes de los AA y AGD son los AGL, los cuales, en contenidos elevados han sido relacionados con una menor absorción de los AG, especialmente los AGS, con la consiguiente reducción del valor energético [35,75]. En nuestro estudio, los valores más bajos de energía fueron obtenidos para las muestras del grupo PFAD, para todas las edades y ambas especies (Tabla S2, Anexo 8.2 [147]), lo que podría explicarse además de por el alto grado de saturación, porque presentaron altos contenidos en AGL (mediana AC= 86,3 g/100g). Además, como era de esperar, tras la corrección con el valor de MIU (estimación de fracción no energética de la grasa) se produjo una reducción de la energía por el efecto diluyente de estos componentes (hecho que también observaron Wealleans et al. [94]), así como una mayor variabilidad en los resultados de energía obtenidos tras la corrección, debido a la variabilidad en contenido de MIU que presentaron estos subproductos.

5.2.2. Efecto del almacenamiento y sus condiciones en piensos fabricados con fuentes grasas y procesos tecnológicos diferentes

Los resultados del estudio en piensos (sección 5.1.4) sobre el análisis de los parámetros de composición y degradación (considerando en esta última tanto hidrólisis como estabilidad oxidativa) mostraron cómo la calidad del pienso se ve afectada por distintos factores, siendo el tipo de fuente grasa añadida (CS, soja cruda; AS, aceite ácido de soja; RS, soja refinada; CP, palma cruda; PFAD, ácidos grasos destilados de palma y RP, palma refinada), el proceso de fabricación (piensos en forma de harinas, granulados o extrusionados) y el tiempo de almacenamiento (0, 30 y 60 días) los más determinantes, mientras que el efecto observado para la temperatura de almacenamiento (temperatura ambiente y 35 °C) no resultó ser relevante para la mayoría de los parámetros estudiados.

El hecho de modificar la fuente grasa y con ello la composición y calidad de esta, permitió obtener una visión general de las diferencias y similitudes en cuanto a la composición y calidad del pienso obtenido, comparando las grasas convencionales en alimentación animal (crudas), las refinadas y los subproductos también destinados a alimentación animal (AA y AGD). Se llevó a cabo una caracterización de las 6 grasas añadidas al pienso, evaluando parámetros de

composición y calidad de las mismas (Table 3). A grandes rasgos, la composición en AG, T y T3 estuvo en concordancia con la típica composición de este tipo de fuentes grasas según el *Codex Alimentarius*, siendo la soja, una fuente más rica en AGI (C18:2 n-6 y C18:3 n-3) y T (especialmente γ-T y δ-T), mientras que la palma en AGS (16:0 y 18:0), AGMI (18:1 n-9) y T3 (especialmente γ-T3 y α-T3) [149]. Por otro lado, AS presentó el valor más elevado de M (Tabla 3), con valores medios similares a los observados en la caracterización llevada a cabo en la presente Tesis (sección 5.1.2). Sin embargo, los valores medios del parámetro U en AS estuvieron por debajo de los obtenidos para AA representativos del mercado español (sección 5.1.2). Además, el PFAD, presentó valores medios de I incluso más bajos en comparación con las medias obtenidas en la caracterización de los AGD (sección 5.1.2). De hecho, considerando la suma de M, I y U, cabe destacar que ambos subproductos presentaron un valor de MIU ligeramente superior al 5% (límite recomendado en las Normas FEDNA tanto para AA como AGD [71]) lo que indica una calidad aceptable de los dos subproductos utilizados en este estudio (AS y PFAD) si se compara a la presentada normalmente por los AA y AGD representativos del mercado español (datos obtenidos en la caracterización, sección 5.1.2).

Tabla 3. Composición de las grasas añadidas a los piensos experimentales (n = 2).

	CS	AS	RS	CP	PFAD	RP
M (g/100 g)	0,20 ± 0,01	1,43 ± 0,02	ND	0,03 ± 0,00	0,01 ± 0,04	ND
I (g/100 g)	1,46 ± 0,06	1,57 ± 0,36	1,27 ± 0,04	0,80 ± 0,03	3,76 ± 0,23	0,59 ± 0,09
U (g/100 g)	0,95 ± 0,07	2,34 ± 0,03	0,99 ± 0,16	0,65 ± 0,05	1,34 ± 0,02	0,21 ± 0,03
AGS (%)	16,0 ± 0,02	19,9 ± 0,22	15,8 ± 0,02	49,4 ± 0,12	55,1 ± 0,19	50,6 ± 0,47
cis-AGMI (%)	25,6 ± 0,06	23,1 ± 0,01	27,1 ± 0,01	39,9 ± 0,10	35,9 ± 0,14	39,4 ± 0,40
cis-AGPI (%)	58,3 ± 0,05	57,0 ± 0,21	57,0 ± 0,01	10,7 ± 0,02	8,8 ± 0,06	9,8 ± 0,07
TAG (%)	95,1 ± 0,16	25,3 ± 1,79	96,3 ± 0,24	82,2 ± 0,14	4,3 ± 0,51	92,5 ± 0,47
DAG (%)	3,1 ± 0,04	13,5 ± 0,52	3,2 ± 0,35	10,3 ± 0,14	3,2 ± 0,18	7,5 ± 0,47
MAG (%)	ND	ND	ND	ND	ND	ND
AGL (%)	1,7 ± 0,13	61,2 ± 1,28	0,5 ± 0,11	7,5 ± 0,28	92,5 ± 0,69	0,0 ± 0,00
T (mg/kg)	1651,1 ± 82,45	1464,2 ± 111,74	1007,3 ± 116,47	166,6 ± 9,09	42,8 ± 9,01	199,4 ± 1,55
T3 (mg/kg)	ND	ND	ND	609,7 ± 28,86	52,6 ± 3,96	431,9 ± 6,72
T+T3 (mg/kg)	1651,1 ± 82,45	1464,2 ± 111,74	1007,3 ± 116,47	776,4 ± 37,94	95,4 ± 12,97	631,3 ± 8,27
PV (meq O_2/kg)	1,0 ± 0,03	1,2 ± 0,00	0,7 ± 0,03	2,9 ± 0,09	3,3 ± 0,16	0,5 ± 0,03
p-AnV	1,1 ± 0,19	10,8 ± 2,18	3,1 ± 0,68	6,5 ± 0,59	54,3 ± 1,44	6,4 ± 0,39

[1] Media ± desviación estándar. Abreviaciones: CS, aceite crudo de soja; AS, aceite ácido de soja; RS, aceite refinado de soja; CP, aceite crudo de palma; PFAD, ácidos grasos destilados de palma; RP, aceite de palma refinado; M, humedad y materia volátil; I, impurezas insolubles (expresado en peso húmedo); U, materia insaponificable (expresado en peso húmedo); AGS, AGS, ácidos grasos saturados (suma de C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0 y C24:0); *cis*-AGMI, *cis*-ácidos grasos monoinsaturados (suma de C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9); *cis*-AGPI, *cis*-ácidos grasos poliinsaturados (suma de C18:2 n-6 y C18:3 n-3); TAG, triacilgliceroles; DAG, diacilgliceroles; MAG, monoacilgliceroles; AGL, ácidos grasos libres; T, suma de α-, β-, γ- y δ-tocoferoles; T3, suma de α-, β-, γ- y δ-tocotrienoles; T+T3, suma de tocoferoles y tocotrienoles; PV, índice de peróxidos; *p*-AnV, índice de *p*-anisidina; ND, no detectado.

También, de acuerdo con los resultados de Nuchi et al. [13], los subproductos (AS y PFAD) fueron las fuentes grasas con los mayores contenidos en AGL y menores en TAG a diferencia de los aceites crudos y refinados (Tabla 3), siendo los contenidos de AGL más elevados en PFAD que en AS debido a que durante la obtención de los AGD el proceso de destilación favorece su acumulación [147]. En cuanto a los parámetros de oxidación, para todas las fuentes grasas (CS, AS, RS, CP, PFAD y RP) los valores de PV estuvieron por debajo del límite recomendado por FEDNA para AA, AGD y aceites crudos [71] y en el caso del *p*-AnV, fue superior en ambos subproductos respecto al resto de fuentes grasas (Tabla 3), siendo especialmente elevado en PFAD, lo que coincide con los resultados de Nuchi et al. [13].

Con respecto a los piensos, inicialmente se analizó la composición en AG de los piensos en forma de harina recién preparados (t0, tiempo 0 de almacenamiento). Los resultados de la Tabla 4 muestran cómo la composición en AG de las diferentes grasas añadidas en la formulación se vio reflejada en el perfil y contenidos de AG de los piensos en forma de harina recién preparados (t0). De hecho, las fuentes grasas fueron añadidas al 6% y los piensos presentaron valores medios de grasa extraída del 7,9%, lo que permite explicar la gran influencia de la grasa añadida.

Tabla 4. Composición en ácidos grasos (expresada como normalización interna de áreas, %) de las grasas añadidas al pienso y de los piensos recién producidos en forma de harinas.

	Grasas añadidas al pienso[1]						Piensos en forma de harina a t0[1]					
	CS[1]	AS	RS	CP	PFAD	RP	CS	AS	RS	CP	PFAD	RP
C16:0 (%)	11,3 ± 0,03	14,9 ± 0,25	11,0 ± 0,01	43,1 ± 0,11	46,6 ± 0,16	43,9 ± 0,48	12,4 ± 0,23	15,3 ± 0,01	12,0 ± 0,01	39,3 ± 0,02	41,5 ±0,22	39,4 ± 0,13
C18:0 (%)	3,3 ± 0,00	3,5 ± 0,01	3,5 ± 0,00	4,3 ± 0,01	6,6 ± 0,03	4,6 ± 0,04	3,4 ± 0,06	3,5 ± 0,03	3,5 ± 0,00	4,2 ± 0,00	6,2 ± 0,02	4,5 ± 0,01
C18:1 n-9 (%)	23,7 ± 0,05	21,1 ± 0,02	25,1 ± 0,01	38,9 ± 0,10	35,0 ± 0,15	38,4 ± 0,37	23,1 ± 0,03	20,8 ± 0,00	24,4 ± 0,01	35,8 ± 0,10	33,1 ± 0,04	36,0 ± 0,07
C18:2 n-6 (%)	52,2 ± 0,02	51,7 ± 0,18	51,7 ± 0,00	10,4 ± 0,02	8,5 ± 0,05	9,7 ± 0,07	51,8 ± 0,26	51,6 ± 0,08	51,4 ± 0,03	16,5 ± 0,13	14,9 ± 0,20	15,8 ± 0,04
C18:3 n-3 (%)	6,1 ± 0,03	5,3 ± 0,03	5,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,00	0,1 ± 0,00	5,8 ± 0,02	5,2 ± 0,01	5,1 ± 0,00	1,0 ± 0,02	1,0 ± 0,03	0,9 ± 0,01
trans-C18:1	0,03 ± 0,00	0,04 ± 0,00	0,04 ± 0,00	0,03 ± 0,00	0,22 ± 0,01	0,08 ± 0,00	0,04 ± 0,00	0,04 ± 0,01	0,04 ± 0,01	0,03 ± 0,00	0,19 ± 0,00	0,09 ± 0,01

[1] Media ± desviación estándar (n = 2, correspondiente tanto a los replicados de las grasas añadidas como a los replicados de los piensos en forma de harina). Abreviaciones: CS, aceite crudo de soja; AS, aceite ácido de soja; RS, aceite refinado de soja; CP, aceite crudo de palma; PFAD, ácidos grasos destilados de palma; RP, aceite refinado de palma; *trans*-C18:1 (suma de isómeros posicionales).

De forma resumida, de acuerdo con el perfil de AG típico de ambos tipos de aceite (soja y palma), los piensos cuya formulación incluía fuentes de soja fueron ricos en AGI (como el C18:2 n-6 y C18:3 n-3), mientras que los piensos fabricados con palma presentaron mayores contenidos de AGS (como el C16:0 y C18:0), así como de AGMI (C18:1 n-9) (Tabla 4). También cabe remarcar la presencia de *trans*-C18:1 en % más elevados en el subproducto PFAD, tanto en

la fuente grasa como en el pienso fabricado con esta (Tabla 4), lo que puede explicarse por las altas temperaturas en la etapa de obtención de dicho subproducto (desodorización) en el proceso físico, las cuales pueden favorecer las reacciones de isomerización [8,51]. De esta forma, la composición en AG de la fuente grasa añadida determina el perfil de AG del pienso que, a su vez, puede afectar a las reacciones de oxidación como se verá a continuación.

Al inicio del almacenamiento (t0, n = 18) también se estudió el efecto del proceso de producción del pienso (considerando harinas, granulados y extrusionados) sobre la composición y estado oxidativo de los piensos y se observó que tanto el tipo de grasa añadida como el proceso de producción del pienso afectaban a varios de los parámetros analizados en dichos piensos. De esta forma, los contenidos de T y T3 dependieron en parte de la composición lipídica de la fuente grasa añadida (Figura 8a): siendo los piensos elaborados con aceites de soja los más ricos en T, mientras que los piensos elaborados con aceite de palma (exceptuando PFAD) mostraron mayores contenidos en T3. Sin embargo, hay que señalar que, el contenido de estos antioxidantes naturales en piensos también se vio influenciado por los contenidos de estos en los cereales de la dieta basal, así como, por la suplementación de vitamina E en forma de α-Tocoferol acetato (donde el premix de vitaminas y minerales incluido en la dieta basal contenía 45 mg/ kg de pienso de vitamina E en forma de α-Tocoferol acetato). Por ejemplo, en el caso de las grasas añadidas a los piensos, los T3 estaban presentes únicamente en las fuentes de palma; sin embargo, debido a algunos cereales incluidos en la formulación como el trigo y la cebada, los cuales, son especialmente ricos en T3 [111,112], éstos vieron aumentado su contenido en todos los piensos (incluyéndose las sojas). También, aunque en todas las fuentes grasas de soja el principal T encontrado fue el γ-T y en las fuentes de palma fue el γ-T3, en todos los piensos el α-T fue el mayoritario, seguido del γ-T en las sojas y del α-T3 en el caso de las palmas. Así, el perfil final de T y T3 en todos los piensos fue el resultado de estos tres componentes, quedando en cierto modo enmascarado el efecto de la fuente grasa como tal. Además, los contenidos de T y T3 también variaron en función del tipo de proceso de elaboración del pienso, observándose una reducción del contenido de estos antioxidantes debido a los tratamientos térmicos aplicados durante los procesos de granulación y extrusión (Figura 8b).

El tipo de grasa añadida y el proceso de producción del pienso afectaron también al contenido de las distintas clases lipídicas en los piensos recién preparados (t0, n = 18). Por un lado, los piensos fabricados con grasas refinadas (RS y RP) y crudas (CS y CP) fueron los que presentaron los mayores contenidos de TAG mientras que los elaborados con subproductos reflejaron el alto contenido de AGL que caracteriza a la composición de los AA y AGD (Figura 8c). Como se ha mencionado previamente, la gran influencia del factor tipo de fuente grasa se

entiende al comparar la grasa añadida al pienso (6%) y el porcentaje medio de grasa extraído de los piensos (7,9%), reflejándose así en el pienso la composición en clases lipídicas de las fuentes grasas. Al estudiar el efecto del proceso tecnológico de fabricación del pienso en las clases lipídicas AGL y TAG, los contenidos de AGL fueron mayores en las harinas frente a los granulados y extrusionados (Figura 8d), lo que podría explicarse por la acción de las lipasas endógenas presentes en algunos ingredientes de los piensos, como por ejemplo los cereales. Estas enzimas, debido a los elevados tratamientos térmicos aplicados en la extrusión (entre 80-200 °C según bibliografía [150]) y en la granulación (generalmente entre 60-90 °C, [151]) pueden quedar inactivadas, reduciéndose así la hidrólisis de los TAG en AGL [102]. Como ya se ha explicado anteriormente en otros apartados, los AGL se relacionan con un impacto negativo en la digestión y absorción cuando están presentes en contenidos elevados en la dieta [35,75]. Sin embargo, estudios actuales con AA y AGD en pollos en fases de crecimiento-terminación indican que contenidos de AGL de hasta un 35% no afectan de forma negativa la utilización de los AG por parte del animal [77] o incluso porcentajes de hasta 56.3% de AS (con una relación I/S = 3.88) tampoco afectaron a la utilización de AG [78].

En cuanto a los parámetros de oxidación a t0 considerando todos los piensos en forma de harinas, granulados y extrusionados, el PV se vio influenciado de forma significativa por la fuente grasa, siendo más elevado en los piensos fabricados con grasas insaturadas (CS seguido de AS y RS) respecto a los fabricados con fuentes más saturadas (CP, PFAD y RP) (Figura 8e); sin embargo, el VC se vio afectado significativamente por el proceso tecnológico de fabricación del pienso, donde en algún caso se acumularon estos productos de oxidación secundaria (de forma significativa en el caso del pentanal), mientras que en otros se observó una reducción significativa en sus contenidos (hidrocarburos, cetonas y alcoholes) (Figura 8f). Las temperaturas de estos procesos podrían haber contribuido a la descomposición de los hidroperóxidos formándose compuestos volátiles que se acumularían en el pienso, pero, por otro lado, los tratamientos térmicos elevados también podrían ser la causa de su pérdida por volatilización. Por tanto, el resultado final encontrado en los piensos en cuanto a su VC se trata de un balance entre la formación y la pérdida por volatilización de dichos productos de oxidación secundaria. En el caso de los alcoholes como éstos pueden estar contenidos en ingredientes como los cereales, podría predominar su pérdida por volatilización frente a su formación por autooxidación de los AG, lo que explica el alto contenido encontrado en las harinas frente a los granulados y extrusionados.

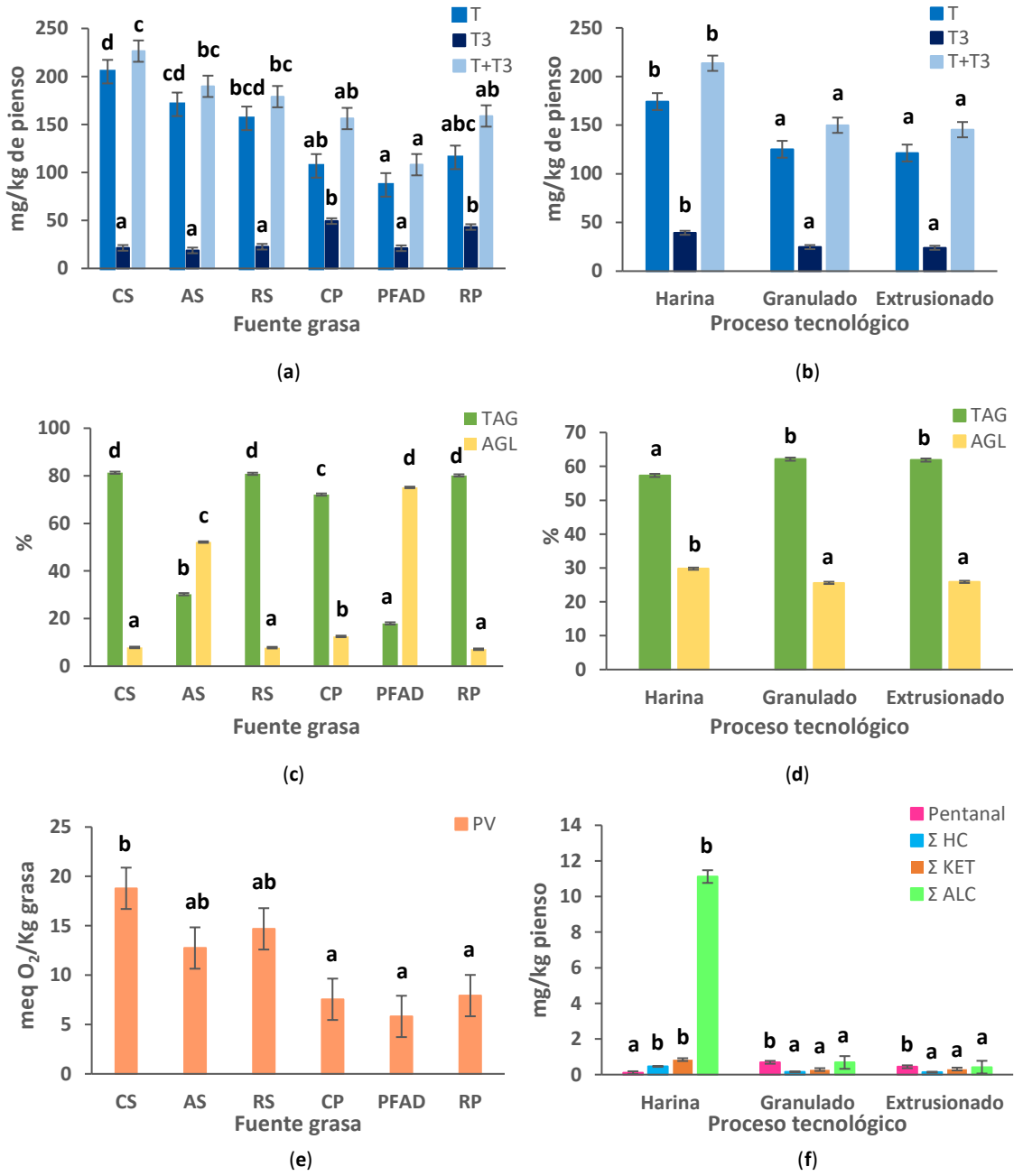
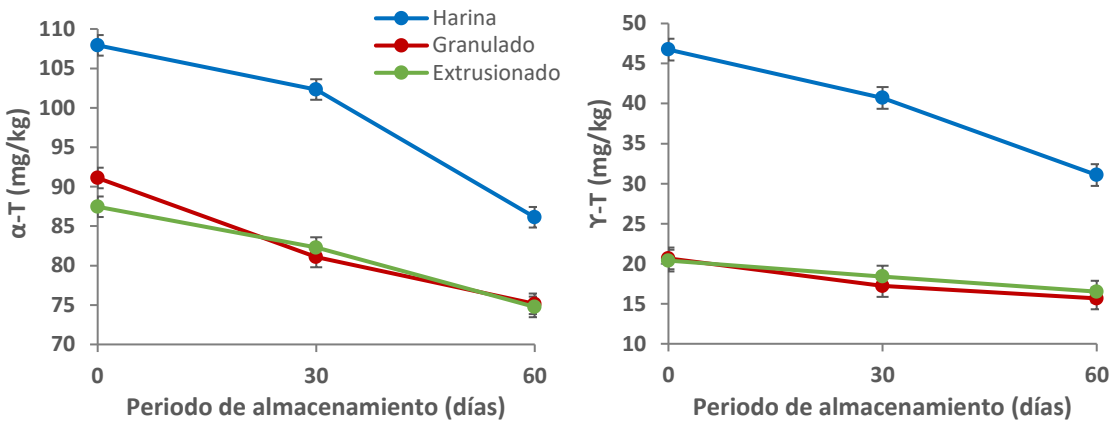


Figura 8. Efectos principales de los factores fuente grasa añadida (CS, aceite crudo de soja; AS, aceite ácido de soja; RS, aceite refinado de soja; CP, aceite crudo de palma; PFAD, ácido graso destilado de palma; RP, aceite refinado de palma) y proceso tecnológico de fabricación (harina, granulado y extrusionado) de todos los piensos a tiempo cero de almacenamiento. (**a**) y (**b**) contenido de tocoferoles (T), tocotrienoles (T3) y la suma de tocoferoles y tocotrienoles (T+T3) ($p \leq 0{,}001$ para T, T3 y T+T3 según la fuente grasa y $p = 0{,}003$ (T), $p = 0{,}001$ (T3) y $p \leq 0{,}001$ (T+T3) según el proceso de fabricación), (**c**) y (**d**) ácidos grasos libres (AGL) y triacilgliceroles (TAG) ($p \leq 0{,}001$ para ambos factores y variables) y (**e**) y (**f**) el índice de peróxidos (PV) ($p = 0{,}010$), pentanal ($p = 0{,}001$) y sumatorios volátiles ($p \leq 0{,}001$). Las barras corresponden a las medias agrupadas ± error estándar de dichas medias obtenidos del ANOVA multifactorial (n = 18 resultante de 6 fuentes grasas x 3 procesos de fabricación). Dentro de cada variable, los grupos según fuente grasa o proceso de fabricación con letras distintas (a-d) son medias significativamente diferentes ($p \leq 0{,}05$) de acuerdo con el test post-hoc de Tukey. Abreviaciones: Σ HC, Σ Hidrocarburos (suma de hexano y decano); Σ KET, Σ cetonas (suma de 1-penten-3-one, 2-heptanona, 3-octanona, 2-octanona y 1-octen-3-ona) y Σ ALC, Σ Alcoholes (suma de 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol, 1-octen-3-ol y 1-nonanol.

Durante el almacenamiento, varios parámetros analizados en los piensos sufrieron cambios en mayor o menor grado según el proceso de elaboración del pienso (harinas, granulados y extrusionados) y/o el tipo de grasa. Por ejemplo, cabe destacar la interacción significativa entre el proceso tecnológico empleado en la fabricación del pienso y el periodo de almacenamiento del pienso (0, 30 y 60 días) en la variables T y T3 (destacando especialmente los T mayoritarios, α-T y γ-T). El pienso en forma de harina se comportó de forma diferente durante el periodo de almacenamiento, donde se observó una reducción marcada de α-T y γ-T a partir del día 30 en las harinas en comparación a los granulados y extrusionados (Figura 9). Esto podría deberse a la gelatinización del almidón alcanzada durante la extrusión y granulación, a diferencia de las harinas, la cual impediría la penetración del oxígeno en los piensos granulados y extrusionados mejorando así la estabilidad oxidativa de los T y T3.



(**a**) (**b**)

Figura 9. Efecto de la interacción entre el proceso tecnológico de fabricación del pienso (harina, granulado y extrusionado) y el periodo de almacenamiento (0, 30, 60 días) en los contenidos de α-Tocoferol (α-T) y γ-Tocoferol (γ-T). (medias agrupadas ± error estándar de dichas medias obtenidos del ANOVA multifactorial, n = 108): (**a**) α-T ($p \leq 0{,}001$) y (**b**) γ-T ($p \leq 0{,}001$).

Considerando las variables TAG y AGL en la interacción respectiva a la fuente de grasa añadida (CS, AS, RS, CP, PFAD y RP) y el proceso de fabricación de los diferentes piensos (harinas, granulados o extrusionados), se observó que, según la composición inicial de la grasa añadida al pienso, los AGL y TAG se vieron afectados de forma distinta, con menores % de TAG y mayores % de AGL en todas las harinas, siendo las diferencias más acusadas en el caso de las grasas crudas y refinadas frente a los subproductos. De hecho, la hidrólisis de los TAG durante el almacenamiento de harinas formuladas con grasas crudas y refinadas podría suponer un problema en animales jóvenes, los cuales tienen mayor dificultad para la digestión y absorción de los AGL. Al estudiar también la interacción de la fuente grasa añadida (CS, AS, RS, CP, PFAD y RP) y la evolución en el tiempo de almacenamiento (0, 30 y 60 días) para las mismas variables,

en los piensos con grasas crudas y refinadas se produjo un aumento de los AGL y una reducción de los TAG durante el almacenamiento, mientras que esta modificación de la composición no fue tan importante en los subproductos que ya partieron de mayores contenidos en AGL. En la Figura 10, se observa la evolución de los AGL según el tipo de grasa, el proceso de fabricación, el tiempo y la temperatura de almacenamiento.

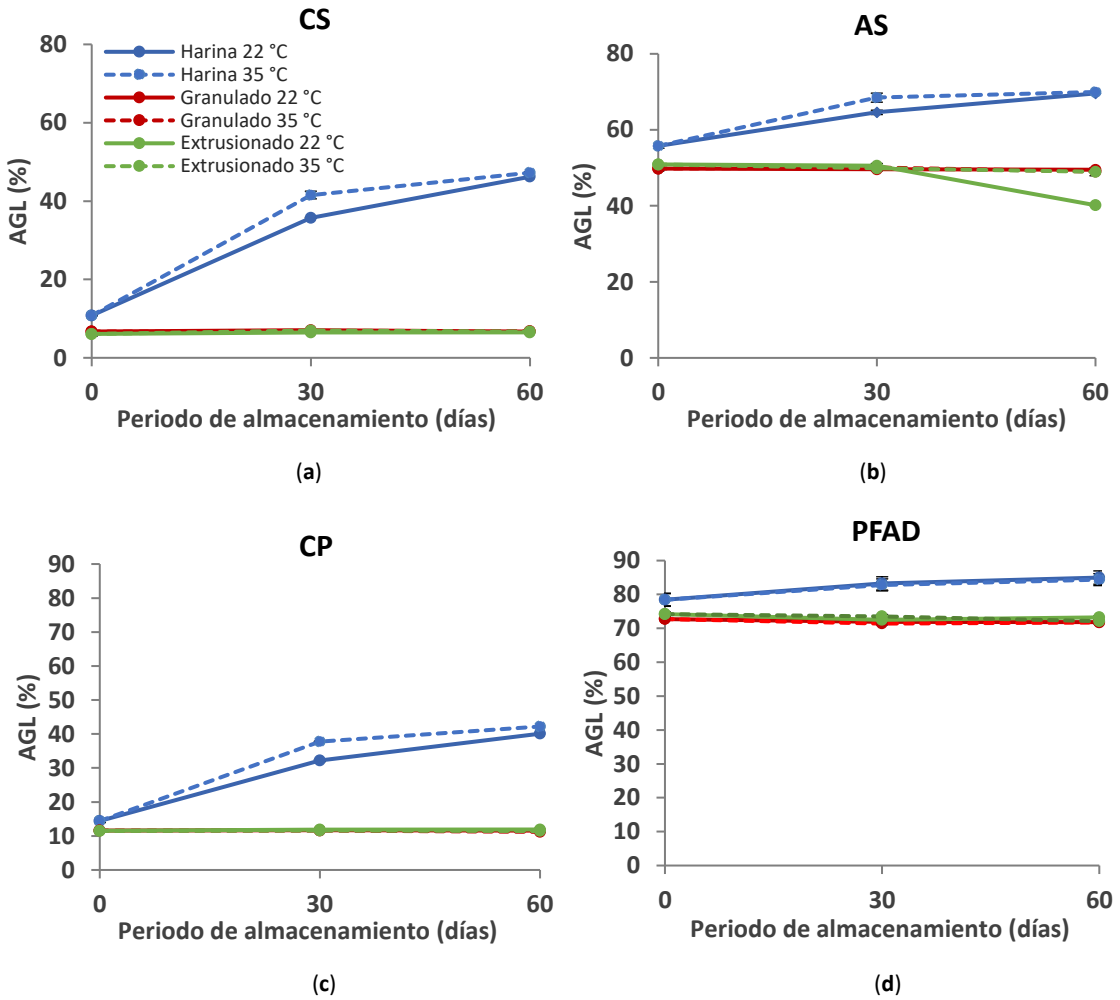


Figura 10. **Evolución de los ácidos grasos libres durante el almacenamiento de los piensos en función del proceso tecnológico de fabricación y de su temperatura de almacenamiento para piensos elaborados con distintas grasas.** (**a**) CS, aceite crudo de soja, (**b**) AS, aceite ácido de soja, (**c**) CP, aceite crudo de palma y (**d**) PFAD, ácidos grasos destilados de palma.

Se evidencia que este incremento de los AGL que ocurre durante el almacenamiento en los piensos con grasas crudas (lo mismo sucede con grasa refinada, Figura S1, sección 8.4) se da de forma más importante en las harinas que en los granulados y extrusionados, sin que la temperatura de almacenamiento influya de manera relevante. Para explicar las diferencias observadas para el contenido en AGL durante el periodo de almacenamiento, éstas se basan principalmente en el diferente comportamiento en el caso de las harinas, debido a que mantienen la actividad de las lipasas, lo que supone un aumento en el contenido de AGL tras la

hidrólisis de los TAG. Sin embargo, los piensos en forma de granulado y extrusionado, donde las lipasas quedan inactivas por los tratamientos térmicos aplicados en ambos procesos, los contenidos de AGL permanecen estables en todos los casos, tanto aceites crudos como subproductos. Según estos datos, los procesos de granulación y extrusión evitan la actividad de las lipasas y, por lo tanto, la hidrólisis de los TAG en piensos almacenados hasta 60 días. Por otro lado, la temperatura de almacenamiento del pienso (22 o 35°C) no influyó en este proceso probablemente debido a las pequeñas diferencias entre la actividad de las lipasas a estas dos temperaturas.

A su vez, según la Figura 10, los resultados sugieren que la utilización de una grasa cruda de soja (CS) almacenada durante 60 días podría ser comparable, en cuanto a presencia de AGL en el pienso (46,2% de AGL), a la utilización de un pienso fabricado con su correspondiente subproducto (AS), siempre que se utilice recién preparado (t0) (55,8% de AGL), ya que con el tiempo éste vería incrementado su contenido en AGL. Este hecho, no ocurre igual en el caso del subproducto de la palma (PFAD) ya que éste, aunque mantiene bastante estables los contenidos de AGL con el tiempo, contenía inicialmente (t0) % muy superiores (78.4% de AGL) respecto a la grasa cruda de palma (CP) al final del periodo de almacenamiento (40.1% de AGL), de forma que el pienso PFAD recién elaborado presentó prácticamente el doble de AGL al compararse con el pienso CP una vez pasado un periodo de 60 días.

Por último, los parámetros de oxidación evaluados (PV y VC) a lo largo del período de almacenamiento se relacionaron en general con una alta estabilidad oxidativa en todos los piensos estudiados. En cuanto a la interacción del proceso tecnológico de fabricación con el tiempo de almacenamiento, cabe destacar una reducción significativa del PV a los 30 días de almacenamiento especialmente en el caso de piensos granulados y extrusionados (Figura S2, sección 8.4). Esto se relacionó tanto con el predominio en la descomposición frente a la formación de los hidroperóxidos lipídicos, así como con la moderada reducción de T y T3 durante el almacenamiento de los piensos elaborados por procesos de granulación y extrusión.

5.2.3. Efectos sobre la calidad de la carne de pollo tras la utilización de aceite ácido de soja y ácidos grasos destilados de palma en las dietas

Respecto a los resultados obtenidos en el estudio de la calidad de la carne (sección 5.1.5), se destaca que, en general, la composición de la carne (AG, T y T3) así como la oxidación primaria (contenido LHP) se vieron mayormente influenciados por el origen botánico de las grasas y no por la utilización de subproductos en comparación grasas crudas o refinadas. Por otro lado, el tratamiento tecnológico (cocinado y almacenamiento en refrigeración tras el

cocinado) incrementó de forma significativa los parámetros de oxidación secundaria, y al igual que antes de forma independiente al tipo de grasa utilizada (crudas, subproductos o refinadas). Hay que tener en cuenta, como se ha discutido anteriormente que la calidad de los subproductos utilizados en este estudio (los mismos que en el estudio de piensos cuyos resultados y discusión sobre su caracterización se explican en el apartado anterior) fue aceptable si se compara con otros AA y AGD disponibles en el mercado, o según las recomendaciones FEDNA para el parámetro MIU [71].

Observando de forma más detallada los resultados, la composición en AG de las fuentes grasas añadidas (considerando el origen, es decir, fuentes de soja vs fuentes de palma), se vio reflejada en la composición del pienso, y a su vez, como era de esperar, ésta influenció los valores encontrados en la carne. Así, los piensos que contuvieron fuentes de soja fueron ricos en C18:2 n-6, C18:3 n-3 y *cis*-AGPI, al igual que también lo fueron las muestras de carne fresca de pollos alimentados con sojas (CS, RS y AS), sin embargo, aquellos piensos que contenían fuentes de palma en su composición presentaron los valores más altos de C16:0, C18:0, AGS, C18:1 n-9 y *cis*-AGMI, como también la carne fresca obtenida de pollos alimentados con palmas (CP, PFAD y RP) presentó los mayores contenidos en AGS y *cis*-AGMI (Tabla 5 y sección 5.1.5).

Tabla 5. Composición en ácidos grasos (expresada como normalización interna de áreas, %) de los piensos en forma de harinas y de las carnes frescas.

	Piensos en forma de harinas[1]						Carne fresca[2]					
	CS	AS	RS	CP	PFAD	RP	CS	AS	RS	CP	PFAD	RP
AGS	17,3 ± 0,33	20,4 ± 0,07	17,0 ± 0,01	45,5 ± 0,05	49,6 ± 0,27	46,0 ± 0,12	26,1 ± 0,50^{a}	27,7 ± 0,54^{b}	25,4 ± 0,68^{a}	34,9 ± 0,73^{cd}	34,4 ± 1,39^{c}	35,8 ± 0,63^{d}
cis-AGMI	25,0 ± 0,04	22,9 ± 0,02	26,4 ± 0,01	37,0 ± 0,10	34,3 ± 0,04	37,2 ± 0,08	36,7 ± 1,27^{a}	35,8 ± 1,00^{a}	36,9 ± 1,27^{a}	51,1 ± 0,23^{b}	51,6 ± 1,39^{b}	50,5 ± 0,63^{b}
cis-AGPI	57,6 ± 0,29	56,7 ± 0,09	56,5 ± 0,04	17,5 ± 0,15	15,9 ± 0,23	16,7 ± 0,05	37,1 ± 1,27^{b}	36,5 ± 0,92^{b}	37,7 ± 1,52^{b}	13,9 ± 0,84^{a}	13,9 ± 0,74^{a}	13,6 ± 0,40^{a}

[1] Media ± desviación estándar (n = 2, replicados por pienso). [2] Media ± desviación estándar del ANOVA de una vía (n = 6, replicados por tratamiento). Las medias de carnes frescas con letras diferentes indican diferencias significativas ($p \leq 0.05$) de acuerdo con el test post-hoc de Tukey. Abreviaciones: CS, aceite crudo de soja; AS, aceite ácido de soja; RS, aceite refinado de soja; CP, aceite crudo de palma; PFAD, ácidos grasos destilados de palma; RP, aceite refinado de palma. Considerando los piensos: AGS, ácidos grasos saturados (suma de C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0; C22:0, C23:0 y C24:0); *cis*-AGMI, *cis*-ácidos grasos monoinsaturados (suma de C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7 and C20:1 n-9); *cis*-AGPI, *cis*-ácidos grasos poliinsaturados (suma de C18:2 n-6 y C18:3 n-3). Considerando la carne fresca: AGS, ácidos grasos saturados (suma de C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, C23:0 and C24:0); *cis*-AGMI, *cis*-ácidos grasos monoinsaturados (suma de C14:1, C16:1 n-9, C16:1 n-7, C18:1 n-9, C18:1 n-7, C20:1 n-9 y C22:1 n-9); *cis*-AGPI, *cis*-ácidos grasos poliinsaturados (suma de C18:2 n-6, C18:3 n-6, C20:2 n-6, C20:3 n-6, C20:4 n-6, C18:3 n-3, C20:3 n-3, C20:5 n-3 y C22:6n-3).

Además, señalar que los contenidos de AGPI de cadena larga como el ácido araquidónico, ácido eicosapentaenoico y ácido docosahexaenoico encontrados en las carnes

frescas (incluidos en el sumatorio de AGPI, Tabla 5) fueron superiores en el caso de animales alimentados con fuentes grasas de soja ya que estos AGPI se sintetizan a partir del linoleico o linolénico, ambos más abundantes en estas fuentes que en las fuentes grasas de palma. Cabe destacar que al comparar entre ellas las diferentes fuentes grasas de soja (CS, AS y RS) no se encontraron grandes diferencias, en cuanto a magnitud, en los contenidos de los AG, ni en las fuentes grasas, ni en la composición en AG de los distintos piensos ni dentro de las carnes (frescas, F). Lo mismo sucedió al comparar entre ellas las diferentes fuentes grasas de palma (CP, PFAD y RP) (Tabla 5 y sección 5.1.4).

A su vez, como se ha comentado en el apartado anterior (5.2.2), varios factores afectaron al contenido y perfil de T y T3 en los piensos, donde se incluyen tanto el perfil de la grasa añadida como los contenidos en T y T3 de otros ingredientes de la dieta (cereales) [111] o, por último, la suplementación de vitamina E, estrategia que puede mejorar la estabilidad oxidativa de la carne [118,152]. Así, finalmente el α-T fue el mayoritario tanto en los piensos fabricados con las fuentes de soja como de palma, el cual, a su vez, también presentó los contenidos más elevados en ambos tipos de carne, las de pollos alimentados con fuentes de soja como con fuentes de palma. A pesar de que el γ-T se detectó en contenidos abundantes en los piensos, es ampliamente reconocida la eficiencia del α-T de ser transferido a la circulación mediante la proteína transportadora hepática del α-T que presenta una mayor preferencia para este compuesto que para el resto de T y T3 [153]. De hecho, todas las carnes frescas (incluyendo los 6 tratamientos dietéticos) presentaron contenidos elevados de α-T en cantidades totales (T+T3) por encima de 20 mg/kg, siendo este compuesto el principal contribuyente a la suma de T+T3 en carnes.

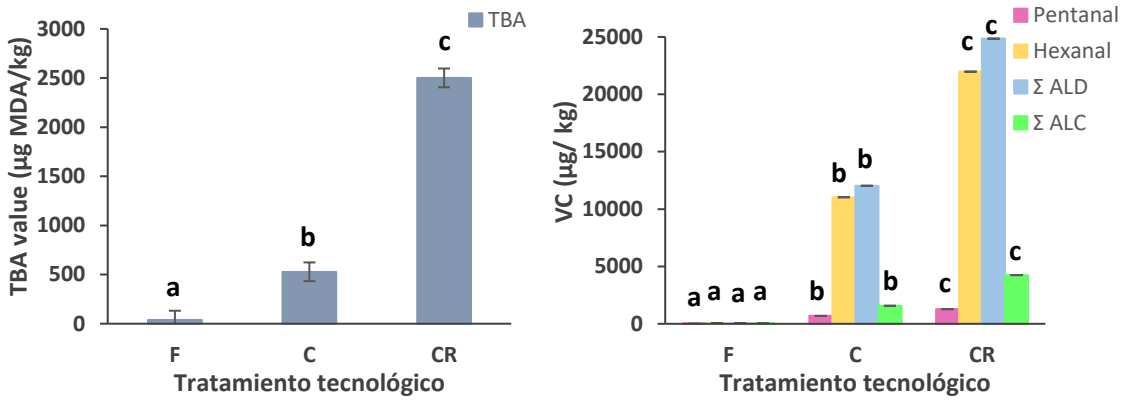
En lo respectivo a los parámetros de oxidación, tanto en los piensos (PV y VC) como en las carnes frescas (contenido de LHP, TBA y VC), de forma general éstos no fueron elevados. Siguiendo con los resultados de carnes frescas, a pesar de las diferencias en base al tratamiento dietético (CS, AS, RS, CP, PFAD y RP) encontradas para el contenido LHP, estas diferencias no fueron relevantes en magnitud. Además, la susceptibilidad a la oxidación (valor final LHP) también fue baja en todos los casos para estas carnes (F). Esta baja oxidación podría explicarse, según los contenidos de T y T3 en los piensos, donde los piensos fabricados con las diferentes fuentes grasas presentaron contenidos superiores a 150 mg/kg (excepto PFAD que fue ligeramente inferior) siendo ésta cantidad, una recomendación general en suplementación de piensos (en forma de α-tocoferol acetato), la cual, permite obtener una adecuada estabilidad oxidativa en carne de pollo fresca de animales alimentados con grasas vegetales, al igual que las grasas utilizadas en este estudio [118].Y, a su vez, los contenidos de T y T3 en las carnes frescas

también explican la baja oxidación encontrada en estas muestras, donde la suma T y T3 se mantuvo en un rango entre 22.6 y 33.3 mg/kg, sin observarse diferencias significativas al comparar los diferentes tratamientos dietéticos en el caso del T mayoritario (α-T).

Considerando el total de muestras (n = 108) y los dos factores principales del estudio, tanto el tratamiento dietético recibido por el animal (CS, AS, RS, CP, PFAD y RP) como el proceso tecnológico al que se somete la carne (fresca, F; cocida, C, y cocida y almacenada en refrigeración, CR) se valoró su posible influencia en el estado oxidativo de la carne, así como sobre su susceptibilidad a la oxidación y se observó una mayor influencia del origen botánico (fuentes de soja vs fuentes de palma) frente al tipo de grasa (cruda, subproducto o refinada) (sección 5.1.5). Respecto al efecto del tratamiento tecnológico, el parámetro de oxidación primaria que mide el contenido inicial de LHP se vio incrementado significativamente en el siguiente orden F< C< CR, lo que se explica debido a la aplicación de tratamientos térmicos que promueven las reacciones de oxidación lipídica en la carne [40,43]. La formación de hidroperóxidos lipídicos durante el cocinado se relaciona con la ruptura de las membranas celulares facilitándose el contacto entre sustratos (AG) y agentes prooxidantes (liberación de hierro…), a la vez que se inactivan enzimas con actividad antioxidante [40,42], lo que conduce al aumento en la oxidación lipídica de la carne, reflejada en ese incremento de los productos de oxidación primaria. Por tanto, procesos como el cocinado promueven las reacciones de oxidación, continuando estas reacciones durante la refrigeración. Sin embargo, además del efecto del tratamiento tecnológico, este incremento del contenido LHP se vio afectado por el tratamiento dietético con un mayor incremento en el caso de las carnes procedentes de fuentes de soja, ricas en AGPI (especialmente en la CS) respecto a las procedentes de fuentes de palma, ya que las grasas más insaturadas son los sustratos principales de la oxidación lipídica (interacción significativa en ANOVA multifactorial, sección 5.1.5). Considerando el valor final de LHP, la interacción entre los dos factores estudiados reflejó una tendencia diferente (F< C> CR) en el caso de las carnes procedentes de animales alimentados con fuentes grasas de palma, donde la susceptibilidad de la muestra a la oxidación fue menor en el caso de las CR frente a las C, especialmente bajo en las muestras de RP.

En cuanto a los parámetros de oxidación secundaria (TBA y VC como hexanal y pentanal, ΣALD y ΣALC), presentaron un incremento significativo en sus contenidos al comparar F respecto a C y, esta última respecto al almacenamiento en refrigeración (CR) donde este aumento fue incluso mayor (Figura 11). Sin embargo, este incremento en la oxidación secundaria especialmente en el caso de las muestras CR, no vino acompañado de una modificación en los contenidos de T o T3, siendo únicamente significativa su disminución tras la refrigeración en el

contenido de T3. De esta forma, aunque los contenidos de T y T3 evitaron que los parámetros de oxidación fueran elevados en las muestras F, las reacciones de oxidación ocurrieron en las muestras de C y CR debido a los tratamientos térmicos aplicados en el cocinado.



(**a**) (**b**)

Figura 11. Efecto principal del factor tratamiento tecnológico aplicado en la carne (F, carne fresca; C, carne cocida y CR, carne cocida y almacenada en refrigeración) considerando todas las muestras de carne del estudio (n = 108). (**a**) TBA, índice del ácido tiobarbitúrico ($p \leq 0{,}001$) y (**b**) pentanal, hexanal y sumatorios de compuestos volátiles ($p \leq 0{,}001$, en todos los casos). Las barras corresponden a las medias agrupadas ± error estándar de dichas medias obtenidos del ANOVA multifactorial (n = 36 para cada tipo de carne). Dentro de cada variable, los grupos según proceso de fabricación con letras distintas (a-c) son medias significativamente diferentes ($p \leq 0{,}05$) de acuerdo con el test post-hoc de Tukey. Abreviaciones: Σ ALD, Σ Aldehídos (suma de pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal y 2,4-nonadienal) Σ ALC, Σ Alcoholes (suma de 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol, 1-octen-3-ol y 1-nonanol).

Por tanto, según una visión general de estos resultados, el uso en alimentación de pollos de los subproductos (AA y PFAD) con una calidad parecida a los ensayados en este estudio, no debería dar lugar a deterioros relevantes en la composición o estabilidad oxidativa de la carne, siendo mayor el efecto del origen botánico de las fuentes grasas usadas en la formulación (carnes obtenidas de animales alimentados con fuentes de soja respecto a las obtenidas de animales alimentados con fuentes de palma) que el tipo de grasa (cruda, subproducto o refinada). Por otro lado, el proceso tecnológico claramente afectó a los parámetros de oxidación primaria (contenido LHP) y secundaria (TBA y VC) de la carne, especialmente en la carne cocinada y almacenada en refrigeración que presentó los valores más elevados referidos a la oxidación secundaria. Sin embargo, únicamente para el contenido LHP se observó interacción entre el tratamiento dietético y el tratamiento tecnológico. En general, en el caso del valor final LHP, la susceptibilidad a la oxidación fue mayor en las carnes procedentes de las fuentes de soja que en las carnes procedentes de las fuentes de palma.

6. CONCLUSIONES

6. Conclusiones

- ***Conclusiones relativas a la adaptación de los métodos analíticos y a la caracterización de los aceites ácidos (AA) y ácidos grasos destilados (AGD):***

1. La adaptación de los métodos analíticos disponibles para grasas y aceites crudos o refinados para ser aplicados al análisis de AA y AGD, requirió mínimas modificaciones en las determinaciones de composición en AG, del contenido en T y T3, U, AC y PV. Sin embargo, para las determinaciones de M, I, composición en clases lipídicas y el *p*-AnV fueron necesarias modificaciones más relevantes. Los métodos analíticos adaptados a los AA y AGD suponen una herramienta de gran utilidad para la mejora del control de calidad de dichos ingredientes en alimentación animal.

2. Los AA y AGD presentaron una elevada variabilidad en composición y calidad lo que refleja la falta de estandarización de estos subproductos. Algunos parámetros de composición como la AC, la composición en clases lipídicas o la M y parámetros de oxidación como el *p*-AnV y el contenido en POL difirieron principalmente debido al tipo de proceso de refinación del que procedían los subproductos mientras que los T y T3, el U y la composición en AG se vieron más influenciados por el origen botánico del aceite crudo del que proceden los subproductos.

3. Gran parte de las muestras de AA y AGD disponibles en el mercado español presentaron valores de MIU, parámetro de interés nutricional y energético, por encima del límite indicado por las pocas recomendaciones que existen para el control de calidad de estos subproductos, por lo que sería necesaria una revisión de los límites recomendados para su uso en alimentación animal.

4. Para una evaluación global del grado de oxidación de los AA y AGD, sería adecuado estudiar la necesidad de incluir en las recomendaciones del control de calidad de estos subproductos además del ya considerado PV como parámetro de oxidación primaria, otros parámetros de oxidación secundaria como el *p*-AnV, o el contenido de POL.

- ***Conclusiones relativas al estudio del efecto de las condiciones de fabricación y almacenamiento sobre la composición y estabilidad oxidativa de piensos elaborados con AA o AGD o sus correspondientes grasas crudas y refinadas:***

5. La hidrólisis lipídica se vio afectada por el proceso tecnológico de fabricación del pienso, observándose un rápido incremento del contenido en AGL de los piensos en forma de harina en comparación con los piensos granulados y extrusionados, en los cuales el contenido en AGL no aumentó a lo largo del almacenamiento.

6. Este aumento en AGL es atribuible a la acción de lipasas endógenas sobre los TAG y es especialmente relevante en los piensos en forma de harina elaborados con grasas crudas y refinadas. Esto es debido a que, a diferencia de la granulación y extrusión, la elaboración de piensos en forma de harina no implica tratamientos térmicos que puedan desnaturalizar estas enzimas y, por otra parte, la formulación con aceites crudos y refinados implica una mayor disponibilidad de TAG que la formulación con subproductos ricos en AGL (AA y AGD).
7. En general, todos los piensos del estudio mostraron una elevada estabilidad oxidativa durante el periodo y condiciones de almacenamiento.
8. En comparación con las correspondientes grasas crudas y refinadas, la estabilidad oxidativa durante el almacenamiento no se vio afectada de forma relevante por la utilización de subproductos ricos en AGL en la formulación de piensos.

- ***Conclusiones relativas al estudio de la composición y estabilidad oxidativa de la fracción lipídica de la carne de pollos alimentados con AA o AGD o sus correspondientes grasas crudas y refinadas:***

9. La composición en AG, los contenidos en T y T3, así como, los parámetros de oxidación primaria (contenido LHP) y estabilidad oxidativa (valor final LHP) en carnes se vieron más afectados por el tipo de grasa añadida en cuanto a origen (soja o palma) que por su calidad (cruda, subproducto o refinada), lo que pudo relacionarse con las mínimas diferencias en composición en AG y en contenido en T y T3 entre los piensos formulados con un subproducto frente a los formulados con la correspondiente grasa cruda o refinada.
10. El tratamiento tecnológico del cocinado de la carne incrementó los contenidos de parámetros de oxidación primaria y secundaria, y el hecho de almacenar en refrigeración después del cocinado incrementó aún más la oxidación secundaria. En ambos casos, el incremento se vio principalmente influenciado por el origen botánico de la grasa añadida al pienso (soja o palma) y no por su calidad (cruda, subproducto o refinada).
11. El uso de los subproductos de la refinación de aceites vegetales no dio lugar a deterioros relevantes en la composición o estabilidad de la carne al comparar su uso con las correspondientes grasas crudas y refinadas.

7. REFERENCIAS

7. Referencias

1. Mohdaly, A.A.R.; Seliem, K.A.H.; Maher, A.M.; Mahmoud, A.A.T. Effect of refining process on the quality characteristics of soybean and otton seed oils. *Int. J. Curr. Microbiol. App. Sci.* **2017**, *6*, 207–222. doi:10.20546/ijcmas.2017.601.026.
2. Gotor, A.A.; Rhazi, L. Effects of refining process on sunflower oil minor components : A review. *OCL (Oilseeds &fats Crops and Lipids)* **2016**, *23*, D207. doi:10.1051/ocl/2016007.
3. Suliman, T.E.M.A.; Jiang, J.; Liu, Y. Chemical refining of sunflower oil: effect on oil stability, total tocopherol, free fatty acids and colour. *Int. J. Engineering Sci. Technol.* **2013**, *5*, 449–454.
4. Ghazani, S.M.; Marangoni, A.G. Minor components in canola oil and effects of refining on these constituents: A Review. *J. Am. Oil Chem. Soc.* **2013**, *90*, 923–932. doi:10.1007/s11746-013-2254-8.
5. Leeson, S.; Summers, J.D. *Commercial Poultry Nutrition,* 3rd ed.; Nottingham University Press: Nottingham, UK, 2008.
6. Naz, S.; Sherazi, S.T.H.; Talpur, F.N.; Kara, H.; Uddin, S.; Khaskheli, A.R. Chemical characterization of canola and sunflower oil deodorizer distillates. *Pol. J. Food Nutr. Sci.* **2014**, *64*, 115–120. doi:10.2478/pjfns-2013-0008.
7. Van Heugten, E.; Odle, J.; Boyd, R.D. Feeding value of fat for swine - Concepts and practice. In *Proceedings of the: Milk Specialties Global Conference, Pre-Conference to Minnesota Nutrition Conference*, Minnesota, USA, 2015.
8. FEDIOL, The European Vegetable Oil and Protein Meal Industry Association - Refining of Edible Oils. Disponible online: www.fediol.eu/web/refining/1011306087/list1187970096/f1.htm (Acceso el 20 agosto 2021).
9. Lin, X.; Azain, M.; Odle, J. Lipids and lipid utilization in swine. In *Sustainable Swine Nutrition*, 1st ed.; Wiley-Blackwell Publishing: Oxford, UK, 2013.
10. Kerr, B.J.; Kellner, T.A.; Shurson, G.C. Characteristics of Lipids and Their Feeding Value in Swine Diets. *J. Anim. Sci. Biotechnol.* **2015**, *6*, 30. doi:10.1186/s40104-015-0028-x.

11. Dumont, M.J.; Narine, S.S. Soapstock and deodorizer distillates from north American vegetable oils: review on their characterization, extraction and utilization. *Food Res. Int.* **2007**, *40*, 957–974. doi:10.1016/j.foodres.2007.06.006.
12. Commission Regulation (EU) 68/2013 of 16 January 2013 and Its Amendments on the Catalogue of Feed Materials*. Off. J. Eur. Union* **2013**, 29, 1-64. Disponible online: http://data.europa.eu/eli/reg/2013/68/oj (Acceso el 10 agosto 2021).
13. Nuchi, C.; Guardiola, F.; Bou, R.; Bondloli, P.; Bella, L.D.; Codony, R. Assessment of the levels of degradation in fat co-and byproducts for feed uses and their relationships with some lipid composition parameters. *J. Agric. Food Chem.* **2009**, *57*, 1952–1959. doi:10.1021/jf803369h.
14. Kochhar, P. *Functional dietary lipids. Food formulation, consumer issues and innovation for health,* 1st ed.; Woodhead: Cambridge, UK, 2016. doi:10.1016/C2013-0-16501-4.
15. Baião, N.C.; Lara, L.J.C. Oil and fat in broiler nutrition. *Revista Brasileira de Ciência Avícola* **2005**, *7*, 129–141. doi:10.1590/s1516-635x2005000300001.
16. Riaz, M.N.; Alam, M.S. Fats and oils in feedstuffs and pet foods, *Bailey's Industrial Oil and Fat Products*, 7th ed.; John Wiley & Sons Ltd.: Texas, USA, 2020. doi:10.1002/047167849x.bio074.pub2.
17. Çetingül, İ.S.; Yardimci, M. The importance of fats in farm animal nutrition. *Kocatepe Vet. J.* **2008**, *1*, 77–81.
18. Bindari, Y.R.; Shrestha, S.; Shrestha, N.; Gaire, T.N. Effects of nutrition on reproduction-A review. *Adv. Appl. Sci. Res.* **2013**, *4*, 421–429.
19. Funston, R.; Filley, S. Effects of Fat Supplementation on Reproduction in Beef Cattle. In *Proceedings of the Applied Reproductive Strategies in Beef Cattle* Workshop, Manhattan, USA, 2002.
20. Atteh, J.O.; Leeson, S. Effects of dietary saturated or unsaturated fatty acids and calcium levels on performance and mineral metabolism of broiler chicks. *Poult. Sci.* **1984**, *63*, 2252–2260. doi:10.3382/ps.0632252.
21. Wohl, G.R.; Loehrke, L.; Watkins, B.A.; Zernicke, R.F. Effects of high-fat diet on mature bone mineral content, structure, and mechanical properties. *Calcif. Tissue Int.* **1998**, *63*, 74–79. doi:10.1007/s002239900492.

22. Mulik, J. Poultry Industry. Rendering technology and rendered by-products. Disponible online: https://en.engormix.com/poultry-industry/articles/fat-animal-feed-t35105.htm (acceso el 2 julio 2021).
23. Ilo, S.; Schoenlechner, R.; Berghofe, E. Role of lipids in the extrusion cooking processes. *Grasas Aceites* **2000**, *51*, 97–110. doi:10.3989/gya.2000.v51.i1-2.410.
24. Doppenberg, J.; Van der Aar, P.J. *Facts about Fats, A review of the feeding value of fats and oils in feeds for swine and poultry,* 1st ed.; Wageningen Academic Publishers: Wageningen, The Netherlands, 2010.
25. Sørensen, M. A. Review of the effects of ingredient composition and processing conditions on the physical qualities of extruded high-energy fish feed as measured by prevailing methods. *Aquacult. Nutr.* **2012**, *18*, 233–24. doi:10.1111/j.1365-2095.2011.00924.x.
26. Rokey, G.J.; Plattner, B.; De Souza, E.M. Feed extrusion process description. *R. Bras. Zootec.* **2010**, *39*, 510–518. doi:10.1590/S1516-35982010001300055.
27. Economic Research Service, U.S. Department of agriculture. Oil Crops Data: Yearbook Tables, 2021. https://www.ers.usda.gov/data-products/oil-crops-yearbook/.
28. Ravindran, V.; Tancharoenrat, P.; Zaefarian, F.; Ravindran, G. Fats in poultry nutrition: digestive physiology and factors influencing their utilisation. *Anim. Feed Sci. Technol.* **2016**, *213*, 1–21. doi:10.1016/j.anifeedsci.2016.01.012.
29. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. III. Effect of chemical composition on metabolisable energy of by-products of vegetable oil refining. *Br. Poult. Sci.* **1996**, *37*, 131–144. doi:10.1080/00071669608417843.
30. Wiseman, J.; Powles, J.; Salvador, F. Comparison between pigs and poultry in the prediction of the dietary energy value of fats. *Anim. Feed Sci. Technol.* **1998**, *71*, 1–9. doi:10.1016/S0377-8401(97)00142-9.
31. Wealleans, A.L.; Bierinckx, K.; Benedetto, M. Fats and oils in pig nutrition: Factors affecting digestion and utilization. *Anim. Feed Sci. Technol.* **2021**, *277*, 114950. doi:10.1016/j.anifeedsci.2021.114950.
32. Ramírez, M.; Amate, L.; Gil, A. Absorption and distribution of dietary fatty acids from different sources. *Early Hum. Dev.* **2001**, *65*, S95–S101. doi:10.1016/S0378-3782(01)00211-0.

33. Vilarrasa, E.; Guardiola, F.; Codony, R.; Esteve-Garcia, E.; Barroeta, A.C. Use of combinations of re-esterified oils, differing in their degree of saturation, in broiler chicken diets. *Poult. Sci.* **2015**, *94*, 1539–1548. doi:10.3382/ps/pev140.
34. Sklan, D. Digestion and absorption of lipids in chicks fed triglycerides or free fatty acids: synthesis of monoglycerides in the intestine. *Poult. Sci.* **1979**, *58*, 885–889. doi:10.3382/ps.0580885.
35. Wiseman, J.; Salvador, F.; Craigon, J. Prediction of the apparent metabolizable energy content of fats fed to broiler chickens. *Poult. Sci.* **1991**, *70*, 1527–1533. doi:10.3382/ps.0701527.
36. Wiseman, J.; Edmunds, B. K.; Shepperson, N. The apparent metabolisable energy of sunflower oil and sunflower acid oil for broiler chickens. *Anim. Feed Sci. Technol.* **1992**, *36*, 41–51. doi:10.1016/0377-8401(92)90084-J.
37. O'Brien, R.D. Fats and oils processing. In *Fats and Oils: Formulating and Processing for Applications*, 2nd ed.; CRC Press LLC: Boca Raton, FL, USA, 2004.
38. Shahidi, F.; Zhong, Y. Lipid oxidation and improving the oxidative stability. *Chem. Soc. Rev.* **2010**, *39*, 4067–4079. doi:10.1039/b922183m.
39. Frankel, E.N. Lipid oxidation: mechanisms, products and biological significance. *J. Am. Oil Chem. Soc.* **1984**, *61*, 1908–1917. doi:10.1007/BF02540830.
40. Min, B.; Ahn, D.U. Mechanism of lipid peroxidation in meat and meat products - A review. *Food Sci.Biotechnol.* **2005**, *14*, 152–163.
41. Choe, E.; Min, D.B. Mechanisms and factors for edible oil oxidation. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 169–186.
42. Amaral, A.B.; Solva, M.V. da; Lannes, S.C.D.S. Lipid oxidation in meat: mechanisms and protective factors - A review. *Food Sci. Technol.* **2018**, *38*, 1–15. doi:10.1590/fst.32518.
43. Domínguez, R.; Pateiro, M.; Gagaoua, M.; Barba, F.J.; Zhang, W.; Lorenzo, J.M.A. Comprehensive review on lipid oxidation in meat and meat products. *Antioxidants* **2019**, *8*, 429. doi:10.3390/antiox8100429.
44. Snyder, J.M.; Frankel, E.N.; Selke, E. Capillary gas chromatographic analyses of headspace volatiles from vegetable oils. *J. Am. Oil Chem. Soc.* **1985**, *62*, 1675–1679. doi:10.1007/BF02541664.

45. Choe, E.; Min, D.B. Comprehensive reviews in food science and food safety mechanisms and factors for edible oil oxidation. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 169–186. doi:10.1111/j.1541-4337.2006.00009.x.
46. Chang, P.L.; Dean Boyd, R.; Zier-Rush, C.; Rosero, D.S.; Van Heugten, E. Lipid peroxidation impairs growth and viability of nursery pigs reared under commercial conditions. *J. Anim. Sci.* **2019**, *97*, 3379–3389. doi:10.1093/jas/skz183.
47. Gunstone, F.D. *Vegetable Oils in Food Technology Composition, Properties and Uses*, 2nd ed.; Willey-Blackwell Publishing Ltd.: Oxford, UK, 2011.
48. de Greyt, W. Edible oil refining: current and future technologies in edible oil processing. In *Edible Oil Processing,* 2nd ed.; John Wiley & Sons, Ltd., 2013.
49. Bockisch, M. *Fats and Oils Handbook,* 1st ed.; Academic Press and AOCS Press: Champaign, IL, USA, 1998.
50. Vaisali, C.; Charanyaa, S.; Belur, P.D.; Regupathi, I. Refining of edible oils: a critical appraisal of current and potential technologies. *Int. J. Food Sci. Technol.* **2015**, *50*, 13–23. doi:10.1111/ijfs.12657.
51. Gibon, V.; De Greyt, W.; Kellens, M. Palm oil refining. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 315–335. doi:10.1002/ejlt.200600307.
52. Dijkstra, A.J. Recent developments in edible oil processing. *Eur. J. Lipid Sci. Technol.* **2009**, *111*, 857–864. doi:10.1002/ejlt.200900124.
53. Verhé, R.; Verleyen, T.; Van Hoed, V.; De Greyt, W. Influence of refining of vegetable oils on minor components. *J. Oil Palm Res.* **2006**, 168–179.
54. Tasan, M.; Demirci, M. Trans FA in sunflower oil at different steps of refining. *J. Am. Oil Chem. Soc.* **2003**, *80*, 825–828. doi:10.1007/s11746-003-0779-9.
55. Xie, D.; Zhou, H.; Jiang, X. Effect of chemical refining on the levels of bioactive components and hazardous substances in soybean oil. *J. Food Meas. Charact.* **2019**, *13*, 1423–1430. doi:10.1007/s11694-019-00058-y.
56. Woerfel, J.B. Soybean oil processing byproducts and their utilization. In *Practical Handbook of Soybean Processing and Utilization,* 1st ed.; Academic Press and AOCS Press, 1995. doi:10.1016/C2015-0-04084-9.

57. Verleyen, T.; Sosinska, U.; Ioannidou, S.; Verhe, R.; Dewettinck, K.; Huyghebaert, A.; Greyt, W. Influence of the vegetable oil refining process on free and esterified sterols. *J. Am. Oil Chem. Soc.* **2002**, *79*, 947–953. doi:10.1007/s11746-002-0585-4.

58. Tasan, M.; Demirci, M. Total and individual tocopherol contents of sunflower oil at different steps of refining. *Eur. Food Res. Technol.* **2005**, *220*, 251–254. doi:10.1007/s00217-004-1045-8.

59. Piloto-Rodriguez, R.; Melo, E.A.; Tobio, I.; Goyos, L.; Verhelst, S. By-products from the vegetable oil industry as a feasible source for biofuels production and pollution reduction. In *Proceedings of the International Conference on Renewable Energies and Power Quality (ICREQ´14)*, Córdoba, Spain, 2014.

60. Pawar, D.M.; Marathe, A.B. Critical review on value added products from cracking of waste vegetable oil. *International Journal of Scientific & Engineering Research* **2015**, *6*, 1063.

61. Chang, A.S.; Sherazi, S.T.H.; Kandhro, A.A.; Mahesar, S.A.; Chang, F.; Shah, S.N.; Laghari, Z.H.; Panhwar, T. Characterization of palm fatty acid distillate of different oil processing industries of Pakistan. *J. Oleo Sci* **2016**, *65*, 897–901. doi:10.5650/jos.ess16073.

62. Bondioli, P. Refining by-products as a source of compounds of high-added value. *Grasas y Aceites* **2006**, *57*, 116–125. doi:10.3989/gya.2006.v57.i1.27.

63. Wang, T. Soybean Oil. In *Vegetable Oils in Food Technology: Composition, Properties and Uses,* 2nd ed.; Blackwell Publishing Ltd.: Oxford, UK, 2011.

64. Qin, L.; Zhang, J.; Cheng, H.; Chen, L.; Qi, Z.; Yuan, W. Selection of imidazolium-based ionic liquids for vitamin E extraction from deodorizer distillate. *ACS Sustainable Chem. Eng.* **2016**, *4*, 583–590. doi:10.1021/acssuschemeng.5b01330.

65. Verleyen, T.; Verhe, R.; Garcia, L.; Dewettinck, K.; Huyghebaert, A.; De Greyt, W. Gas chromatographic characterization of vegetable oil deodorization distillate. *J. Chromatogr. A* **2001**, *921*, 277–285. doi:10.1016/S0021-9673(01)00881-0.

66. Shi, L.; Zheng, L.; Liu, R.; Chang, M.; Huang, J.; Jin, Q.; Wang, X. Quantification of polycyclic aromatic hydrocarbons and phthalic acid esters in deodorizer distillates obtained from soybean, rapeseed, corn and rice bran oils. *Food Chem.* **2019**, 206–213. doi:10.1016/j.foodchem.2018.09.119.

67. Chen, Y.; Thiyam-Hollander, U.; Barthet, V.J.; Aachary, A.A. Value-added potential of expeller-pressed canola oil refining: characterization of sinapic acid derivatives and tocopherols from byproducts. *J. Agric. Food. Chem.* **2014**, *62*, 9800–9807. doi:10.1021/jf502428z.

68. Commission Regulation (EU) 277/2012 of 28 March and Its Amendments on the Annexes I and II to Directive 2002/32/EC of the European Parliament and of the Council as Regards Maximum Levels and Action Thresholds for Dioxins and Polychlorinated Biphenyls. *Off J. Eur. Union,* **2012***.*

69. Real Decreto 465/2003 (BOE) de 25 de abril 2003, sobre las sustancias indeseables en la alimentación animal. *Boletín Oficial del Estado*, España, **2003**.

70. Ábalos, M.; Parera, J.; Abad, E.; Rivera, J. PCDD/Fs and DL-PCBs in feeding fats obtained as co-products or by-products derived from the food chain. *Chemosphere* **2008**, *71*, 1115–1126. doi:10.1016/j.chemosphere.2007.10.034.

71. FEDNA (Fundación Española Para El Desarrollo de La Nutrición Animal). Normas FEDNA de Control de Calidad de Ingredientes para Piensos-Especificación Técnica de Materia Prima, Grasas y Aceites. 2002. Disponible online: http://www.fundacionfedna.org/sites/default/files/8_Grasas_y_aceites.pdf (acceso el 16 agosto 2021).

72. Gunawan, S.; Yi-Hsu, J. Vegetable oil deodorizer distillate: characterization, utilization and analysis. *Sep. Purif. Rev.* **2009**, *38*, 207–241. doi:10.1080/15422110903095151.

73. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. I. Influence of age, rate of inclusion and degree of saturation on fat digestibility and metabolisable energy of acid oils. *Br. Poult. Sci.* **1996**, *37*, 105–117. doi:10.1080/00071669608417841.

74. Wiseman, J.; Salvador, F. The influence of free fatty acid content and degree of saturation on the apparent metabolizable energy value of fats fed to broilers. *Poult. Sci.* **1991**, *70*, 573–582. doi:10.3382/ps.0700573.

75. Vilà, B.; Esteve-Garcia, E. Studies on acid oils and fatty acids for chickens. II. Effect of free fatty acid content and degree of saturation of free fatty acids and neutral fat on fatty acid digestibility. *Br. Poult. Sci.* **1996**, *37*, 119–130. doi:10.1080/00071669608417842.

76. Rodriguez-Sanchez, R.; Tres, A.; Sala, R.; Garcés-Narro, C.; Guardiola, F.; Gasa, J.; Barroeta, A.C. Effects of dietary free fatty-acid content and saturation degree on lipid-

class composition and fatty-acid digestibility along the gastrointestinal tract in broiler starter chickens. *Poult. Sci.* **2019**, *98*, 4929–4941. doi:10.3382/ps/pez253.

77. Rodriguez-Sanchez, R.; Tres, A.; Sala, R.; Soler, M.D.; Guardiola, F.; Barroeta, A.C. Effects of free-fatty-acid content and saturation degree of the dietary oil sources on lipid-class content and fatty-acid digestibility along the gastrointestinal tract in broilers from 22 to 37 days of age. *Poultry Science* **2021**, *100*, 101261. doi:10.1016/j.psj.2021.101261.
78. Jimenez-Moya, B.; Barroeta, A.C.; Tres, A.; Soler, M.D.; Sala, R. Replacement of palm oil with soybean acid oil in broiler chicken diet: fat digestibility and lipid class content along the intestinal tract. *Animals* **2021**, *11*, 2586. doi:10.3390/ani11041035.
79. Jimenez-Moya, B.; Barroeta, A.C.; Tres, A.; Soler, M.D.; Sala, R. Soybean oil replacement by palm fatty acid distillate in broiler chicken diets: fat digestibility and lipid-class content along the intestinal tract. *Animals* **2021**, *11*, 1035. doi:10.3390/ani11041035.
80. Rostagno, H.S.; Albino, L.F.T.; Hannas, M.I.; Donzele, J.L.; Sakomura, N.K.; Perazzo, F.G.; Saraiva, A.; Teixeira de Abreu, M.L.; Rodrigues, P.B.; Oliveira, R.F.; Luiz de Toledo Barreto, S., Brito, C.O. *Tablas Brasileñas para Aves y Cerdos: Composición de Alimentos y Requerimientos Nutricionales,* 4th ed.; Rostagno, H.S., Ed.; Universidad Federal de Viçosa, Brasil, 2017. ISBN 978-85-8179-122-7.
81. Bontems, V.; Chapoutot, P.; Doreau, B.; Jondrevill, C.; Kaushik, S.J.; Lessire, M.; Martin-Rosset, W.; Meschy, F.; Noblet, J.; Perez, J.M.; Peyraud, J.L.; Rulquin, H.; Sauvant, D.; Sève, B.; Tran, G. *INRAE-CIRAD-AFZ Feed Tables*, *Composition and Nutritive Values of Feeds for Cattle, Sheep, Goats, Pigs, Poultry, Rabbits, Horses and Salmonids*, 2017. https://feedtables.com.
82. Spek, J.W.; Van Wesemael, D. *CVB Feed Table, Chemical Composition and Nutritional Values of Feedstuffs*, 2021.
83. Pritchard, S.; Garland, P.; Lawlor, E. *Premier Nutrition E-Atlas*, 2019.
84. WPSA (World´s Poultry Science Association). *European Table of Energy Values for Poultry Feedstuffs*, 3rd Ed.; Spelderholt Centre for Poultry Research, Beekbergen, The Netherlands, 1989. ISBN 90-71463-00-0.
85. National Research Council. *Nutrient Requirements of Poultry,* 9th ed.; The National Academies Press: Washington, USA, 1994.

86. Alvarenga, R.R.; Rodrigues, P.B.; Zangeronimo, M.G.; Oliveira, E.C.; Mariano, F.C.M.Q.; Lima, E.M.C.; Garcia, A.A.P.; Naves, L.P.; Nardelli, N.B.S. Validation of prediction equations of energy values of a single ingredient or their combinations in male broilers. *Asian Australas. J. Anim. Sci.* **2015**, *28*, 1335–1344. doi:10.5713/ajas.14.0339.

87. Wiseman, J. *Fats in Animal Nutrition*. 1st ed.; Butterworth-Heinemann: 1984.

88. Rodriguez-Sanchez, R.; Tres, A.; Sala, R.; Guardiola, F.; Barroeta, A.C. Evolution of lipid classes and fatty acid digestibility along the gastrointestinal tract of broiler chickens fed different fat sources at different ages. *Poult. Sci.* **2019**, *98*, 1341–1353. doi:10.3382/ps/pey458.

89. FEDNA (Fundación Española Para El Desarrollo de La Nutrición Animal). Necesidades Nutricionales Para Avicultura: Normas FEDNA, 2nd ed.; Madrid, Spain, 2018. (acceso el 5 agosto 2021).

90. FEDNA (Fundación Española Para El Desarrollo de La Nutrición Animal). Necesidades Nutricionales Para Ganado Porcino: Normas FEDNA, 2nd ed.; Madrid, Spain, 2013. (acceso el 1 agosto 2021).

91. Camire, M.E.; Camire, A.; Krumhar, K. Chemical and nutritional changes in foods during extrusion. *Crit. Rev. Food Sci. Nutr.* **1990**, *29*, 35–57. doi:10.1080/10408399009527513.

92. Dekker, M. *Food Chemistry*, 3rd ed.; Marcel Dekker Inc.: New York, USA, 1996.

93. Miyashita, K.; Takagi, T. Study on the oxidative rate and prooxidant activity of free fatty acids. *J. Am. Oil Chem. Soc.* **1986**, *63*, 1380–1384. doi:10.1007/BF02679607.

94. Wealleans, A.L.; Bierinckx, K.; Witters, E.; Benedetto, M.; Wiseman, J. Assessment of the quality, oxidative status and dietary energy value of lipids used in non-ruminant animal nutrition. *J. Sci. Food Agric.* **2021**, *101*, 4266–4277. doi:10.1002/jsfa.11066.

95. Shurson, G.C.; Kerr, B.J.; Hanson, A.R. Evaluating the quality of feed fats and oils and their effects on pig growth performance. *J. Anim. Sci. Biotechnol.* **2015**, *6.* doi:10.1186/s40104-015-0005-4.

96. Yang, P.; Wang, H.; Zhu, M.; Ma, Y. Evaluation of extrusion temperatures, pelleting parameters, and vitamin forms on vitamin stability in feed. *Animals* **2020**, *10*, 894. doi:10.3390/ani10050894.

97. Morin, P.; Gorman, A.; Lambrakis, L. A literature review on vitamin retention during the extrusion of dry pet food. *Anim. Feed Sci. Technol.* **2021**, *277*, 114975. doi:10.1016/j.anifeedsci.2021.114975.
98. Lampi, A.M.; Damerau, A.; Li, J.; Moisio, T.; Partanen, R.; Forssell, P.; Piironen, V. Changes in lipids and volatile compounds of oat flours and extrudates during processing and storage. *J. Cereal Sci.* **2015**, *62*, 102–109. doi:10.1016/j.jcs.2014.12.011.
99. Winkler–Moser, J.K.; Hwang, H.-S.; Kerr, B.J. Changes in markers of lipid oxidation and thermal treatment in feed-grade fats and oils. *J. Sci. Food and Agric.* **2020**, *100*, 3328–3340. doi:10.1002/jsfa.10364.
100. Gidenne, T.; Garreau, H.; Drouilhet, L.; Aubert, C.; Maertens, L. Improving feed efficiency in rabbit production, a review on nutritional, technico-economical, genetic and environmental aspects. *Anim. Feed Sci. Technol.* **2017**, *225*, 109–122. doi:10.1016/j.anifeedsci.2017.01.016.
101. Ayed, H.B.; Attia, H.; Ennouri, M. Effect of oil supplemented diet on growth performance and meat quality of broiler chickens. *Adv. Tech. Biol. Med.* **2015**, *4*, 1000156. doi:10.4172/2379-1764.1000156.
102. Dierick, N.A.; Decuypere, J.A. Endogenous lipolysis in feedstuffs and compound feeds for pigs: effects of storage time and conditions and lipase and/or emulsifier addition. *Anim. Feed Sci. Technol.* **2002**, *102*, 53–70. doi:10.1016/S0377-8401(02)00224-9.
103. Shannon, D.W.F. The effect of level of intake and free fatty acid content on the metabolizable energy value and net absorption of tallow by the laying hen. *J. Agric. Sci.* **1971**, *76*, 217–221. doi:10.1017/S0021859600025569.
104. Lin, S.; Hsieh, F.; Huff, H.E. Effects of lipids and processing conditions on lipid oxidation of extruded dry pet food during storage. *Anim. Feed Sci. Technol.* **1998**, *71*, 283–294. doi:10.1016/S0377-8401(97)00157-0.
105. Kinsella, J.E.; Shimp, J.L.; Mai, J. The proximate and lipid composition of several species of freshwater fishes. *New York'S Food and Life Sciences Bulletin* **1978**, *69*, 1–20.
106. Bharathi, D.; Rajalakshmi, G. Microbial lipases: an overview of screening, production and purification. *Biocatal. Agric. Biotechnol.* **2019**, 22, 101368. doi:10.1016/j.bcab.2019.101368.

107. Maciorowski, K.G.; Herrera, P.; Jones, F.T.; Pillai, S.D.; Ricke, S.C. Effects on poultry and livestock of feed contamination with bacteria and fungi. *Anim. Feed Sci. Technol.* **2007**, *133*, 109–136. doi:10.1016/j.anifeedsci.2006.08.006.
108. Jørgensen, H.; Fernández, J.A. Chemical composition and energy value of different fat sources for growing pigs. *Acta Agric. Scand. Sect A* **2000**, *50*, 129–136. doi:10.1080/090647000750014250.
109. Overholt, M.F.; Dilger, A.C.; Boler, D.D.; Kerr, B.J. Influence of feeding thermally peroxidized soybean oil on growth performance, digestibility, and gut integrity in finishing pigs. *J. Anim. Sci.* **2018**, *96*, 2789–2803. doi:10.1093/jas/sky091.
110. Seppanen, C.M.; Song, Q.; Saari Csallany, A. The antioxidant functions of tocopherol and tocotrienol homologues in oils, fats, and food systems. *J. Am. Oil Chem. Soc.* **2010**, *87*, 469–481. doi:10.1007/s11746-009-1526-9.
111. Granda, L.; Rosero, A.; Benešová, K.; Pluháčková, H.; Neuwirthová, J.; Cerkal, R. Content of selected vitamins and antioxidants in colored and nonpigmented varieties of quinoa, barley, and wheat grains. *J. Food Sci.* **2018**, *83*, 2439-2447. doi:10.1111/1750-3841.14334.
112. Nielsen, M.M.; Hansen, Å. Rapid high-performance liquid chromatography determination of tocopherols and tocotrienols in cereals. *Cereal Chem.* **2008**, *85*, 248–251. doi:10.1094/CCHEM-85-2-0248.
113. Anderson, J.S.; Sunderland, R. Effect of extruder moisture and dryer processing temperature on vitamin c and e and astaxanthin stability. *Aquaculture* **2002**, *207*, 137–149. doi:10.1016/S0044-8486(01)00787-6.
114. Andersen, H.J.; Oksbjerg, N.; Young, J.F.; Therkildsen, M. Feeding and meat quality - a future approach. *Meat Sci.* **2005**, *70*, 543–554. doi:10.1016/j.meatsci.2004.07.015.
115. Scaife, J.R.; Moyo, J.; Galbraith, H.; Michie, W.; Campbell, V. Effect of different dietary supplemental fats and oils on the tissue fatty acid composition and growth of female broilers. *Br. Poult. Sci.* **1994**, *35*, 107–118. doi:10.1080/00071669408417675.
116. Wood, J.D.; Enser, M.; Fisher, A.V.; Nute, G.R.; Sheard, P.R.; Richardson, R.I.; Hughes, S.I.; Whittington, F.M. Fat deposition, fatty acid composition and meat quality: A review. *Meat Sci.* **2008**, *78*, 343–358. doi:10.1016/j.meatsci.2007.07.019.

117. Zaki, E.F.; El Faham, A.I.; Nematallah, G.M. Fatty acids profile and quality characteristics of broiler chicken meat fed different dietary oil sources with some additives. *Int. J. of Health, Anim. Sci. Food Safe.* **2018**, *5*, 40–50. doi:10.13130/2283-3927/9581.
118. Bou, R.; Codony, R.; Tres, A.; Decker, E.A.; Guardiola, F. Dietary strategies to improve nutritional value, oxidative stability, and sensory properties of poultry products. *Crit. Rev. Food Sci.* **2009**, *49*, 800–822. doi:10.1080/10408390902911108.
119. Pinchasov, Y.; Nir, I. Effect of dietary polyunsaturated fatty acid concentration on performance, fat deposition, and carcass fatty acid composition in broiler chickens. *Poult. Sci.* **1992**, *71*, 1504–1512. doi:10.3382/ps.0711504.
120. Kouba, M.; Mourot, J. A review of nutritional effects on fat composition of animal products with special emphasis on n-3 polyunsaturated fatty acids. *Biochimie* **2011**, *93*, 13–17. doi:10.1016/j.biochi.2010.02.027.
121. Teye, G.A.; Sheard, P.R.; Whittington, F.M.; Nute, G.R.; Stewart, A.; Wood, J.D. influence of dietary oils and protein level on pork quality. 1. effects on muscle fatty acid composition, carcass, meat and eating quality. *Meat Sci.* **2006**, *73*, 157–165. doi:10.1016/j.meatsci.2005.11.010.
122. Wood, J.D.; Richardson, R.I.; Nute, G.R.; Fisher, A.V.; Campo, M.M.; Kasapidou, E.; Sheard, P.R.; Enser, M. Effects of fatty acids on meat quality: A review. *Meat Sci.* **2004**, *66*, 21–32. doi:10.1016/S0309-1740(03)00022-6.
123. Wood, J.D.; Nute, G.R.; Richardson, R.I.; Whittington, F.M.; Southwood, O.; Plastow, G.; Mansbridge, R.; da Costa, N.; Chang, K.C. Effects of breed, diet and muscle on fat deposition and eating quality in pigs. *Meat Sci.* **2004**, *67*, 651–667. doi:10.1016/j.meatsci.2004.01.007.
124. Alagawany, M.; Elnesr, S.S.; Farag, M.R.; Abd El-Hack, M.E.; Khafaga, A.F.; Taha, A.E.; Tiwari, R.; Iqbal Yatoo, M.; Bhatt, P.; Khurana, S.K.; et al. Omega-3 and omega-6 fatty acids in poultry nutrition: effect on production performance and health. *Animals* **2019**, *9*, 573. doi:10.3390/ani9080573.
125. Barroeta, A.C. Nutritive value of poultry meat: relationship between vitamin E and PUFA. *World Poultry Sci. J.* **2007**, *63*, 277–284. doi:10.1017/S0043933907001468.

126. Jensen, C.; Engberg, R.; Jakobsen, K.; Skibsted, L.H.; Bertelsen, G. Influence of the oxidative quality of dietary oil on broiler meat storage stability. *Meat Sci.* **1997**, *47*, 211–222. doi:10.1016/S0309-1740(97)00052-1.

127. Kanazawa, K.; Ashida, H. Dietary hydroperoxides of linoleic acid decompose to aldehydes in stomach before being absorbed into the body. *Biochim. Biophys. Acta* **1998**, *1393*, 349–361. doi:10.1016/S0005-2760(98)00089-7.

128. Hamre, K.; Kolås, K.; Sandnes, K.; Julshamn, K.; Kiessling, A. Feed intake and absorption of lipid oxidation products in atlantic salmon (salmo salar) fed diets coated with oxidised fish oil. *Fish Physiol. Biochem.* **2001**, *25*, 209–219. doi:10.1023/A:1022257928437.

129. Papuc, C.; Goran, G.V.; Predescu, C.N.; Nicorescu, V. Mechanisms of oxidative processes in meat and toxicity induced by postprandial degradation products: A review. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16*, 96–123. doi:10.1111/1541-4337.12241.

130. Falowo, A.B.; Fayemi, P.O.; Muchenje, V. Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Res. Int.* **2014**, *64*, 171–181. doi:10.1016/j.foodres.2014.06.022.

131. Kanner, J. Oxidative processes in meat and meat products: quality implications. *Meat Science* **1994**, *36*, 169–189. doi:10.1016/0309-1740(94)90040-X.

132. Morrissey, P.A.; Sheehy, P.J.A.; Galvin, K.; Kerry, J.P.; Buckley, D.J. Lipid stability in meat and meat products. *Meat Sci.* **1998**, *49*, S73-S86. doi:10.1016/S0309-1740(98)90039-0.

133. Estiasih, T.; Ahmadi, K.; Widyaningsih, T.D.; Maligan, J.M.; Mubarok, A.Z.; Zubaidah, E.; Mukhlisiyyah, J.; Puspitasari, R. Bioactive compounds of palm fatty acid distillate (PFAD) from several palm oil refineries. *Adv. J. of Food Sci. Tech.* **2013**, *5*, 1153–1159. doi:10.19026/ajfst.5.3074.

134. Fontanel, D. *Unsaponifiable Matter in Plant Seed Oils*. Springer: New York, USA, 2013. doi:10.1007/978-3-642-35710-7.

135. Cheng, Y.; Chen, Y.; Li, J.; Qu, H.; Zhao, Y.; Wen, C.; Zhou, Y. Dietary β-sitosterol improves growth performance, meat quality, antioxidant status, and mitochondrial biogenesis of breast muscle in broilers. *Animals* **2019**, *9*, 71. doi:10.3390/ani9030071.

136. Psomiadou, E.; Tsimidou, M. Stability of virgin olive oil. 1. autoxidation studies. *J. Agric. Food. Chem.* **2002**, *50*, 716–721. doi:10.1021/jf0108462.

137. Salami, S.A.; Guinguina, A.; Agboola, J.O.; Omede, A.A.; Agbonlahor, E.M.; Tayyab, U. Review: in vivo and postmortem effects of feed antioxidants in livestock: a review of the implications on authorization of antioxidant feed additives. *Animal* **2016**, *10*, 1375–1390. doi:10.1017/S1751731115002967.

138. Voljč, M.; Frankič, T.; Levart, A.; Nemec, M.; Salobir, J. Evaluation of different vitamin e recommendations and bioactivity of α-tocopherol isomers in broiler nutrition by measuring oxidative stress in vivo and the oxidative stability of meat. *Poultry Science* **2011**, *90*, 1478–1488. doi:10.3382/ps.2010-01223.

139. Naji, T.A.A.; Amadou, I.; Abbas, S.; Zhao, R.Y.; Shi, Y.H.; Le, G.W. Phytosterol supplementation improves antioxidant enzymes status and broiler meat quality. *Pak. J. Food Sci.* **2013**, *23*, 163–171.

140. Zhao, Y.R.; Chen, Y.P.; Cheng, Y.F.; Qu, H.M.; Li, J.; Wen, C.; Zhou, Y.M. Effects of dietary phytosterols on growth performance, antioxidant status, and meat quality in partridge shank chickens. *Poult. Sci.* **2019**, *98*, 3715–3721. doi:10.3382/ps/pez059.

141. Chen, Y.; Gu, Y.; Zhao, H.; Zhang, H.; Zhou, Y. Effects of graded levels of dietary squalene supplementation on the growth performance, plasma biochemical parameters, antioxidant capacity, and meat quality in broiler chickens. *Poult. Sci.* **2020**, *99*, 5915–5924. doi:10.1016/j.psj.2020.08.042.

142. Yang, Y.X.; Kim, Y.J.; Jin, Z.; Lohakare, J.D.; Kim, C.H.; Ohh, S.H.; Lee, S.H.; Choi, J.Y.; Chae, B.J. Effects of dietary supplementation of astaxanthin on production performance, egg quality in layers and meat quality in finishing pigs. *Asian-Aust. J. Anim. Sci.* **2006**, *19*, 1019-1025. doi:10.5713/ajas.2006.1019.

143. Englmaierovà, M.; Bubancovà, I.; Vít, T.; Skøivan, M. The effect of lycopene and vitamin e on growth performance, quality and oxidative stability of chicken leg meat. *Czech J. Anim.Sci.* **2011**, *56*, 536–543. doi:10.17221/4416-cjas.

144. Luciano, G.; Vasta, V.; Monahan, F.J.; López-Andrés, P.; Biondi, L.; Lanza, M.; Priolo, A. antioxidant status, colour stability and myoglobin resistance to oxidation of longissimus dorsi muscle from lambs fed a tannin-containing diet. *Food Chem.* **2011**, *124*, 1036–1042. doi:10.1016/j.foodchem.2010.07.070.

145. Sarojini J.K.A.; Dutta, P.C. Sterols and oxidized sterols in feed ingredients obtained from chemical and physical refining processes of fats and oils. *J. Am. Oil Chem. Soc.* **2009**, *86*, 595–604. doi:10.1007/s11746-009-1388-1.

146. Varona, E.; Tres, A.; Rafecas, M.; Vichi, S.; Barroeta, A.C.; Guardiola, F. Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding. *MethodsX* **2021**, *8*, 101334. doi:10.1016/j.mex.2021.101334.

147. Varona, E.; Tres, A.; Rafecas, M.; Vichi, S.; Barroeta, A.C.; Guardiola, F. Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding. *Animals* **2021**, *11*, 196. doi:10.3390/ani11010196.

148. Varona, E.; Tres, A.; Rafecas, M.; Vichi, S.; Sala, R.; Guardiola, F. Oxidative quality of acid oils and fatty acid distillates used in animal feeding. *Animals* **2021**, *11*, 2559. doi:10.3390/ani11092559.

149. Codex Alimentarius. Standard for named vegetable oils (CXS 210-1999). In *Codex Alimentarius, International Food Standards; Codex Alimentarius: Rome, Italy, 1999; Adopted in 1999. Revised in 2001, 2003, 2009, 2017, 2019. Amended in 2005, 2011, 2013, 2015,* 2019.

150. Boroojeni, F.G.; Svihus, B.; Graf von Reichenbach, H.; Zentek, J. The effects of hydrothermal processing on feed hygiene, nutrient availability, intestinal microbiota and morphology in poultry—A review. *Anim. Feed Sci. Tech.* **2016**, *220*, 187-215. doi:10.1016/j.anifeedsci.2016.07.010.

151. Samarasinghe, K.; Messikommer, R.; Wenk, C. Activity of supplemental enzymes and their effect on nutrient utilization and growth performance of growing chickens as affected by pelleting temperature. *Arch. Anim. Nutr.* **2000**, *53*, 45-58. doi:10.1080/17450390009381937.

152. Pogorzelska-Nowicka, E.; Atanasov, A.G.; Horbańczuk, J.; Wierzbicka, A. Bioactive compounds in functional meat products. *Molecules* **2018**, *23*, 307. doi:10.3390/molecules23020307.

153. Schneider, C. Chemistry and biology of vitamin E. *Mol. Nutr. Food Res.* **2005**, *49*, 7-30. doi:10.1002/mnfr.200400049.

8. ANEXOS

8. Anexos

8.1. Información suplementaria de la publicación "Methods to determine the quality of acid oils and fattyacid distillates used in animal feeding"

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F. *MethodsX* 2021, *8*, 101334, doi:10.1016/j.mex.2021.101334

INDEX

SUPPLEMENTARY MATERIALS
Methods to determine the quality of acid oils and fatty acid distillates used in animal feeding

INTRODUCTION

This compendium of methods is the result of applying different methods to 92 samples, 79 acid oils (AO) and 13 fatty acid distillates (FAD), intended for animal feeding. These methods are based on methods described in the literature for crude or refined fats and oils. However, there is little information about their application to by-products of edible oil refining, such as AO and FAD. As these samples are very heterogeneous, the application of these methods rises several drawbacks that must be overcome in order to obtain reliable results. The objective of this compendium is to give a detailed description of the methods, including how to overcome the drawbacks. This compendium will contribute to improve the quality control of these samples.

METHOD S1- PREPARATION OF THE TEST SAMPLE

1. SUBJECT

This protocol specifies the procedures for the preparation of the samples before the analysis. Applicable to vegetable acids oils from the chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats.

2. PRINCIPLE

A representative sample of about 1 L of AO or FAD was obtained from fat and oil producers or feed manufacturers. This sample is homogenized and aliquoted in glass vials of different volumes according to the amount of sample necessary for the subsequent analyses.

3. PREPARATION OF TEST SAMPLE

For samples that are liquid at room temperature, normally those coming from seed oils, the first step consists of shaking vigorously the container containing the representative sample. If complete homogenization is achieved, the sample is immediately aliquoted into vials. If on the contrary, just stirring is not enough for a complete homogenization, it is necessary to heat the sample. In this case, the sample is heated in a water bath to the minimum temperature necessary for a complete melting while it is vigorously shacked every 5 minutes to achieve homogenization (Table S1.1). Once sample is homogeneous it is poured into 1 L glass beaker, making sure that the entire sample falls into it. The beaker is heated in a plate to the same temperature (Table S1.1, controlled by a probe immersed in the oil) with constant agitation (magnetic stirring). Finally, the sample is aliquoted into vials as fast as possible.

In the case of samples that are solid at room temperature, generally FAD from palm, coconut and palm kernel oil, it is necessary to heat the samples to homogenize them. The FAD sample is heated in a water bath (Table S1.1), vigorously shaken at intervals of 5 min during warming to achieve complete homogenization and transferred completely into a 1 L glass beaker. The beaker is heated in a plate to the same temperature (Table S1.1, controlled by a probe immersed in the oil) with constant agitation (magnetic stirring). Finally, the sample is aliquoted into vials as fast as possible.

Sample vials are filled with N_2 in their head-space, capped and stored at -20 ºC until analysis. Before weighing the sample for analysis, the vial is thawed by immersing it in a water bath according to the temperatures established in Table S1.1. To achieve the complete homogenization the vial is shaken vigorously from time to time. As each determination is carried out in duplicate, it should be checked that the sample in the vial remains homogeneous throughout the weighing operations.

The heating temperatures and times are always the minimum necessary to avoid any alteration of the samples by oxidation or polymerization.

Table S1.1

Heating temperatures for the samples according to refining process and botanical origin of the oils.

Refining process	Botanical origin	Heating temperature[a]
CHEMICAL REFINING (Acid oils, AO)	Blends of AO from seed oils, cocoa butter and palm oil	65 ºC
	Blends of AO from seed and palm oils	65 ºC
	AO from olive pomace oils and blends of AO from olive pomace and olive oils	From RT[b] to 35/40 ºC
	Blends of AO from seed oils	If traces of palm, 65 ºC, if not, from RT to 35/40 ºC
	AO from sunflower oil	
	Blends of AO from sunflower and soybean oils	From RT to 35/40 ºC
	AO from soybean oil	
PHYSICAL REFINING (Fatty acid distillates, FAD)	FAD from coconut oil and blends of FAD from coconut and palm kernel oils	45 ºC
	FAD from palm oil	65 ºC
	FAD from olive pomace and olive oils	From RT to 35/40 ºC

[a] Heating temperature is applied the minimum time needed to melt and homogenize the sample.
[b] Room temperature.

METHOD S2- DETERMINATION OF FATTY ACID COMPOSITION

1. SUBJECT

This method describes a procedure for the determination of the fatty acid profile (expressed as %) by gas chromatography (GC) in vegetable acid oils (AO) and fatty acid distillates (FAD) of vegetable oils and fats. The method described below is an adaptation of the method described by Guardiola et al. [1].

2. PRINCIPLE

To determine fatty acid composition, a double methylation in methanolic medium is carried out, first with sodium methoxide and later with boron-trifluoride, to ensure that free fatty acids are completely methylated. Then, fatty methyl esters (FAME) are separated by GC-FID and identified by means of comparison of retention times with those of a standard mixture.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Test tubes with screw caps, 10 mL.
3.3 Positive displacement micropipettes suitable to dispense exact volumes of volatile solvents or solutions (MICROMAN®, 100 and 1000 µL from Gilson, Middleton, USA).
3.4 Water bath B-491 from Buchi (Flawil, Switzerland).
3.5 Volumetric pipettes, 3 mL.
3.6 Graduated pipettes, 10 mL.
3.7 Gas chromatograph 4890D from Agilent Technologies (Santa Clara, USA) equipped with flame ionization detector (FID) and split/splitless injector, set at 300 and 270 ºC, respectively. The split ratio is 1:30. Chromatographic separation is performed on a fused-silica capillary column SP-2380 (Merck, Darmstadt, Germany) (60 m × 0.25 mm i.d.) coated with 0.2 µm of a stationary phase of 90% biscyanopropyl- plus 10% cyanopropylphenyl-polysiloxane. Hydrogen, at 25 psi, is used as a carrier gas. The program of the oven is: firstly 1 min at 150 ºC, then increases the temperature by 1.5 ºC/min to 180 ºC at which point it is held for 0.5 min; then by 14.5 ºC/min to 220 ºC at which point it is held for 3 min, and then finally the temperature is increased by 9.9 ºC/min to 250 ºC and held for 9 min and 30 s. The sample volume injected is 1 µL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Diethyl ether free from peroxides for analysis, ACS, ISO grade, stabilized with approx. 7 ppm of 2,6-Di-tert-butyl-4-methylphenol (BHT) from Scharlau (Sentmenat, Spain).

5.2 Sodium methoxide 0.5 M solution in methanol, ACS grade, from Sigma-Aldrich (St. Louis, USA).
5.3 Boron trifluoride-methanol complex (20% in methanol), for synthesis, from Merck (Darmstadt, Germany).
5.4 Methanol for analysis, ACS, ISO grade, from Scharlau (Sentmenat, Spain).
5.5 Phenolphthalein indicator, ACS grade, from Scharlau (Sentmenat, Spain).
5.5.1 Phenolphtalein indicator solution, 1% in methanol.
5.6 n-Hexane EMSURE® for analysis, ACS grade, from Merck (Darmstadt, Germany).
5.7 Sodium chloride, Pharmpur®, Ph Eur, BP, USP from Scharlau (Sentmenat, Spain).
5.8 Sodium sulphate anhydrous, powder, for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).
5.9 Standard mixture "Supelco 37 component FAME Mix" from Merck (Darmstadt, Germany).

6. PROCEDURE

6.1 Approximately weigh 50 mg of test sample (AO or FAD) into a 10 mL test tube.
6.2 Add 100 µL of diethyl ether using a positive displacement micropipette, to help the dissolution of fat and to favor the subsequent extraction of FAME with n-hexane. Diethyl ether remains until the end of the procedure when FAME are extracted with n-hexane. At this point the diethyl ether increases the polarity of the n-hexane fraction facilitating the extraction of the more polar FAME coming from short chain FA.
6.3 Add 1.25 mL of sodium methoxide 0.5 M using a positive displacement micropipette. Mark the level of the solvent mixture on the tube.
6.4 Put the tube in a boiling water bath for 20 min. Mix the tube gently after 5 min heating. Check that there is no evaporation of the solvents. If it is the case, add methoxide again until reaching the expected volume.
6.5 Cold the tube to room temperature before uncapping them by placing them in tap water.
6.6 Add 2-3 drops of phenolphthalein 1% in methanol. The solution must become pink.
6.7 Add 1.5 mL of boron trifluoride-methanol complex (20%) in methanol. The tube will become colorless. Mark the level of the solvent mixture on the tube.
6.8 Cap the tube and put into the boiling water bath again for 15 min. Check that there is no evaporation of the solvents during heating.
6.9 Cool down the tube by placing it in tap water. The tube needs to be colorless. If otherwise it is pink, more boron trifluoride-methanol must be added, and the tube needs to be placed in the boiling water bath again for 15 min.
6.10 Add 3 mL of n-hexane EMSURE®. Shake the tube turning upside down for 10 s.
6.11 Add 2 mL of sodium chloride saturated solution and turn the tube upside down for 10 s.
6.12 Wait until the two phases have been separated.
6.13 Transfer the hexane upper phase to another tube containing sodium sulphate anhydrous.
6.14 After 1 h, transfer the hexane to a GC vial and inject into GC.

7. CALCULATIONS

Fatty acid methyl esters are quantified by peak area normalization (peak area percentage):

$$\% \text{ FA} = \frac{\text{FA peak area}}{\text{Total peak areas}} \times 100$$

Where:

FA peak area: is the area of each FAME peak identified.

Total peak areas: is the sum of areas of all the identified FAME peaks.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the results obtained for an AO were: for C16:0, 11.66% (RSD = 0.84%); for C18:0, 4.45% (RSD = 0.35%); for C18:1 n-9, 30.46% (RSD = 0.17%); for C18:2 n-6, 46.58% (RSD = 0.13%) and for C18:3 n-3, 1.89% (RSD = 0.25%). For a FAD the results were: for C16:0, 47.53% (RSD = 1.31%); for C18:0, 4.28 % (RSD = 0.98%); for C18:1 n-9, 35.74% (RSD = 0.73%); for C18:2 n-6, 8.99% (RSD = 0.79%); and for C18:3 n-3, 0.35% (RSD = 1.03%).

9. REFERENCES

[1] F. Guardiola, R. Codony, M. Rafecas, J. Boatella, A. López, Fatty acid composition and nutritional value of fresh eggs, from large- and small-scale farms, J. Food Comp. Anal. 7 (1994) 171–188, https://doi.org/10.1006/jfca.1994.1017

METHOD S3- DETERMINATION OF TOCOPHEROL AND TOCOTRIENOL CONTENT

1. SUBJECT

This method describes a procedure for the determination of the amounts of tocopherols (T) and tocotrienols (T3) as measured by HPLC and expressed in mg/kg. This method assesses the eight homologs of vitamin E: α-, β-, γ-, and δ-T and their corresponding T3 in vegetable acid oils from chemical refining (AO) and fatty acids distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the methods described by Aleman et al. [1] and Hewavitharana et al. [2].

2. PRINCIPLE

The sample is subjected to saponification. The unsaponifiable matter is extracted with petroleum ether, filtered, evaporated, and dissolved in n-hexane prior to injection in a high performance liquid chromatograph equipped with a silica column and a fluorescence detector.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Volumetric flasks, 50 mL.
3.3 Beaker, 250 mL.
3.4 Glass centrifuge tubes with screw caps, 50 mL.
3.5 Graduated pipettes, 5 and 10 mL.
3.6 Positive displacement micropipettes suitable to dispense exact volumes of volatile solvents or solutions (MICROMAN®, 100, 250 and 1000 µL from Gilson, Middleton, USA).
3.7 Vortex mixer.
3.8 Graduated cylinder, 20 mL.
3.9 Centrifuge Meditronic-BL-S from J.P. Selecta®, with a rotor radius of 10 cm (Abrera, Spain).
3.10 Syringe filter PTFE membrane, 25 mm diameter and 0.45 µm of pore from VWR™ International (Llinars del Vallés, Spain).
3.11 Round bottom flasks, 200 mL.
3.12 Glass test tubes with screw caps, 10 mL capacity.
3.13 Water bath B-491 from Buchi (Flawil, Switzerland).
3.14 Rotary evaporator Rotavapor® R-210 from Buchi (Flawil, Switzerland).
3.15 Dry block heater Tembloc from J.P. Selecta® (Abrera, Spain).
3.16 HPLC system 1100 series from Agilent Technologies (Santa Clara, USA) with the following characteristics:

Mobile phase: n-Hexane/ Dioxane (95/5, v/v)
HPLC isocratic pump, pulseless, with a flow of 1.0 mL/min
Injection valve with a 20 µL loop
Precolumn: security guard system with silica cartridge of 3.0 mm I.D. x 4 mm.
Phenomenex Luna Silica (2) column, 4.6 mm I.D. x 150 mm, 3 µm diameter of the particles, pore size: 100 Å (Phenomenex, Torrance, CA).

Detector: fluorescence (FLD) with excitation wavelength set at 290 nm and emission wavelength set at 320 nm.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Butylated hydroxytoluene (BHT), ACS grade, from Sigma-Aldrich (St. Louis, USA).
5.2 Anhydrous citric acid, ACS grade, from Scharlau (Sentmenat, Spain).
5.3 Pyrogallol, ACS grade, from Sigma-Aldrich (St. Louis, USA).
5.4 Potassium hydroxide (KOH) 85%, ACS grade, from Acros Organic (Geel, Belgium).
5.5 Ethanol absolute, ACS grade, from Panreac (Castellar del Vallés, Spain).
5.6 Methanol, ACS, ISO grade, from Scharlau (Sentmenat, Spain).
5.7 α-, β-, γ-, δ- Tocopherol standards from Calbiochem® from Merck (Darmstadt, Germany).
5.8 1,4-Dioxane, HPLC grade, stabilized with BHT from Carlo Erba Reagents (Val de Reuil, France).
5.9 n-Hexane 99%, HPLC grade, from Scharlau (Sentmenat, Spain).
5.10 Preparation of the antioxidant solution: weigh 0.006 ± 0.0005 g of BHT (*Reagent 5.1*), 0.20 g ± 0.05 g of citric acid (*Reagent 5.2*) and 0.50 g ± 0.05 g of pyrogallol (*Reagent 5.3*), transfer to a 50 mL volumetric flask and dissolve with absolute ethanol. This solution must be prepared extemporaneously.
5.11 Preparation of the potassium hydroxide 1.5 M (approximately) solution: weigh 26.5 g of KOH (*Reagent 5.4*) and transfer it to a 250 mL beaker using methanol. This solution can be used for 1 week.
5.12 Petroleum ether, boiling point 40-60 ºC, ACS grade, from Scharlau (Sentmenat, Spain).

6. PROCEDURE

All the process must be carried out under attenuated lighting conditions (warm white LED light with nil emission at UV region is adequate).

6.1 Accurately weigh 0.20-0.25 g (± 0.0001g) of AO or FAD into a 50 mL centrifuge tube, immediately add 5 mL of the antioxidant solution using a graduated pipette. Mix gently and put the tube in an ice bath. Weigh the next sample and add immediately the antioxidant solution.
6.2 Once every sample is weighted and added the antioxidant solution, add 10 mL of methanolic KOH 1.5 M using a graduated pipette.
6.3 Mix with a vortex mixer for 1 min.
6.4 Put the tubes in a water bath at 70 ºC under gentle agitation during 30 min. Every 10 min mix the tubes manually and put them back in the water bath.
6.5 After exactly 30 min take the tubes from the water bath and put them in an ice bath for 7 min.
6.6 Once the tubes are cold, add 15 mL of distilled water with a graduated cylinder.
6.7 Add 10 mL of petroleum ether with a graduated cylinder.
6.8 Mix the tubes with a vortex mixer for 1 min.

6.9 Centrifuge at 591.42 g for 7 min.
6.10 Take the upper layer with a Pasteur pipette and transfer it to a round bottom flask.
6.11 Add another 10 mL of petroleum ether to the centrifuge tube and repeat steps 6.8-6.10 separating the upper layer using the same Pasteur pipette as before. Repeat this step once more until the total volume of petroleum ether used for the extraction is 30 mL.
6.12 After the three extractions are done, rinse the Pasteur pipette with petroleum ether and collect it in the round bottom flask.
6.13 Using a rotary vacuum evaporator evaporate the solvent at 30 ºC near to dryness.
6.14 Dissolve the dry residue in the round bottom flask with petroleum ether (5 mL) and filter it through a PTFE syringe filter (0.45 µm). Collect the filtrate in a screw-capped test tube.
6.15 Rinse the round bottom flask and the syringe + filter various times with 1-2 mL of petroleum ether each time, collecting the filtrate in the same tube, until approximately 10 mL are collected. To help the filtration process, connect the manifold to the vacuum pump until the whole volume has been filtered and collected in the test tubes.
6.16 Transfer the test tubes to a dry block heater at 30 ºC and evaporate under a nitrogen stream until dryness. Close the tubes and keep them under refrigeration until injection.
6.17 Re-dissolve the final extract in exactly 1 mL of n-hexane and inject in the HPLC system.
6.18 Dilute or concentrate the extract according to the total T concentration (see Table S3.1) and re-inject to do the final quantification.

Table S3.1.

Dilution or concentration of the final extract according to the total concentration of tocopherols (sum of the four tocopherol homologs) in the sample.

Volume of n-hexane added to dissolve the final extract (V) (step 6.17)	Dilution/ concentration factor (f) (step 6.18)[a]	mg/kg of ∑T
1 mL	0.5 or 0.33	2000-8500
1 mL	1	500-2000
1 mL	2, 3, 4 or 5	100-500

Abbreviations: ∑T, sum of the four tocopherol homologs (α-, β-, γ- and δ-tocopherol).
[a] For example, a dilution factor of 0.5 was carried out by taking 200 µL of sample plus 200 µL of n-hexane, and a concentration factor of 2 was carried out by taking 400 µL of sample, evaporating it and re-dissolving it in 200 µL of n-hexane.

7. CALCULATIONS

When T and T3 are identified, the areas are passed through calibration curves previously built with the α-, β-, γ- and δ-T standards. The calibration curves are constructed by plotting values of peak areas versus known concentrations of tocopherols in the standard solutions. Each T3 is quantified using the calibration curve for the corresponding T analogue. Then, the concentrations of T and T3 (expressed as mg/kg) are calculated considering the sample weight and the final dilution of the injected extract, using the following formula for each homolog:

$$\text{Tocol content } \left(\frac{\text{mg}}{\text{kg}}\right) = \frac{(\text{A} - \text{b}) \times (\text{V/f})}{\text{a} \times \text{m}}$$

Where:

A = is the peak area.
a, b = are the coefficients of the calibration curve: y = ax + b (x: µg tocopherol/mL of hexane; y: peak area).
V = is the volume of n-hexane used to re-dissolve the unsaponifiable fraction in mL. It is always 1 mL.
f = is the dilution/concentration factor, if necessary.
m= sample weight, in grams.

Last, to obtain the real T and T3 concentrations in samples, recovery percentages must be applied to the concentrations of T and T3 (see below).

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Eight determinations of two samples, one AO and one FAD, were carried out to study repeatability of the method, by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions, for AO the RSD were 4.5, 8.4, 4.7 and 6.1% for α-, β-, γ- and δ-T (means = 188.03, 6.72, 158.43 and 5.80 mg/kg respectively, sample weight = 0.20 g) and for FAD were 10.6, 11.0, 13.5 and 7.8% for α-, β-, γ- and δ-T (means = 6.05, 6.02, 5.06 and 18.68 mg/kg respectively, sample weight = 0.20 g).

9. RECOVERY OF THE METHOD

Recovery was evaluated by analyzing six times the same two samples used for repeatability spiked with a mixture of the four tocopherol standards, by the same analyst within a day, using the same reagents, equipment and instruments. The recovery results obtained (mean % ± SD) were for AO 87 ± 0.02%, 82 ± 0.02%, 78 ± 0.03% and 36 ± 0.01% for α-, β-, γ- and δ-T, respectively, and in case of FAD were 96 ± 0.05%, 93 ± 0.04%, 93 ± 0.04% and 63 ± 0.03% for α-, β-, γ- and δ-T, respectively.

10. REFERENCES

[1] M. Aleman, C. D. Nuchi, R. Bou, A. Tres, J. Polo, F. Guardiola, R. Codony, Effectiveness of antioxidants in preventing oxidation of palm oil enriched with heme iron: A model for iron fortification in baked products, Eur. J. Lipid Sci. Technol. 112 (2010) 761–769, https://doi.org/10.1002/ejlt.200900220

[2] A. K. Hewavitharana, M.C. Lanari, C. Becu, Simultaneous determination of vitamin E homologs in chicken meat by liquid chromatography with fluorescence detection, J. Chromatogr. A. 1025 (2004) 313-317, https://doi.org/10.1016/j.chroma.2003.10.052

METHOD S4- DETERMINATION OF MOISTURE AND VOLATILE MATTER

1. SUBJECT

This method determines the moisture and any other volatile material under the conditions of the test. Applicable to vegetable acids oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the AOCS official method Ca 2d-25 [1] and AOAC official method 926.12 [2].

2. PRINCIPLE

The sample is subjected to heat and vacuum and the % loss in weight is reported as moisture and volatile matter.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Vacuum oven (VO200 from Memmert, Schwabach, Germany).
3.4 Desiccator, containing an efficient desiccant (silica gel with moisture indicator and phosphorus pentoxide, both used to capture the accumulated moisture inside the desiccator).
3.5 Aluminium moisture dishes (55 mm of internal diameter x 35 mm of internal height) from Humboldt (Elgin, USA).

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. PROCEDURE

5.1 Accurately (± 0.0001g) weigh about 5 g of test sample into a tared moisture dish, previously dried under the stablished conditions in a vacuum oven including its lid (see Fig. S4.1): pressure = 66 mbar (approximately 50 mm Hg); temperature = 58 ºC; min/max temperature = 56-60 ºC. Always use powder-free gloves to handle the moisture dishes.

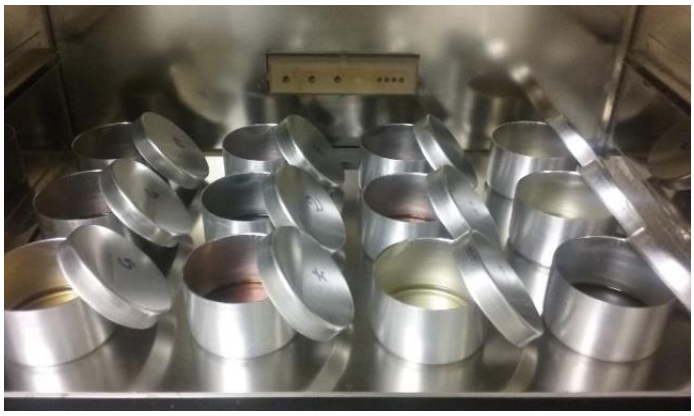


Fig. S4.1. Position of the dishes and lids inside the vacuum oven.

5.2 After 1 h of drying, the dish is lidded, cooled in the desiccator for 30 min and weighed.

5.3 Dry the sample to constant weight in a vacuum oven under the same conditions described above (drying period = 1 h).

5.4 Quickly lid the dish, remove it from the oven, cool to room temperature in a desiccator for 30 min and weigh.

5.5 Repeat the procedure (5.2 and 5.3) until constant weight is attained (when successive 1 h drying periods show additional loss of ≤ 0.05%).

NOTES:

- Some AO samples can give problems due to an excess of moisture, so they can explode producing splattering inside the vacuum oven if they are subjected to the stablished conditions. To avoid that, once the sample has been weighed into the tared dish, the uncovered dish is kept overnight (16 h) into the desiccator under a progressive vacuum until reaching 10 mm Hg to remove part of the moisture and volatiles from the sample. After this operation, the determination follows the procedure described above (5.2 and subsequent steps).

- Lauric fats contain a significant amount of C6:0-C12:0 fatty acids. In the case of the lauric FAD coming from the physical refining of coconut or palm kernel oils most of the fatty acids (65-82%) are in free form. It has been reported that some of these free fatty acids, mainly C6:0 (boiling point at 50 mm Hg = 135 ºC), can be volatilized in the vacuum oven. This volatilization under our working conditions (constant weight achievement) conducts to an overestimation of the moisture and volatile content. This overestimation was proven in several lauric FAD samples by comparing the results obtained as described above and following the Karl Fischer method (ISO 8534:2017) [3]. As it can be observed in Table S4.1, if constant weight is attained, the overestimation is considerable; however, if only one 1 h-drying period is applied, the difference with the Karl Fischer results is minimum. These results agree to the fact that during the first drying period, water (boiling point at 50 mm Hg = 37.5 ºC) and the most volatile compounds are lost. Thus, for lauric FAD only one drying period of 1 h was applied to obtain the results of moisture and volatile compounds.

- The application to these samples of the hot plate methods, AOCS official methods Ca 2b-38 [4] and Tb 1a-64 [5] gives erroneous results due to the presence of a high amount of C6:0-C12:0 free fatty acids.

Table S4.1.

Determination of moisture (%) in lauric FAD samples by different methods (determinations in duplicate).

	Karl Fisher (ISO 8534:2017)	Vacuum oven	
		One drying period	Constant weight (5 drying periods)
Sample 1	0.28	0.32	0.47
Sample 2	0.16	0.14	0.36
Sample 3	0.18	0.21	0.57

6. CALCULATIONS

The percentage of moisture and volatile matter is given by the formula:

$$\text{Moisture and volatile matter}, \% = \frac{\text{loss in mass, g}}{\text{mass of test portion, g}} \times 100$$

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

7. INTRALABORATORY REPEATIBILITY

Eight determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 0.96% (mean = 0.98%, sample weight = 5 g), 12.80% for a non-lauric FAD (mean = 0.07%, sample weight = 5 g) and 8.43% for a lauric FAD (mean = 0.21%, sample weight = 5 g).

8. REFERENCES

[1] AOCS official method Ca 2d-25. Moisture and volatile matter, vacuum oven method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[2] AOAC official method 926.12. Moisture and volatile matter in oils and fats, vacuum oven method. Official methods of analysis of the Association of Official Analytical Chemists, 21st ed. AOAC Press, Champaign, IL. 2019.

[3] International Standard ISO 8534:2017. Animal and vegetable fats and oils, determination of water content, Karl Fischer method (pyridine free). 3rd ed. 2017.

[4] AOCS official method Ca 2b-38. Moisture and volatile matter, hot plate method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[5] AOCS official method Tb 1a-64. Moisture and volatile matter fatty acids, hot plate method. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S5- DETERMINATION OF INSOLUBLE IMPURITIES

1. SUBJECT

This method describes a procedure for the determination of the content of insoluble impurities in vegetable acids oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method determines dirt and other foreign substances insoluble in light petroleum ether. The method is based on the ISO 663:2017 method [1] and the AOCS official method Ca 3a-46 [2].

2. PRINCIPLE

A test portion is treated with an excess of petroleum ether, then the obtained solution is filtered. The filter and residue are washed with the same solvent, then dried at 103 ºC in an air oven and weighed to determine the percentage of insoluble impurities.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Erlenmeyer flask with ground glass stopper, 250 mL.
3.3 Desiccator, containing an efficient desiccant [silica gel with moisture indicator and phosphorus pentoxide, both used to capture the accumulated moisture inside the desiccator, and last, paraffin M.P. 51-53 ºC pellets from Panreac (Castellar del Vallés, Spain) that captures solvents inside the desiccator].
3.4 Filter crucible, glass filter ROBU® Glasfilter-Geraete GmbH (Hattert, Germany). Capacity of filter crucible: 50 mL; filter plate diameter: 40 mm; and pore: 4, which is equivalent to P16 [10-16 µm of pore size].
3.5 Glass centrifuge tubes with screw caps, 50 mL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2). In addition, AO and FAD must be dried to constant weight in a vacuum oven as described in this compendium of methods (Method S4, pages 14-17).

5. REAGENTS

5.1 Petroleum ether 40-60 ºC ROTIPURAN® for analysis, ACS, ISO grade from Carl Roth (Karlsruhe, Germany). Having a distillation range between 30 ºC and 60 ºC and having a bromine value of less than 1.
5.2 Celite® Hyflo Super Cel® filter aid (kieselguhr) from Carl Roth (Karlsruhe, Germany).
5.3 Cleaning reagents: isooctane for analysis and chromic acid solution both from Scharlau (Sentmenat, Spain).

6. PROCEDURE

6.1 Prepare the filter crucible: in a 100 mL glass beaker, prepare a slurry consisting of 2 g of kieselguhr and approximately 30 mL of petroleum ether 40-60 ºC. Pour the

mixture into the filter crucible under reduced pressure to obtain a homogeneous layer of kieselguhr on the glass filter (according to safety instructions for glassware ROBU® do not exceed 735.56 mm Hg of vacuum). Let the crucible dry for 1 h in the oven at 103 ºC, and then allow it to cool in the desiccator (30 min) and weigh it (m1). Repeat the drying operation until constant weight (maximum difference between weights of 0.003 g) usually constant weight is obtained after the first drying operation, especially when the same filter crucibles and kieselguhr batch are used). Always use powder-free gloves to handle the filter crucibles.

6.2 Accurately weigh (± 0.0001g) about 5 g for AO or FAD (m0, see notes below) into a tared moisture dish and determine the moisture and volatile matter.

6.3 Transfer the dried sample from the moisture dish to a 250 mL Erlenmeyer flask, using 10 mL of petroleum ether.

6.4 Stopper the flask and shake to assure the complete sample dissolution. Filter slowly through the filter crucible, using suction.

6.5 Repeat the 6.3 step 4 times, so that in total 50 mL of petroleum ether are used to transfer the sample.

6.6 Rinse the flask and wash thoroughly the filter crucible with five fractions of 5 mL of warm petroleum ether (warm the solvent to 50 ºC in a capped centrifuge tube during 10 minutes before to use it) to ensure that all impurities are washed into the crucible.

6.7 Allow most of the solvent remaining in the filter to evaporate in air under fume hood and complete the evaporation in the oven set at 103 ºC for 15 min. Then, remove from the oven, allow to cool in the desiccator (45 min) and weigh. Repeat procedure until constant weight (m2) (this is when the lost in weight does not exceed 0.004 g; normally two weights are needed to achieve constant weigh).

NOTES:

- Samples with a high percentage of insoluble impurities may be difficult to filter in the following steps of the procedure. For such samples, 2 g can be weighed (m0) without significantly affecting the precision of the method. According to our results, in AO samples with impurities percentages higher than 10% it is necessary to reduce the sample weight to 2 g instead of 5 g, in order to carry out the method without any problem in the filtration step nor in transferring the entire sample from the dish to the filter. In the case that some remains do not dissolve in the ether and remain in the moisture dish, they must be weighed and this value must be added to the total weight for the calculation of the impurities. In addition, we observed a sample with a low percentage of insoluble impurities whose filtration was problematic due to the consistency of the sample itself. In this case the weight was reduced to 2.5 g.

7. CALCULATIONS

The insoluble impurities content, expressed as a percentage by mass, is given by the next formulas. The first one is expressed on wet weight and the other one on dry weight.

$$\text{Insoluble impurities, \% (on wet weight)} = \frac{m2 - m1}{m0} \times 100$$

$$\text{Insoluble impurities, \% (on dry weight)} = \frac{m2 - m1}{m0 \times (\frac{100 - \text{moisture\%}}{100})} \times 100$$

Where:

m0: is the mass of the test portion, in grams.
m1: is the mass of the kieselguhr and the filter crucible, in grams.
m2: is the mass of the kieselguhr, the filter crucible and dry residue, in grams.
Moisture %: moisture and volatile matter content of the sample.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. CLEANING FILTER CRUCIBLES

8.1 Remove all residues from the filter crucible (kieselguhr and insoluble impurities) and dispose of in the appropriate waste container.
8.2 Clean the filter using appropriate solvents under vacuum filtration: first isooctane and then distilled water.
8.3 Let the filter crucible dry and soak it overnight in chromic acid solution.
8.4 Wash the filter thoroughly with water and soap and then rinse with distilled water.
8.5 Dry the filter crucibles 1 h at 103 ºC.

9. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions, the RSD obtained for four different samples of AO were: RSD of 10.85% for the first (mean = 0.51% on wet weight, sample weight = 5 g), RSD of 11.97% for the second (mean = 3.65% on wet weight, sample weight = 5 g), RSD of 12.75% for the third (mean = 10.24% on wet weight, sample weight = 2 g) and RSD of 12.65% for the fourth (mean = 1.57% on wet weight, sample weight = 2 g). For one FAD sample the RSD was 7.72% (mean = 2.85% on wet weight, sample weight = 5 g).

10. REFERENCES

[1] International Standard ISO 663:2017. Animal and vegetable fats and oils, determination of insoluble impurities content. 5th ed. 2017.
[2] AOCS official method Ca 3a-46. Insoluble impurities. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S6- DETERMINATION OF UNSAPONIFIABLE MATTER USING DIETHYL ETHER EXTRACTION

1. SUBJECT

This method describes a procedure for the determination of unsaponifiable matter in vegetable acid oils from chemical refining (AO) and fatty acids distillates from physical refining (FAD) of vegetable oils and fats. Unsaponifiable matter includes those substances frequently found dissolved in fats and oils that cannot be saponified by the usual caustic treatment and that are not volatile under the specified operating conditions, but that are soluble in diethyl ether. The method described below is a slight adaptation of the AOCS official method Ca 6b-53 [1], the AOAC Official Method 933.08 [2] and the ISO 3596:2000 method [3].

2. PRINCIPLE

A test portion is saponified with an ethanolic potassium hydroxide solution. The unsaponifiable matter is extracted from the soap solution by diethyl ether. The solvent is then evaporated, and the residue is weighed after drying.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Glass centrifuge tubes with screw caps, 50 mL.
3.3 Erlenmeyer flasks, 250 mL.
3.4 Glass separatory funnels, 250 mL and 500 mL.
3.5 Glass beakers, 500 mL.
3.6 Water bath B-491 from Buchi (Flawil, Switzerland).
3.7 Rotary evaporator Rotavapor® R-210 from Buchi (Flawil, Switzerland).
3.8 Graduated pipette, 10 mL.
3.9 Volumetric flask, 100 mL, to prepare the sodium hydroxide solution 0.01 N.
3.10 Burette, capacity 10 mL, graduated in 0.05 mL.
3.11 Desiccator, containing an efficient desiccant (silica gel with moisture indicator and phosphorus pentoxide, both used to capture the accumulated moisture inside the desiccator, and last, paraffin M.P. 51-53 ºC pellets from Panreac (Castellar del Vallés, Spain) that captures solvents inside the desiccator).
3.12 Air oven capable of operating at 105 ºC.
3.13 Vacuum oven VO200 from Memmert (Schwabach, Germany).

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Ethanol 96% reagent grade, from Panreac (Castellar del Vallés, Spain).
5.2 Potassium hydroxide (KOH) 85%, ACS grade, from Acros Organic (Geel, Belgium).

5.2.1 Potassium hydroxide, 1.5 M (approximately) ethanolic solution: prepared by dissolving 70.6 g of potassium hydroxide in 40 mL of water and dilute to 700 mL with 96% ethanol. The solution should be colorless or straw-yellow.

5.2.2 Potassium hydroxide aqueous solution, approximately 0.5 M: prepared by dissolving 35.3 g of potassium hydroxide in 1 L of distilled water.

5.3 Diethyl ether free from peroxides for analysis, ACS, ISO grade, stabilized with approx. 7 ppm of 2,6-Di-tert-butyl-4-methylphenol (BHT) from Scharlau (Sentmenat, Spain).

5.4 Phenolphthalein indicator, ACS grade, from Scharlau (Sentmenat, Spain)

5.4.1 Phenolphtalein indicator 1% (w/v) solution in ethanol 96%.

5.5 Extemporaneous preparation of a neutralized solvent mixture of ethanol (*Reagent 5.1*) and diethyl ether (*Reagent 5.3*) (20:4, v/v): add phenolphthalein as an indicator (0.3 mL of phenolphthalein by 100 mL of mixture) and neutralize it to the phenolphthalein endpoint.

5.6 Sodium hydroxide (NaOH) 0.1 M solution from Scharlau (Sentmenat, Spain), standardized volumetric solution, factor 0.999-1.001, checked by means of potentiometric methods using Scharlau´s potassium hydrogen phthalate volumetric standard.

5.6.1 Extemporaneous preparation of the sodium hydroxide 0.01 M solution: pipet exactly 10 mL of sodium hydroxide solution 0.1 M into a volumetric flask of 100 mL and dilute to volume with distilled water. Freshly prepare this solution for each batch of samples.

5.7 Acetone reagent grade, from Scharlau (Sentmenat, Spain).

6. PROCEDURE

6.1 Accurately (± 0.0001g) weigh about 2.0 g of test sample into a 50 mL centrifuge tube. Add 26 mL of potassium hydroxide, approximately 1.5 M ethanolic solution, and heat in a water bath for 45 min at 85 ºC, with occasional swirling. No loss of ethanol should occur during saponification, thus, mark the level of the saponification medium in the tubes to check it.

6.2 Transfer the tube content while warm to the 250 mL separatory funnel using two fractions of 25 mL of water. Wash the centrifuge tube with two fractions of 25 mL of diethyl ether and add to the separatory funnel.

6.3 Insert the stopper and shake vigorously for 1 min and wait until both layers are clear. Draw off the lower aqueous layer into a 250 mL Erlenmeyer flask, including a small amount of diethyl ether layer as well. Transfer the upper diethyl ether layer to a 500 mL separatory funnel. Transfer the aqueous layer from the Erlenmeyer to the same 250 mL separatory funnel to continue the extraction.

6.4 Repeat the extraction three more times, using 50 mL portions of diethyl ether each time and shaking vigorously for 1 min. Combine all the diethyl ether extracts in the 500 mL separatory funnel. AO and FAD are by-products with a high content of unsaponifiable matter, which may require more than four extractions for complete extraction of the unsaponifiable matter. Test, if necessary, by making a further extraction and evaporating this extract. If unsaponifiable matter is found by gravimetry, dissolve the residue in two fractions of 5 mL of diethyl ether and add back to the combined extracts. Continue with the extractions until the extract has no unsaponifiable matter.

6.5 Gently rotate during 20 s the combined diethyl ether extracts with 20 mL of water. Avoid violent agitation at this step since it may result in emulsions that are difficult

to separate. Allow the layers to separate completely and discard the lower aqueous layer into the 250 mL Erlenmeyer flask. Always let some aqueous phase stay in the separatory funnel containing the diethyl ether phase, instead it is otherwise indicated. Wash the diethyl ether layer two more times, using 20 mL of water each time.

6.6 Wash the diethyl ether extracts in the separatory funnel three times with 20 mL of KOH 0.5 M (aqueous) each time, shaking vigorously during 20 s and follow each alkali wash by a wash with 20 mL of water.

6.7 After the third wash with 0.5 M KOH, wash the diethyl ether extracts with successive 20 mL portions of water until the washes are no longer alkaline to phenolphthalein 1%. When the last wash is carried out, do the separation eliminating all water from the ether extract.

6.8 Transfer the diethyl ether to a 500 mL flat bottom flask (previously tared after heating at 105 ºC during 30 min and cooling in desiccator). Rinse the separatory funnel using two fractions of 20 and 10 mL of diethyl ether and add the rinses to the solution in the flask.

6.9 Evaporate up to about 1 mL with a rotary vacuum evaporator at 25 ºC and then, under a stream of nitrogen.

6.10 When almost all of diethyl ether has been evaporated, add 3 mL of acetone. The flask will remain in the desiccator all night.

6.11 Complete the drying to constant weight in a vacuum oven for 1 h at 58 ºC, 56/60 ºC min/max temperature and 66 mbar (approximately 50 mm Hg). Cool in a desiccator to room temperature (45 min) and weight. Usually three 1 h-drying steps are necessary to achieve the constant weigh. The result become "A" in the calculations.

6.12 After weighing the residue, dissolve it in 24 mL of a neutralized solvent mixture (ethanol/diethyl ether, 20:4, v/v, containing phenolphthalein) (*Reagent 5.5*).

6.13 To correct the residue for the free fatty acid content, titrate with 0.01 M NaOH solution to a faint pink color (although these by-products are rich in free fatty acids, many of these are washed away, so it is decided to use a 0.01 M NaOH solution and a 10 mL-capacity burette graduated in 0.05 mL). Correct the weight of the residue for free fatty acid content, using the following relationship: 1 mL NaOH 0.01 M is equivalent to 0.00282 g oleic acid. The grams of fatty acid determined by this titration become "B" in the calculations. Carry out a blank titration with only 24 mL of the neutralized solvent mixture (*Reagent 5.5*) and correct, if necessary, the free fatty acid content ("B").

6.14 Correct the weight of the residue ("A") for any possible reagent residue doing a blank by conducting the whole unsaponifiable matter procedure without any fat or oil present. The blank determined by this procedure becomes "C" in the calculations. Using the described reagents, the blank was always zero, but if this residue exceeds 1.5 mg investigate the reagents and the procedure.

7. CALCULATIONS

The unsaponifiable matter, expressed as a percentage, is given by the formula:

$$\text{Unsaponifiable matter, \%} = \frac{\mathrm{A} - (\mathrm{B} + \mathrm{C})}{\mathrm{m}} \times 100$$

Where:

A: is the mass of the residue, in grams.

B: is the mass of the free fatty acids, in grams.

C: is the mass of unsaponifiable blank, in grams.

m: is the mass of the test portion, in grams.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 5.35% (mean = 5.02%, sample weight = 2 g) and for a FAD, 3.46% (mean 1.22%, sample weight = 2 g).

9. REFERENCES

[1] AOCS official method Ca 6b-53. Unsaponifiable matter. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[2] AOAC official method 933.08. Unsaponifiable matter. Official methods of analysis of the Association of Official Analytical Chemists', 21st ed. AOAC Press, Champaign, IL. 2019.

[3] International Standard ISO 3596:2000. Animal and vegetable fat and oils, determination of unsaponifiable matter, method using diethyl ether extraction. 1st ed. 2000, reviewed and confirmed in 2016.

METHOD S7- DETERMINATION OF ACIDITY AND ACID VALUE

1. SUBJECT

This method describes a procedure for the determination of the content of free fatty acids in vegetable acid oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the ISO 660:2009 method [1] and considering some aspects described in the AOCS official method Cd 3d-63 [2].

2. PRINCIPLE

The sample is dissolved in a suitable solvent mixture, and the acids present are titrated with an 0.1 M ethanolic solution of potassium hydroxide. While the acidity or free fatty acid content is expressed as mass percent of lauric, palmitic, erucic or oleic acids, according to the fatty acid composition of the sample (see Table S7.1), the acid value is expressed as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in 1 g of fat.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Burette, capacity 25 mL, graduated in 0.05 mL, class A.
3.3 Erlenmeyer flasks with ground glass stopper, 250 mL.
3.4 Erlenmeyer flasks with ground glass stopper, 1 L, to prepare the solvent mixture.
3.5 Volumetric flask, 100 mL, to prepare the phenolphthalein indicator solution 1% in ethanol 96%.
3.6 Graduated cylinder, 100 mL.
3.7 Magnetic stirring device.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Solvent A: Ethanol 96 % (v/v) for analysis, ACS grade, from Panreac (Castellar del Vallés, Spain).
5.2 Solvent B: Toluene for analysis, ACS grade, from Scharlau (Sentmenat, Spain).
5.3 Solvent mixture: mix equal volumes of solvents A and B. Prepare the total quantity depending on the number of samples. Neutralize just before use, under constant agitation, by adding 0.1 M potassium hydroxide ethanolic solution (*Reagent 5.4*) in the presence of 0.3 mL of the phenolphthalein solution (*Reagent 5.5.1*) per 100 mL of solvent mixture. The endpoint of the neutralization consists in the appearance of a persistent faint pink color in the whole solvent mixture. The color change must be achieved by the addition of a single drop of the alkali solution. As some samples must be warmed to achieve a complete solution of the sample before titration, the mixture of ethanol-toluene was used for all samples. The mixture of ethanol-diethyl

ether is only appropriate for samples that can be completely dissolved by shaking at room temperature.

5.4 Potassium hydroxide (KOH) 0.1 M solution in ethanol from Carl Roth (Karlsruhe, Germany), standardized volumetric solution, factor 0.996-1.004.

5.5 Phenolphthalein indicator, ACS grade from Scharlau (Sentmenat, Spain).

5.5.1 Phenolphtalein indicator 1% (w/v) solution in ethanol 96 %.

6. PROCEDURE

6.1 Accurately weigh (±0.0001 g) about 0.5 g of test sample into a 250 mL Erlenmeyer flask.

6.2 Add 75 mL of the neutralized solvent mixture (*Reagent 5.3*) by means of a graduated cylinder. Be sure that the sample is completely dissolved. Most samples are dissolved by shaking at room temperature, but some samples need gentle warming to achieve the complete dissolution. The samples more difficult to dissolve are the palm fatty acid distillates that need to be warmed under constant agitation in water bath to maximum of 60 ºC to achieve the complete dissolution.

6.3 Add approximately 1 mL of phenolphtalein indicator solution and titrate with constant magnetic stirring using standardized potassium hydroxide 0.1 M solution in ethanol. The endpoint of the titration is reached when the addition of a single drop of the alkali solution produces a persistent global color change of the reaction medium. The pink color (or orange pink color for dark samples) must persist at least 30 s. To facilitate the observation of the endpoint, make to fall the drops of the titrating solution away from the eddy created by the magnetic stirring. In this way, it is easily observed when the titration approaches the endpoint due to the persistence of the pink color around the drop that has fallen in the reaction mixture. Remember that the titration endpoint is reached when the addition of a single drop of alkali produces a slight but clear color change affecting the whole reaction mixture and persisting for at least 30 s.

7. CALCULATIONS

The acidity or free fatty acid content expressed as a percentage mass fraction is equal to:

$$\text{FFA}\ (\%) = \frac{\text{V} \times \text{c} \times \text{M} \times 100}{1000 \times \text{m}}$$

Where:

V = is the volume, in milliliters, of the standardized volumetric potassium hydroxide solution.

c = is the concentration, in moles per liter, of the standardized volumetric potassium hydroxide solution.

M = is the molar mass, in grams per mole, of the fatty acid chosen to express the results according to the fatty acid composition of the fat type (Table S7.1).

m = sample weigh, in grams.

The FFA content (%) or acidity is expressed according to the length of the major fatty acids in the fat (Table S7.1). Thus, in the AO and FAD samples collected in this study, the acidity is expressed as lauric acid for the FAD coming from coconut and palm

kernel oils, as palmitic acid for the palm FAD, and as oleic acid for the rest of samples where clearly predominate the fatty acids having 18 carbons.

Table S7.1

Fatty acids used to express of acidity (ISO 660:2009).

Type of fat	Expressed as	Molar mass (g/mol)
Coconut oil, palm kernel oil and similar oils	Lauric acid	200
Palm oil	Palmitic acid	256
Oils from certain *Cruciferae*[a]	Erucic acid	338
All other fats	Oleic acid	282

[a] In the case of rapeseed oil having a maximum erucic acid content of 5%, the acidity shall be expressed as oleic acid.

NOTE: If the result is simply as "acidity" without further definition, this is, by convention, expressed as oleic acid.

The acid value expressed as mg of KOH/g of fat is equal to:

$$\text{AV (mg KOH/ g of fat)} = \frac{56.1 \times c \times V}{m}$$

Where:

c = is the concentration, in moles per liter, of the standardized volumetric potassium hydroxide solution.
V = is the volume, in milliliters, of the standardized volumetric potassium hydroxide solution used for the titration.
m = sample weigh, in grams.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 4.04% (mean = 61.98 g oleic acid/100 g or 123.30 mg KOH/g of fat, sample weight = 0.5 g) and for a FAD, 1.06% (mean = 74.77 g lauric acid/100 g or 209.74 mg KOH/g of fat, sample weight = 0.5 g).

9. REFERENCES

[1] International Standard ISO 660:2009. Animal and vegetable fat and oils, determination of acid value and acidity. 3rd ed. 2009, reviewed and confirmed in 2014.

[2] AOCS official method Cd 3d-63. Acid value of fats and oils. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S8- DETERMINATION OF LIPID FRACTIONS: POLYMERIC COMPOUNDS, TRIACYLGLYCEROLS, DIACYLGLYCEROLS, MONOACYLGLYCEROLS AND FREE FATTY ACIDS

1. SUBJECT

This method describes a procedure for the determination of the lipid fractions (expressed as %) in vegetable acid oils from chemical refining (AO) and non-lauric fatty acid distillates from physical refining (FAD) of vegetable oils and fats by liquid chromatography (HPLC), according to the IUPAC 2508 method [1]. This method is not applicable to fats and oils rich in medium- and/or short-chain fatty acids, such as lauric fats.

2. PRINCIPLE

The triacylglycerols, diacylglycerols, monoacylglycerols, FFA and polymeric compounds in AO and FAD are separated by size molecular exclusion chromatography by means of two HPLC columns connected in series and determined through a refractive index detector. The polymeric compounds are mainly formed when radicals of the main lipid fractions (FFA and acylglycerols) react and form new intermolecular bonds.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Test tubes with screw caps, 10 mL.
3.3 Positive displacement micropipettes suitable to dispense exact volumes of volatile solvents or solutions (MICROMAN®, 250 and 1000 µL from Gilson, Middleton, USA).
3.4 Volumetric glass pipettes, 3mL.
3.5 Vortex mixer.
3.6 Nylon filter, 13 mm diameter and 0.45 µm of pore from Scharlau (Sentmenat, Spain).
3.7 Plastic syringe, 2.5 mL.
3.8 HPLC system was an Agilent 1100 series equipped with an isocratic pump, oven (35 ºC) and refractive index detector (RID) from Agilent Technologies (Santa Clara, USA). Two HPLC columns (30 cm x 7.8 mm i.d.) filled with a highly cross-linked styrene-divinylbenzene copolymer of 5 µm particle size (Styragel HR 1 and Styragel HR 0.5) of 100 and 50 Å pore size, respectively, were used connected in series. Tetrahydrofuran is used both to dissolve samples and as mobile phase at 1 mL/min. The analysis time is about 20 min.

This method is only applicable to fats and oils that are basically composed of fatty acids with 16 and 18 carbons. The method is not applicable to fats and oils rich in medium- and/or short-chain fatty acids, such as lauric FAD as their lipid classes have a wide range of molecular weights, which disables the size exclusion columns from separating them. We noted this problem using several triacylglycerol, monoacylglycerol and free fatty acid standards with different molecular weights. For instance, monolaurin is included in the peak corresponding to C16 and C18 free fatty acids (Fig. S8.1). This is because the molecular weight of monolaurin is 274.4 g/mol and of palmitic and oleic acid, respectively, 256.4 and 282.5 g/mol. For the same reason, tricaprin elutes together with

the diacylglycerols in peak 2 (Fig. S8.1). Therefore, we discarded this method for lauric FAD since the separation between lipid classes cannot be achieved as the ranges of molecular weights of different lipid classes are clearly overlapped.

On the contrary, the method separates well the lipid classes in fats and oils mainly composed of fatty acids with 16 and 18 carbons (Fig. S8.2). However, in some AO with low content of polymeric compounds, the peak corresponding to these compounds (peak 1, Fig. S8.2) appears like a shoulder prior the peak corresponding to the triacylglycerols (peak 2).

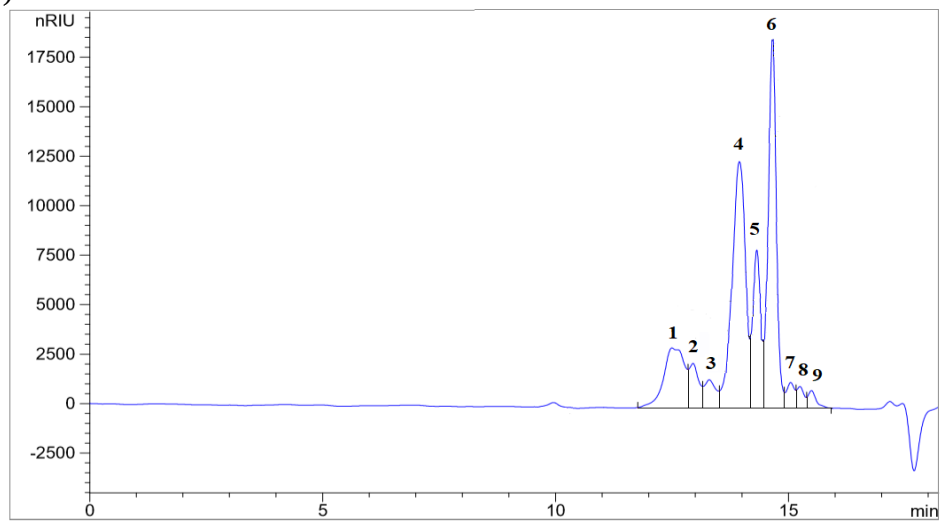


Fig. S8.1. Separation of the lipid classes from a lauric fatty acid distillate. Main compounds corresponding to peaks: 1, triacylglycerols; 2, diacylglycerols; 3, monoacylglycerols; 4, C16 and C18 free fatty acids; 5, myristic acid; 6, lauric acid; 7, capric acid; and 8 and 9, free fatty acids with less than 10 carbons.

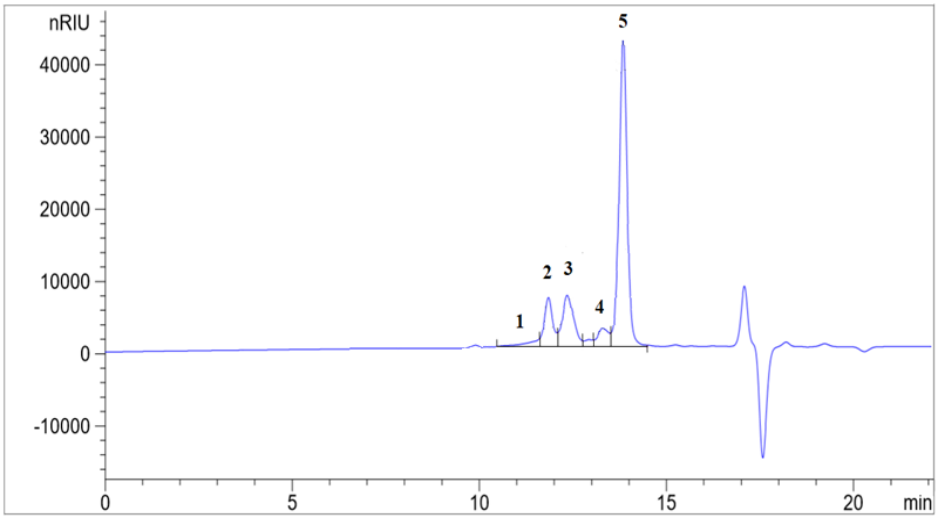


Fig. S8.2. Separation of the lipid classes from an acid oil coming from the refining of olive pomace oil. Main compounds corresponding to peaks: 1, polymeric compounds; 2, triacylglycerols; 3, diacylglycerols; 4, monoacylglycerols; and 5, C16 and C18 free fatty acids.

4. PREPARATION OF THE SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Tetrahydrofuran, HPLC grade, from Scharlau (Sentmenat, Spain).

6. PROCEDURE

6.1 Approximately weigh 150 mg of test sample (AO or FAD) into a 10 mL test tube.
6.2 Add 3 mL of tetrahydrofuran with a volumetric pipette.
6.3 Homogenize using the vortex for 30 s.
6.4 In an empty screw capped tube, add 800 µL of tetrahydrofuran using a positive displacement micropipette.
6.5 Transfer 200 µL of the sample solution prepared in point 6.3, using a positive displacement micropipette, into the tube prepared in point 6.4. Cap the tube and homogenize using the vortex for 10 s.
6.6 Remove the plunger from the plastic syringe and connect the syringe to a Nylon filter (0.45 µm).
6.7 Filter the solution (point 6.5) into a new glass tube or an HPLC vial, using the plunger if necessary.
6.8 Inject in the HPLC-RID system.

7. CALCULATIONS

Each lipid fraction (triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids or polymeric compounds) is quantified by peak area normalization (peak area percentage):

$$\% = \frac{\text{Peak area}}{\text{Total peak areas}} \times 100$$

Where:

Peak area: is the area of each lipid fraction peak.

Total peak areas: is the sum of areas of all peaks corresponding to the different lipid fractions.

The determination of each sample is carried out in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the results obtained for an AO were: for polymeric compounds, 2.51% (RSD = 11.38%); for triacylglycerols, 21.39% (RSD = 0.79%); for diacylglycerols, 17.98% (RSD = 1.13%); for monoacylglycerols, 4.05% (RSD = 5.04%); and for free fatty acids 54.07% (RSD = 0.73%). For a non-lauric FAD the results were: for triacylglycerols, 6.03% (RSD = 6.79%); for diacylglycerols, 5.88% (RSD = 9.83%); for free fatty acids, 88.09% (RSD = 0.93%); the polymeric compounds and the monoacylglycerols were not detected.

9. REFERENCES

[1] International Union of Pure and Applied Chemistry (IUPAC). Determination of polymerized triglycerides in oils and fats by high performance liquid chromatography (method 2508). In Standard methods for the analysis of oils, fats and derivatives, 7th ed.; IUPAC, Blackwell Scientific Publications: Oxford, UK (1991).

METHOD S9- DETERMINATION OF PEROXIDE VALUE

1. SUBJECT

This method describes the determination of peroxide value in vegetable acid oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the method adopted by the European Union for the analysis of olive oils [1].

2. PRINCIPLE

The peroxide value is the quantity of peroxides (expressed in milliequivalents of active oxygen per kg of fat) in the test sample. The test sample is dissolved in chloroform-acetic acid and treated with a solution of potassium iodide. The iodine liberated by oxidation of potassium iodide is titrated with a standard volumetric sodium thiosulfate solution. The oxidant substances under these conditions are generally assumed to be peroxides.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Erlenmeyer flasks with ground glass stopper and totally dry, 250 mL.
3.3 Volumetric flask, 1000 mL, to prepare the sodium thiosulfate solution.
3.4 Graduated pipettes, 10 mL.
3.5 Micropipette, 1000 µL (PIPETMAN™ from Gilson™ Middleton, USA).
3.6 Graduated cylinders, 25 mL and 100 mL.
3.7 Burette, capacity 25 mL, graduated in 0.1 mL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Chloroform stabilized with ethanol for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).
5.2 Glacial acetic acid for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).
5.3 Sodium thiosulfate 0.1 N standardized volumetric solution from Scharlau (Sentmenat, Spain); factor 0.999- 1.001, checked by means of potentiometric methods using Scharlau's potassium iodate volumetric standard.
    5.3.1 Extemporaneous preparation of the sodium thiosulfate 0.001 N solution: pipet exactly 10 mL of sodium thiosulfate 0.1 N solution into a volumetric flask of 1000 mL and dilute to volume with distilled water. Freshly prepare this solution each day.
5.4 Potassium iodide for analysis, ACS, ISO grade from Scharlau (Sentmenat, Spain).
    5.4.1 Extemporaneous preparation of the saturated solution of potassium iodide free from iodine and iodates: in a small baker add a little amount of distilled water and add the necessary amount of potassium iodide (about 10 g KI in 6 mL of water). Shake with a spatula to favor its dissolution. It can be used when the

solution remains saturated (undissolved KI crystals) and colorless. Freshly prepare the solution for each batch of samples (approximately each 30 minutes) and always store in the dark when not in use.

5.5 Starch solution 1% (w/v) from Scharlau (Sentmenat, Spain).

6. PROCEDURE

All the process must be carried out under attenuated lighting conditions (warm white LED light with nil emission at UV region is adequate).

6.1 Accurately (±0.0001 g) weigh by difference about 2.5 g of fat into a 250 mL Erlenmeyer flask. Since some samples are very dark and this makes it difficult the observation of the endpoint of the iodometric titration, no more than 2.5 g should be weighed, even if the expected peroxide value of the sample is much lower than 10 (the 92 samples analyzed had peroxide values lower than 10 and the 95% had peroxide values between 0 and 3).

6.2 Add 10 mL of chloroform using a graduated pipette. Dissolve by shaking the test portion.

6.3 Add 15 mL of glacial acetic acid using a graduated cylinder.

6.4 Pass a nitrogen stream inside the Erlenmeyer flask to remove the O_2 from the solution. Stopper the Erlenmeyer flask.

6.5 Add 1 mL of potassium iodide solution put the caps and shake during 1 min. Then, keep the Erlenmeyer flask in darkness during 5 min at room temperature (between 15-25 ºC). When several samples are analyzed, this step can be done by pairs of Erlenmeyer flasks allowing enough time (around 10 min between pairs) to do the volumetric titration. A maximum of 3 samples in duplicate plus the blank are carried in each batch of samples.

6.6 After the 5 minutes in the darkness, immediately add 75 mL of distilled water.

6.7 Add about 1 mL of starch indicator and titrate with constant and vigorous swirling using sodium thiosulfate 0.001 N solution until the deep blue color disappears.

As the samples showed very low peroxide values and therefore the amount of liberated iodine is low, in most cases, the indicator could be added at the beginning of the titration. If the amount of liberated iodine is high (samples with high peroxide values), start the titration and add the indicator when the iodine yellow color is light. In some cases, there is no apparent liberation of iodine and after adding the indicator no color change is observed at all, which indicates a peroxide value equal to zero.

In the case of FAD samples, the observation of the titration endpoint is clear. However, several AO samples possess very dark colors, hindering the observation of the endpoint. In these samples, the indicator should be added before starting the titration and the constant vigorous shaking should be periodically reduced to gentle shaking to clearly observe the endpoint. The slowdown of the agitation allows the concentration of the sample´s dark color into the chloroform phase and the clear observation of the change of the indicator's color in the aqueous phase.

To facilitate the visualization of the endpoint always use a white background. However, in these samples, remember the importance of the intermittent vigorous shaking during all the titration because the iodine has more affinity for the chloroform phase, but its reduction by thiosulfate to iodide takes place in the aqueous phase.

6.8 Carry out determinations in duplicate and simultaneously perform a blank test for each batch of samples. If the blank test result exceeds 0.1 mL of the sodium thiosulfate 0.001 N, replace the reagents and repeat the determination.

7. CALCULATIONS

The peroxide value is given by the formula:

$$PV = \frac{V \times N \times 1000}{m}$$

Where:
V: is the volume, in milliliters, of sodium thiosulfate solution used for the titration.
N: is the normality of sodium thiosulfate solution.
m: mass of sample, in grams.

All samples were determined in duplicate. Take as the result the arithmetic mean of the two replicates.

8. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for a dark AO sample was 2.14% (mean = 2.63 milliequivalents of active oxygen per kg of fat, sample weight = 2.5 g).

9. REFERENCES

[1] Commission regulation (EEC) 2568/91 of 11 July 1991 on the Characteristics of olive oil and olive-residue oil and on the relevant methods of analysis (and its amendments). Annex III - Determination of the peroxide value, Off. J. Eur. Communities L248 (1991) 1-83.

METHOD S10- DETERMINATION OF *p*-ANISIDINE VALUE

1. SUBJECT

This method describes a procedure for the determination of the amount of aldehydes (principally 2-alkenals and 2,4-dienals) in vegetable acid oils from chemical refining (AO) and fatty acids distillates from physical refining (FAD) of vegetable oils and fats. The method described below is an adaptation of the AOCS official method Cd 18-90 [1].

2. PRINCIPLE

The amount of aldehydes is determined by reaction in an isooctane/acetic acid solution of the aldehydic compounds present in the sample and the *p*-anisidine. The reaction products formed in this reaction absorb at 350 nm. The intensity of the absorption of the reaction products formed depends not only on the amount of aldehydic compounds but also on their structure. A double bond in the carbon chain conjugated with the carbonyl double bond increases the molar absorbance at 350 nm four to five times, so especially 2-alkenals and 2,4-dienals, will contribute substantially to the *p*-anisidine value.

The *p*-anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1 g of the sample in 100 mL of a mixture of solvents and *p*-anisidine reagent.

3. APPARATUS

3.1 Analytical balance, with an accuracy of ± 0.0001 g.
3.2 Pyrex® test tubes with Teflon®-lined screw caps, 10 mL.
3.3 Volumetric flasks of 25 mL, or of 50 mL of capacity in case of high absorbances.
3.4 Volumetric pipettes, 1 mL and 5 mL.
3.5 Spectrophotometer suitable for observation at 350 nm (Shimadzu UV-3600, Kyoto, Japan).
3.6 Glass cuvettes, 1.00 ± 0.01 cm.
3.7 Syringe filters ReZist® PTFE membrane, 30 mm diameter and 5 µm of pore from Whatman® (Cytiva, Little Chalfont, UK).
3.8 Syringe filters OlimPeak® CR (Cellulose regenerated) membrane, 13 mm diameter and 0.45 µm of pore from Teknokroma (Sant Cugat del Vallés, Spain).
3.9 Glass syringes, 30 mL.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. REAGENTS

5.1 Isooctane UV, IR, HPLC grade, from Panreac (Castellar del Vallés, Spain).
5.2 Glacial acetic acid for analysis, ACS grade from Scharlau (Barcelona, Spain).

5.3 *p*-Anisidine ≥ 99% from Sigma-Aldrich (Madrid, Spain). During storage, *p*-anisidine tends to darken because of oxidation. It should be stored at 0-4 ºC in a dark bottle.

5.3.1 Preparation of the p-anisidine solution: Weigh 0.25 g of *p*-anisidine. Transfer *p*-anisidine into a 100 mL volumetric flask. Dissolve and dilute to volume with glacial acetic acid. Measure the absorbance of *p*-anisidine at 350 nm (maximun absorbance 0.20).

6. PROCEDURE

All the process must be carried out under attenuated lighting conditions (warm white LED light with nil emission at UV region is adequate).

6.1 Accurately (±0.0001 g) weigh by difference 0.5-0.8 g for AO and 0.7-1.5 g for FAD into a 25 mL volumetric flask. Most often, the proper weight for AO is 0.8 g, however, when after point 6.2. the solution is very turbid it is necessary to reduce the weight to 0.5 g.

6.2 Dissolve and dilute to volume with isooctane (also, n-hexane was tested with these samples, but it was discarded since more turbid solutions were obtained for some AO).

6.3 Most AO samples show suspended particles after dissolution in isooctane and a filtration is necessary before measuring the absorbance of the solution. The filtration of 10-12 mL of the solution is usually made by a PTFE filter of 5 µm of pore (*Apparatus 3.7*). If after filtration the solution obtained is not limpid the procedure is repeated by weighing 0.8 g of sample and doing the filtration with a CR filter of 0.45 µm of pore (only necessary for 6 samples out of 79 AO samples analyzed) (*Apparatus 3.7*). If the filtrate is still turbid, weigh 0.5 g of sample and filter the solution with a CR filter of 0.45 µm of pore (only necessary for 1 sample out of 79 AO samples analyzed). Sometimes gravity is sufficient to filter at an adequate speed, but if not, gentle pressure is applied by the syringe plunger. Average filtration rate: 12.32 mL/min.

For FAD, limpid solutions are obtained and filtration is not necessary. For these samples usually the appropriate weight is 1.5 g, but when absorbance after the reaction with *p*-anisidine is higher than 3, weight must be reduced up to 0.7 g (see linearity section below).

6.4 Measure the absorbance (Ab) of the fat solutions at 350 nm using isooctane as a blank.

6.5 Pipet exactly 5 mL of isooctane into one Pyrex® tube of 10 mL (blank) and exactly 5 mL of the different fat solutions into Pyrex® tubes of 10 mL (samples). Add exactly 1 mL of the *p*-anisidine reagent to each tube and gently shake to complete the homogenization. If you analyze more than one sample in the same batch of analysis, wait 1-2 min between the addition of *p*-anisidine to each sample tube, in order to accurately control the reaction time before the measurement of the absorbance. A maximum of 3 samples were analyzed by duplicate in each batch (6 sample tubes).

6.6 After exactly 10 min of reaction, measure the absorbance at 350 nm (As) of each sample using as a blank the content of the blank tube (isooctane plus *p*-anisidine reagent).

7. CALCULATIONS

The *p*-anisidine value (*p*-AnV) is given by the formulas:

$$p - \mathrm{AnV} = \frac{25 \times (1.2\mathrm{As} - \mathrm{Ab})}{\mathrm{m}}$$

Where:

As: is the absorbance of the fat solution after reaction with the *p*-anisidine reagent.

Ab: is the absorbance of the fat solution.

m: sample weight, in grams.

All samples were determined in duplicate. Take as the result the arithmetic mean of the two replicates.

8. LINEARITY OF THE RESPONSE

To check the linearity of the response, the *p*-AnV of various AO and FAD samples was determined in duplicate using different weights from 0.5-1.5 g. The linearity of the response was confirmed up to an As value of 3.2, since until this As value the *p*-AnV of several samples was independent of the weight. Thus, in all cases the weight of the samples was adjusted in order to obtain As values equal or lower than 3.0. If when weighing 0.5 g of sample, the As is higher than 3.0, then weight the appropriate amount of sample (> 0.5 g) into a 50 mL volumetric flask and use the following formula (this is very unlike to happen; it did not happen in any of the 92 samples analyzed, but it was close to happen in a couple of samples).

$$p - \mathrm{AnV} = \frac{50 \times (1.2\mathrm{As} - \mathrm{Ab})}{\mathrm{m}}$$

9. INTRALABORATORY REPEATIBILITY

Six determinations were carried by the same analyst within a day, using the same reagents, equipment and instruments. Under these conditions the RSD obtained for an AO was 2.96% (mean = 13.47, sample weight = 0.8 g) and for a FAD, 6.98% (mean = 12.62, sample weight = 1.5 g).

10. REFERENCES

[1] AOCS official method Cd 18-90. *p*-Anisidine value. Official methods and recommended practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

METHOD S11- DETERMINATION OF OXIDATIVE STABILITY BY RANCIMAT INSTRUMENT

1. SUBJECT

This method describes a procedure for the determination of the oxidative stability measured by the Rancimat instrument from Metrohm (Herisau, Switzerland) in vegetable acid oils from chemical refining (AO) and fatty acid distillates from physical refining (FAD) of vegetable oils and fats. The method described below is a slight adaptation of the AOCS official method Cd 12b-92 [1] and ISO 6886:2016 method [2].

2. PRINCIPLE

In the Rancimat instrument, the sample is subjected to an air flow and a constant temperature (between 80-140 ºC depending on the sample oxidability) which produces a deterioration of the sample. The air stream sweeps the highly volatile oxidation products such as alcohols, aldehydes, ketones and carboxylic acids, among others, which are transferred into the measuring vessel containing Milli-Q® water and the electrode that measures the conductivity. The conductivity is continuously registered and increases slowly to a sudden jump, at which point the oxidation accelerates and becomes high very rapid. The time elapsed until this rapid acceleration of the oxidation is the measure of the resistance to oxidation and is referred to as the induction time or induction period, which is a good characteristic of the oxidative stability of the sample. The computer software determines the break point of the conductivity curve by using the maximum of the second derivative of the curve and gives the induction time expressed in hours. The longer this time, the greater the oxidative stability of the sample.

3. APPARATUS

3.1 Precision balance, with an accuracy of ± 0.01 g.

3.2 The 892 Professional Rancimat from Metrohm (Herisau, Switzerland) is equipped with two heating blocks, each one with 4 heating positions and the corresponding measuring positions. The 892 Professional Rancimat is controlled by the StabNet® computer software from Metrohm. The Rancimat instrument has different parts that must be assembled to do the determination (Fig. S11.1):

- Fluorinated ethylene propylene (FEP) tubing: for supplying air into the reaction vessel.
- Polyvinylidene fluoride (PVDF) tubing adapters
- Reaction tube cover.
- Disposable glass reaction tube: where sample is weighed.
- Disposable glass cannula to bubble air through the sample. To properly insert it in the reaction tube cover (3), and to tight it, it is inserted through a nitrile O-ring (which does not appear in Fig. S11.1) placed on the reaction tube cover and it is tighten to the tubing adapter.
- Foam barrier piece: used with highly foaming samples to avoid the foam to pass to the measuring vessel. This barrier is optional but, in our case, as some samples foam heavily, it was used for all the AO and FAD samples. The foam barrier can melt if it is introduced too deeply into the heating block. Thus, ensure that the foam barrier is at least 7 cm above the base of the reaction tube. In our case, it was placed approximately at 9.5 cm.

- Silicone tubing: for connecting the reaction (heating) tubes to the measuring vessel.
- Measuring vessel cover: contains an integrated conductivity electrode protected by a ring.
- Polytetrafluoroethylene (PTFE) cannula to bubble air through the water (measuring solution).
- Measuring vessel: filled with 60-80 mL of milli-Q® water.
- Oil separator: Used if samples have a high content of volatile compounds, to avoid that important amounts of sample are transferred to the measuring vessel in the vapor phase. The use or not of the oil separator was tested in AO and FAD, but finally no differences were observed, so it was decided not to use it.

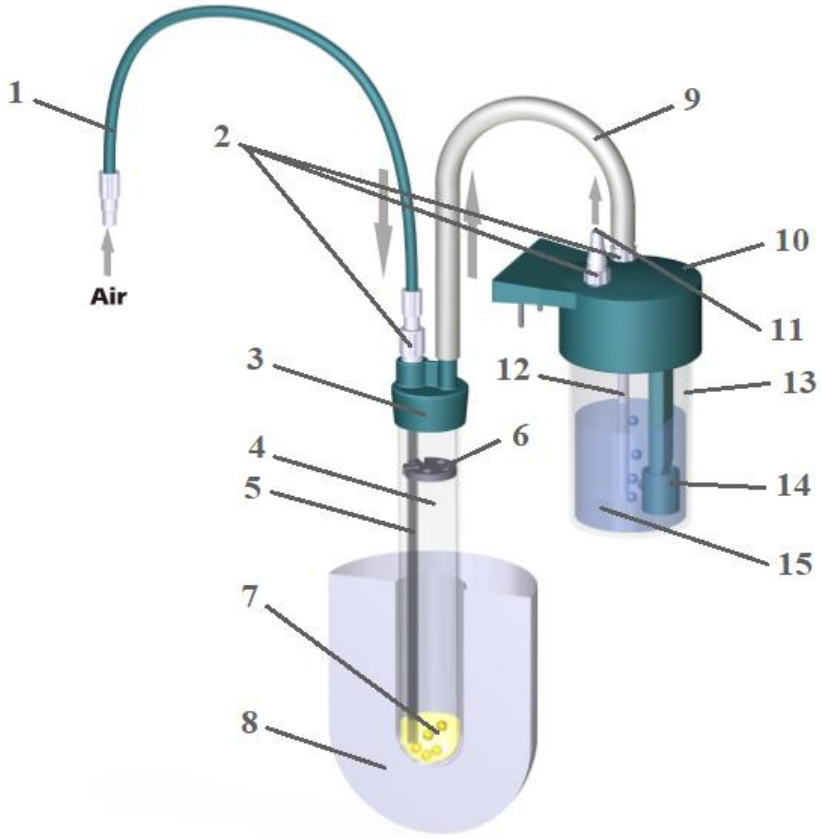


Fig. S11.1. Rancimat parts [3]: 1) Fluorinated ethylene propylene (FEP) tube; 2) Polyvinylidene fluoride (PVDF) tubing adapters; 3) Reaction tube cover made of thermoplastic elastomer-ether-ester (TEEE); 4) Glass reaction tube; 5) Glass cannula; 6) Foam barrier made of polypropylene (PP); 7) Sample; 8) Heating block; 9) Silicone tube; 10) Measuring vessel cover made of PP; 11) Air out hole; 12) Polytetrafluoroethylene (PTFE) cannula; 13) Measuring vessel made of polystyrene (PS); 14) Conductivity electrode protected by a ring; 15) Measuring solution.

4. PREPARATION OF TEST SAMPLE

The test sample was prepared from a representative sample as described in this compendium of methods (Method S1, pages 1-2).

5. PROCEDURE

5.1 The Rancimat instrument is recognized by the StabNet computer program when both are turned on. Select a method already created or create a new one. The setting parameters of the method used in our case are:

- Air flow, 20 L/h.
- Heating temperature, 120 ºC.
- Temperature correction factor, 1.6 (indicates the deviation of the current sample temperature from the temperature of the heating block).
- Evaluation suppression, 1 h (prevents the evaluation of the conductivity curve for 1 h). This option is useful for samples such as some AO and FAD that contain per se a high content of volatile compounds that cause a very early increase in conductivity, which does not correspond to the endpoint (Fig. S11.3).
- Evaluation sensitivity, 1 (the higher is this value, the lower is the sensitivity to detect the endpoint). The default value is 1, but if a false endpoint is found due to oscillations in the conductivity curve, the curve can be reprocessed by increasing the sensitivity in order to find the true endpoint.
- Stop criterion, 300 h. We do not use other stop criteria (conductivity value, automatic stop once found the endpoint) and set the stop at a very long time (300 h) to be able to manually stop the determination once the sudden increase of conductivity is clearly observed in the screen of the computer. This was done in this way to prevent the determination from stopping before reaching the true endpoint, which forces to make a new determination in duplicate for the sample. The automatic stopping is useful when the samples are known and they behave in a similar manner, which was not our case.

5.2 Fill the measuring vessel with 80 mL of Milli-Q® water. The initial specific conductivity of the water in the measuring vessel should not exceed a value of 5 μS/cm. The amount of water must be between 60-80 mL, so mark both levels in the measuring vessel. During the determination, the air stream evaporates water (approximately 7 mL per day). Thus, for samples with long induction times it may be necessary to add Milli-Q® water to maintain the water level of 60 mL and to keep the electrode safely immersed. If more water needs to be added it must be done through the air out hole (Fig. S11.1). In our case, is done by a glass syringe equipped with a stainless-steel needle.

5.3 Weigh the sample material (3 or 3.5 ± 0.1 g) directly in the reaction tube. The usual weight for AO and FAD samples is 3 ± 0.1 g, but for some AO samples that have a very long induction time (> 80 h), 3.5 ± 0.1 g of sample is weighed for the glass cannula immerse deep enough into the sample throughout the determination.

5.4 The temperature defined in the method has to be reached before the reaction tube is inserted in the heating block (at this moment the Rancimat screen will go from red to green color). Connect the reaction tube to the air supply and to the measuring system as represented in Fig. S11.1 (first connect the FPE tube and then the silicone one), introduce the reaction tube into the heater and immediately press the start button to start the data recording. When mounting the system remember that the PTFE cannula must be positioned such as to avoid air bubbles directly reaching the conductivity electrode (Fig. S11.2). Air bubbles at the electrode produce noisy measuring curves that are difficult to evaluate.

5.5 Once the endpoint of the determination is reached, press stop in the StabNet software, disconnect the tubes from the system and remove them from the heating block. Then, the air stream can be turned off (never turn off the air stream before the tubes have been removed and disconnected from the system because the sample could be sucked in and contaminate the FPE tube and finally the equipment.

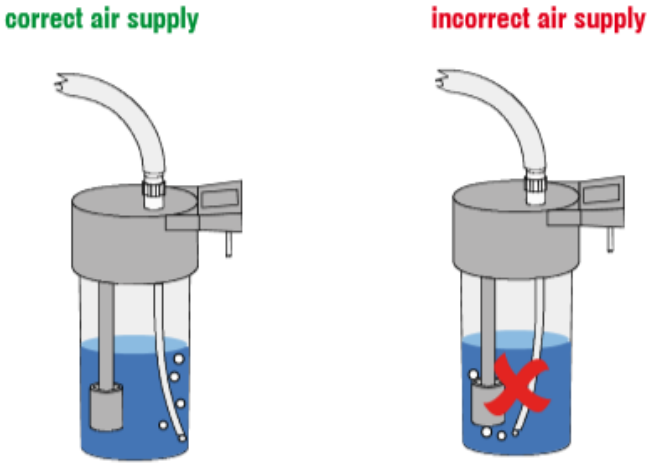


Fig. S11.2. Correct and incorrect position of the PTFE cannula with respect to the electrode [4].

6. CLEANING

Dispose used glass reaction tubes and glass cannulas and use new ones for the next determination. If there is no lipid polymerization during the determination that makes difficult the cleaning, the reaction tubes are cleaned and reused a maximum of two times.

The reaction tubes, measuring vessels and all the accessories represented in Fig. S11.1, except the fluorinated ethylene propylene (FEP) tube, are cleaned as follows:

- First remove the excess of dirt by hand cleaning with dishwashing detergent and suitable laboratory brushes, this is particularly important in the polyvinylidene fluoride (PVDF) tubing adapters, reaction cover tube, reaction tube, foam barrier, silicone tube and polytetrafluoroethylene (PTFE) cannula.
- Afterwards, all the material, except the O-ring and the measuring vessel cover, is cleaned at the dishwasher at 80 ºC. The O-ring and the measuring vessel cover with the incorporated electrode are thoroughly cleaned by hand with dishwashing detergent. To clean the electrode more easily, the protective ring can be removed (the protective ring is cleaned at the dishwasher).
- Afterwards, all the material, except the polystyrene measuring vessel, is rinsed with acetone and thoroughly with Milli-Q® water. The acetone cannot be used for cleaning the measuring vessel because it degrades the polystyrene.
- The following pieces must be dried in the oven at 80 ºC for 2 hours: PVDF tubing adapters, reaction tube cover, nitrile O-ring, glass reaction tube, foam barrier, silicone tube and PTFE cannula. The plastic pieces of the previous list can absorb reaction products during the measurement and to avoid carry over to the next measurement, it is important to dry these accessories at 80 ºC for 2 h.
- The measuring vessel cover with the incorporated electrode and the measuring vessel are dried at room temperature. The FEP tube should only be cleaned if it gets dirty by error (see point 5.5 in the procedure).

7. OPERATION AND MAINTENANCE

The air flow in the 892 Professional Rancimat is usually supplied using the internal air pump, which aspirates laboratory air. For air purification, the following accessories must be checked:

- The molecular sieve serves to adsorb interfering water from the aspirated air. Each 6-8 weeks it is necessary to change it. It is possible to regenerate it in the drying oven at 160 °C for 24 h.
- The dust filter serves to filter the air aspirated through the air pump and must be checked at periodic intervals and replaced in the case of intense contamination (the filter contamination level can be evaluated through the color of the filter in the air intake side).

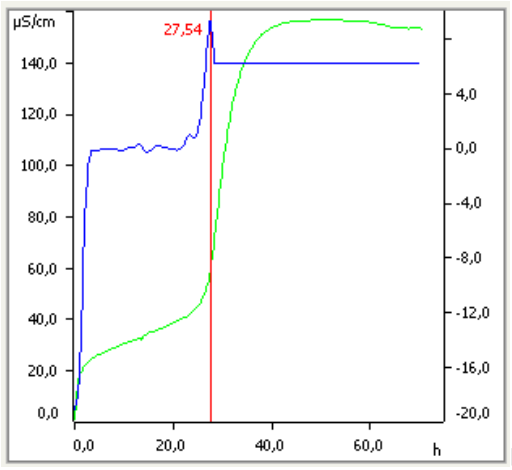
8. CALCULATIONS

The computer software determines the break point of the conductivity curve by using the maximum of the second derivative of the curve and gives the induction time expressed in hours. Below some graphs corresponding to AO and FAD samples are shown (Figs. S11.3 and S11.4). The determination of the induction time has been attempted in a total of 92 samples (79 AO and 13 FAD samples), but in 10 AO samples the conductivity curve did not show a clear jump and the induction time could not be determined.

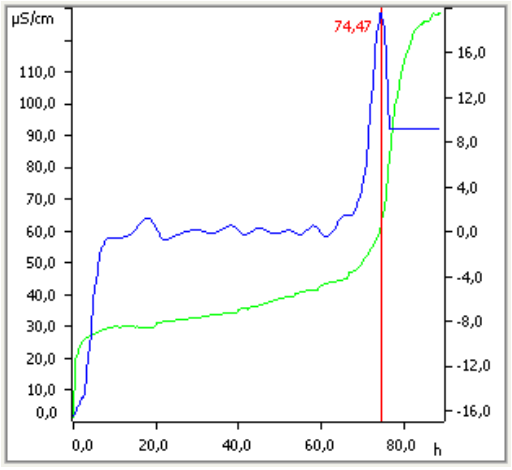
All samples were determined in duplicate. Take as the result the arithmetic mean of the two replicates.

The repeatability of the determination is good since the relative standard deviation (RSD%) was calculated for the duplicates of the 82 samples determined and the median of the RSD was 2.33%

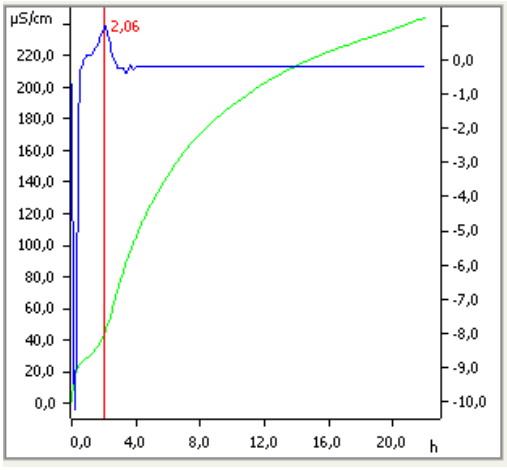
A)



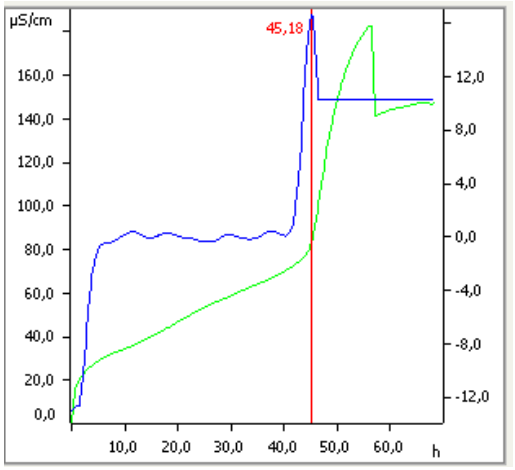
B)



C)



D)



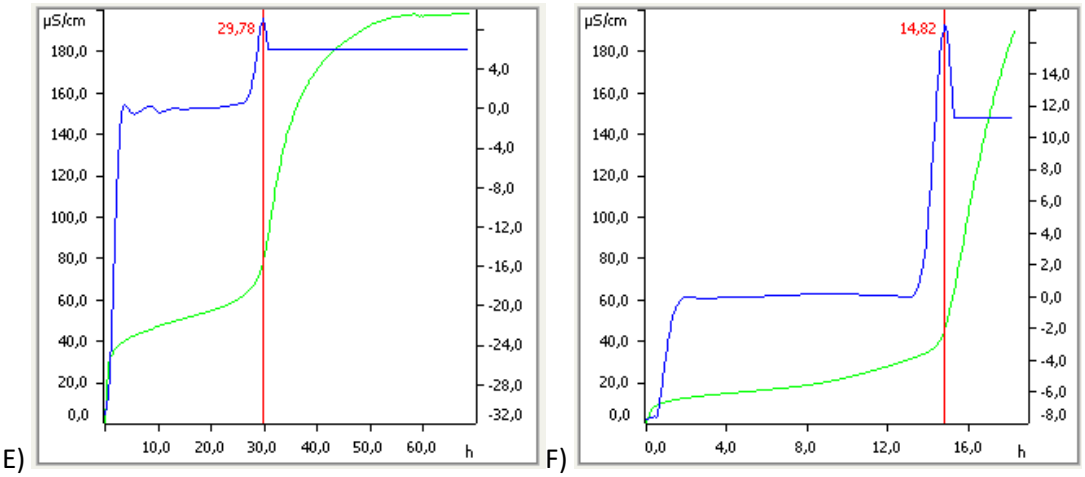


Fig. S11.3. Examples of the conductivity curve (green), its second derivative (blue) and its induction time (red) for some acid oil (AO) samples: A) Blend of AO from seed oils, cocoa butter and palm oil; B) Blend of AO from seed and palm oils; C) AO from olive pomace oil ; D) Blend of AO mainly from seed oils (sunflower, high oleic sunflower, soybean, corn and olive pomace oils); E) AO from sunflower oil; F) Blend of AO from sunflower and soybean oils.

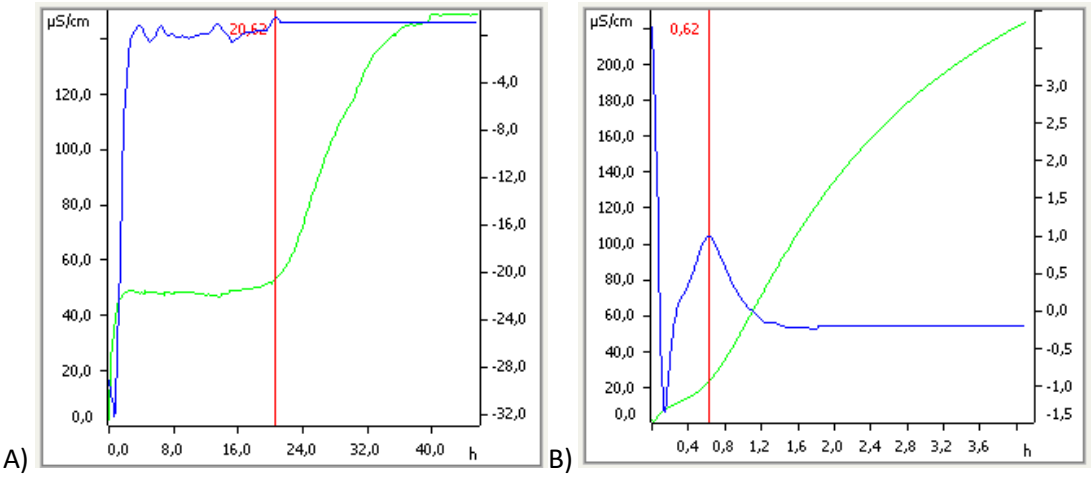


Fig. S11.4. Examples of the conductivity curve (green), its second derivative (blue) and its induction time (red) for some fatty acid distillate (FAD) samples: A) FAD from coconut and palm kernel oils; B) FAD from palm oil.

9. REFERENCES

[1] AOCS official method Cd 12b-92, Oil Stability Index (OSI). Official Methods and Recommended Practices of the American Oil Chemists' Society, 7th ed. AOCS Press, Champaign, IL. 2017.

[2] International Standard ISO 6886:2016. Animal and vegetable fats and oils, determination of oxidative stability (accelerated oxidation test). 3rd ed. 2016.

[3] 892 Professional Rancimat Manual (8.892.8001EN, 2020). Metrohm AG, Herisau, Switzerland. Accessed, 14 August 2020. https://www.metrohm.com/es/documents/88928001.

[4] Metrohm. Tips for measuring the oxidation stability with Rancimats (MI-2008-1-TT-1). Metrohm information Issue 1/2008. Accessed, 14 August 2020. https://www.metrohm.com/en-th/applications/%7B65EBDFA3-8F74-409D-A422-106E4C5E791F%7D.

8.2. Información suplementaria (supplementary material) de la publicación "Composition and nutritional value of acid oils and fatty acid distillates used in animal feeding"

Varona, E., Tres, A., Rafecas, M., Vichi, S., Barroeta, A.C., Guardiola, F. *Animals* 2021, 11, 196, doi:10.3390/ani11010196

Table S1.

Median values for the individual tocopherols, tocotrienols and vitamin E content (mg/kg) according to the refining process and botanical origin.

Botanical origin		α-T	β-TF	ϒ-TF	δ-TF	α-T3	β-T3	ϒ-T3	δ-T3	T	T3	T+ T3	Vitamin E[1]
Chemical refining (AO, n = 79)	SCP	320.9^{a}	11.2^{b}	222.3^{b}	20.7^{b}	7.6^{b}	ND	15.9^{b}	ND	576.3^{b}	23.9^{b}	598.6^{b}	353.6^{a}
	SP	340.3^{ab}	26.4^{c}	237.7^{b}	249.3^{c}	21.5^{b}	ND	43.9^{b}	17.8^{b}	839.1^{c}	83.2^{b}	1081.2^{c}	434.7^{ab}
	O	449.3^{ab}	9.1^{a}	17.2^{a}	ND	ND	ND	ND	ND	477.1^{a}	ND	477.1^{a}	455.0^{a}
	BS	591.9^{bc}	31.0^{c}	236.6^{b}	28.3^{bc}	29.6^{b}	ND	26.2^{b}	ND	1211.4^{c}	55.8^{b}	1239.7^{c}	753.2^{bc}
	SU	883.9^{c}	51.5^{c}	398.5^{b}	115.9^{c}	24.1^{b}	ND	31.3^{b}	ND	1676.5^{c}	55.4^{b}	1699.2^{c}	942.0^{c}
	SU-SO	470.4^{abc}	34.4^{c}	347.3^{b}	195.5^{c}	0.0^{b}	ND	0.0^{b}	ND	1035.1^{c}	0.0^{b}	1098.9^{c}	509.2^{ab}
	SO	578.9^{abc}	36.1^{c}	427.7^{b}	279.5^{c}	7.3^{b}	4.39^{b}	6.3^{b}	ND	1322.1^{c}	18.1^{b}	1340.2^{c}	641.4^{ab}
	p values[2]	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Physical refining (FAD, n = 13)	LFAD	134.7	3.6	39.9	14.7^{b}	90.0	ND	136.9	20.8	186.2	247.7	433.9	168.8
	PFAD	35.2	2.7	2.3	2.6^{a}	21.5	17.5^{b}	78.6	61.6	44.3	184.5	234.2	45.4
	OFAD	94.3	10.5	28.5	12.8^{ab}	ND	ND	0.5	ND	145.9	0.5	146.4	101.4
	p values[3]	0.514	0.076	0.094	0.024	0.231	0.017	0.423	0.152	0.241	0.429	0.676	0.514
	p values[4]	0.000	0.000	0.000	0.026	0.220	0.000	0.125	0.000	0.000	0.065	0.000	0.000

Abbreviations: T, sum of α-, β-, γ- and δ-tocopherol; T3, sum of α-, β-, γ- and δ-tocotrienol; T+T3, sum of tocopherols and tocotrienols; ND, not detected; SCP, blends of AO from seed oils, cocoa butter and palm oil; SP, blends of AO from seed and palm oils; O, AO from olive pomace oil and blends of AO from olive pomace and olive oils; BS, blends of AO from seed oils; SU, AO from sunflower oil; SU-SO, blends of AO from sunflower and soybean oils; SO, AO from soybean oil; LFAD, FAD from coconut oil and blends of FAD from coconut and palm kernel oils; PFAD, FAD from palm oil; OFAD, FAD from olive pomace and olive oils.

[1] The total vitamin E activity (expressed as mg of α-tocopherol/kg) was calculated using the activity conversion factors given by McLaughlin and Weihrauch (1979) for each T and T3.

[2] *p* values were obtained from Kruskal-Wallis U test and post-hoc comparisons for independent samples to compare medians between botanical origin groups within acid oils (AO, n = 79). AO groups bearing different letters (a-c) are significantly different ($p \leq 0.05$).

[3] *p* values were obtained from Kruskal-Wallis U test and post-hoc comparisons for independent samples to compare medians between botanical origin groups within fatty acid distillates (FAD, n = 13). FAD groups bearing different letters (a-b) are significantly different ($p \leq 0.05$).

[4] *p* values were obtained from Mann Whitney U test for independent samples to compare medians between refining process (AO, n = 79 vs FAD, n = 13). $p \leq 0.05$ was considered significant.

Reference:

- Mclaughlin, P.J.; Weihrauch J.L. Vitamin E content of foods. J. Am. Diet Assoc. **1979**, 75, 647-665.

Table S2.

Median values of apparent metabolizable energy (AME, kcal/kg) for broilers and digestible energy (DE, kcal/kg) for pigs of different ages (young and adult) of acid oils and fatty acid distillates. As described in the Material and Methods section of the main article, AME and DE values were obtained according to Wiseman et al. (1998) equation and by applying the MIU (g/100g) correction to it as suggested by Bierinckx (2020).

		Wiseman et al. (1998) equation				Energy corrected by MIU (g/100g)			
		AME (broilers)		DE (pigs)		AME (broilers)		DE (pigs)	
		Young	Adult	Young	Adult	Young	Adult	Young	Adult
Chemical refining (AO, n = 79)	SCP	5691.1^{a}	7141.9^{a}	7211.8^{a}	7440.4^{a}	5423.5^{a}	6806.1^{a}	6872.8^{a}	7090.6^{a}
	SP	6853.6^{b}	7776.4^{b}	7838.1^{b}	7968.3^{b}	6581.4^{b}	7476.0^{bc}	7555.5^{b}	7637.4^{b}
	O	7153.1^{c}	7941.0^{c}	7989.1^{c}	7972.9^{bc}	6610.0^{bc}	7336.8^{b}	7378.0^{b}	7385.6^{b}
	BS	7574.4^{d}	8221.7^{d}	8221.6^{d}	8169.2^{cd}	6813.7^{bcd}	7401.7^{bc}	7403.4^{b}	7363.6^{ab}
	SU	7621.7^{d}	8256.1^{d}	8244.5^{d}	8159.5^{d}	6872.1^{cd}	7483.5^{bc}	7487.1^{b}	7405.0^{b}
	SU-SO	7583.3^{d}	8227.6^{d}	8226.9^{d}	8168.0^{d}	6943.0^{d}	7634.5^{c}	7653.2^{b}	7589.5^{b}
	SO	7357.2^{cd}	8078.8^{cd}	8099.4^{cd}	8034.9^{bcd}	6827.8^{bcd}	7498.5^{bc}	7518.0^{b}	7458.8^{b}
p values[1]		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Physical refining (FAD, n = 13)	LFAD	5918.9^{b}	7244.5^{b}	7333.1^{b}	7555.3^{b}	5747.3^{b}	7045.1^{b}	7132.3^{b}	7350.3^{b}
	PFAD	4932.8^{a}	6683.3^{a}	6794.8^{a}	7013.8^{a}	4560.7^{a}	6216.8^{a}	6327.0^{a}	6530.0^{a}
	OFAD	7166.0^{b}	7968.0^{b}	7985.6^{b}	7835.5^{b}	6631.3^{b}	7374.5^{b}	7391.4^{b}	7253.3^{b}
p values[2]		0.006	0.006	0.006	0.006	0.006	0.006	0.006	0.008
p values[3]		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

See Table 1 in the main article for abbreviations.

[1] *p* values were obtained from Kruskal-Wallis U test and post-hoc comparisons for independent samples to compare medians between botanical origin groups within acid oils (AO, n = 79). AO groups bearing different letters (a-d) are significantly different ($p \leq 0.05$).

[2] *p* values were obtained from Kruskal-Wallis U test and post-hoc comparisons for independent samples to compare medians between botanical origin groups within fatty acid distillates (FAD, n = 13). FAD groups bearing different letters (a-c) are significantly different ($p \leq 0.05$).

[3] *p* values were obtained from Mann Whitney U test for independent samples to compare medians between refining processes.

References:

- Wiseman, J.; Powles J.; Salvador, F. Comparison between pigs and poultry in the prediction of the dietary energy value of fats. *Anim. Feed Sci. Technol*. **1998**, 71, 1-9. doi: 10.1016/S0377-8401(97)00142-9

- Bierinckx K. Variation in dietary lipids potentially influences your poultry production. *Int. Poult. Prod.* **2020**, 28, 27-28. Available online: http://www.positiveaction.info/pdfs/articles/pp28_3p27.pdf.

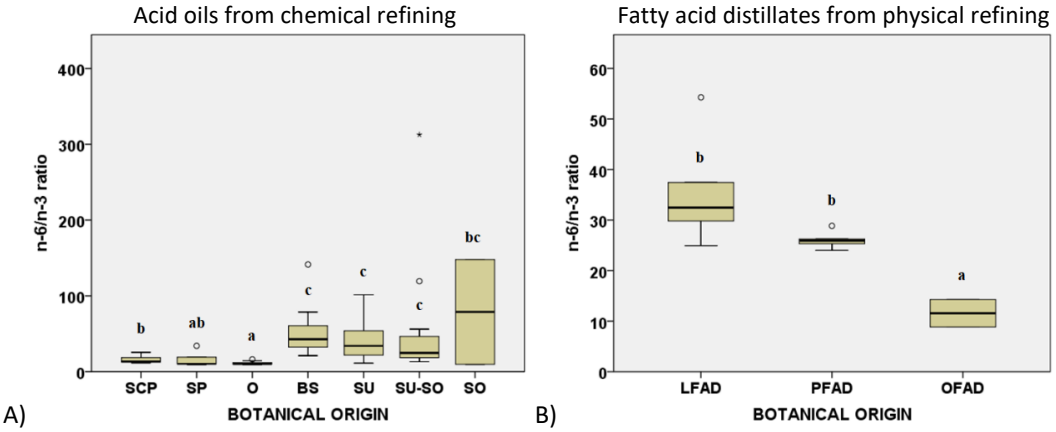


Figure S1. n-6 Polyunsaturated/n-3 polyunsaturated fatty acid ratio (n-6/n-3 ratio) boxplots according to botanical groups for acid oils from chemical refining (n = 79) and fatty acid distillates from physical refining (n = 13). Within each type of refining, botanical groups bearing different letters (a-e) are significantly different according to Kruskal-Wallis test and post-hoc comparisons ($p \leq 0.05$). See Table S1 for botanical group abbreviations.

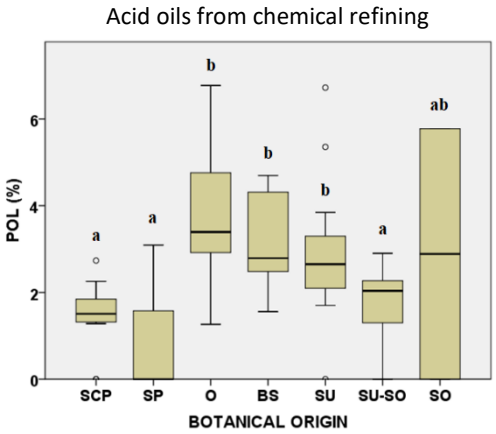


Figure S2. Polymeric compounds (POL) boxplots according to botanical groups for acid oils from chemical refining (n = 79). Botanical groups bearing different letters (a-b) are significantly different according to Kruskal-Wallis test and post-hoc comparisons ($p \leq 0.05$). See Table S1 for botanical group abbreviations. No POL were detected in FAD samples.

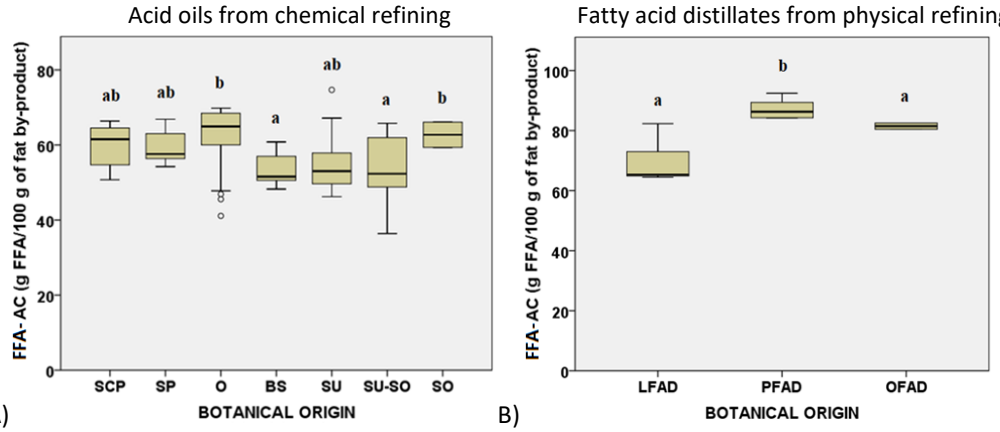


Figure S3. Acidity (FFA-AC, free fatty acids –FFA- were expressed as lauric acid for the FAD coming from coconut and palm kernel oils, as palmitic acid for the palm FAD, and as oleic acid for the rest of samples) boxplots according to botanical groups for A) acid oils from chemical refining (n = 79) and B) fatty acid distillates from physical refining (n = 13). Within each type of refining, botanical groups bearing different letters (a-b) are significantly different according to Kruskal-Wallis test and post-hoc comparisons ($p \leq 0.05$). See Table S1 for botanical group abbreviations.

8.3. Información suplementaria (supplementary material) de la publicación "Oxidative quality of acid oils and fatty acid distillates used in animal feeding"

Varona, E., Tres, A., Rafecas, M., Vichi, S., Sala, R., Guardiola, F. *Animals* 2021, 11, 2559, doi:10.3390/ani11092559

Table S1.

Median values for the individual fatty acids identified and quantified by internal normalization (peak area percentage) according to the type of by-product and the botanical origin.

By-product	Botanical origin	C6:0	C8:0	C10:0	C12:0	C14:0	C16:0	C16:1 n-9	C16:1 n-7	C17:0	C18:0	C18:1 *trans*	C18:1 n-9	C18:1 n-7	C18:2 n-6	C20:0	C18:3 n-3	C20:1 n-9	C20:2 n-6	C22:0	C22:2	C24:0
Acid oils (AO, n = 79)	SCP	0.00^{a}	0.01^{a}	0.01^{a}	0.02^{bc}	0.15^{c}	21.86^{d}	0.05^{b}	0.35^{b}	0.20^{c}	23.38^{c}	0.48^{ab}	37.26^{c}	0.96^{a}	12.67^{b}	0.98^{c}	0.90^{a}	0.18^{a}	0.00^{ab}	0.41^{a}	0.00	0.29^{a}
	SP	0.02^{b}	0.09^{c}	0.04^{c}	0.25^{d}	0.38^{c}	18.25^{c}	0.05^{ab}	0.17^{a}	0.10^{ab}	5.34^{ab}	0.31^{ab}	43.94^{c}	1.24^{b}	24.87^{c}	0.45^{a}	1.77^{b}	0.28^{b}	0.00^{a}	0.47^{b}	0.00	0.31^{ab}
	O	0.01^{b}	0.02^{bc}	0.01^{b}	0.01^{a}	0.06^{a}	13.20^{b}	0.13^{c}	0.75^{c}	0.11^{b}	3.63^{a}	1.66^{c}	64.14^{d}	1.95^{c}	10.83^{a}	0.56^{a}	1.05^{ab}	0.32^{c}	0.09^{b}	0.40^{a}	0.00	0.55^{c}
	BS	0.00^{ab}	0.03^{bc}	0.02^{bc}	0.02^{d}	0.10^{b}	13.06^{ab}	0.06^{b}	0.16^{a}	0.09^{a}	3.41^{ab}	0.66^{abc}	33.24^{bc}	1.04^{ab}	45.81^{d}	0.54^{a}	0.90^{ab}	0.24^{b}	0.00^{ab}	0.66^{bc}	0.00	0.42^{bc}
	SU	0.07^{b}	0.03^{c}	0.02^{c}	0.03^{cd}	0.12^{b}	9.81^{a}	0.07^{b}	0.19^{a}	0.09^{a}	3.87^{ab}	1.01^{bc}	31.66^{b}	1.20^{b}	46.14^{d}	0.79^{b}	1.26^{b}	0.25^{b}	0.00^{ab}	0.93^{d}	0.00	0.50^{c}
	SU-SO	0.00^{ab}	0.02^{bc}	0.01^{ab}	0.01^{ab}	0.11^{b}	11.44^{a}	0.03^{a}	0.12^{a}	0.10^{ab}	4.07^{b}	0.44^{a}	22.36^{a}	1.14^{b}	52.70^{e}	0.54^{a}	1.99^{b}	0.19^{a}	0.00^{a}	0.80^{cd}	0.00	0.44^{bc}
	SO	0.00^{ab}	0.01^{ab}	0.01^{ab}	0.01^{a}	0.10^{b}	11.51^{ab}	0.03^{a}	0.11^{a}	0.09^{ab}	4.09^{ab}	0.55^{ab}	25.17^{a}	1.24^{ab}	51.79^{e}	0.68^{ab}	2.83^{b}	0.19^{ab}	0.01^{ab}	0.93^{cd}	0.00	0.45^{bc}
p values[1]		0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.021	0.000	0.006	0.000	0.108	0.000
Fatty acid distillates (FAD, n = 13)	LFAD	0.53^{b}	6.60^{b}	5.55^{c}	33.50^{c}	18.25^{c}	15.92^{a}	0.01^{a}	0.06^{a}	0.03^{a}	4.58	0.15	9.58^{a}	0.18^{a}	2.52^{a}	0.11^{a}	0.08^{a}	0.05^{a}	0.00	0.03^{a}	0.00	0.02^{a}
	PFAD	0.00^{a}	0.03^{a}	0.02^{b}	0.22^{b}	1.16^{b}	46.31^{b}	0.03^{b}	0.16^{b}	0.11^{b}	4.53	0.21	35.98^{b}	0.64^{b}	8.93^{b}	0.34^{b}	0.35^{b}	0.13^{b}	0.01	0.06^{a}	0.00	0.04^{ab}
	OFAD	0.00^{a}	0.01^{a}	0.00^{a}	0.01^{a}	0.05^{a}	11.78^{a}	0.14^{c}	1.31^{c}	0.07^{a}	1.86	0.22	69.75^{c}	2.83^{c}	10.13^{c}	0.29^{ab}	0.95^{c}	0.29^{c}	0.09	0.10^{b}	0.00	0.05^{b}
p values[2]		0.004	0.008	0.006	0.006	0.006	0.008	0.006	0.006	0.006	0.094	0.493	0.006	0.006	0.006	0.011	0.006	0.006	0.056	0.036	1.000	0.049
AO (n = 79)		0.00	0.02^{a}	0.01^{a}	0.02^{a}	0.11^{a}	13.05^{a}	0.05^{b}	0.30^{b}	0.10	3.88	0.66^{b}	36.57	1.16^{b}	36.83^{b}	0.59^{b}	1.05^{b}	0.23^{b}	0.00	0.55^{b}	0.00	0.45^{b}
FAD (n = 13)		0.00	0.03^{b}	0.03^{b}	0.29^{b}	1.21^{b}	18.90^{b}	0.03^{a}	0.16^{a}	0.08	4.50	0.21^{a}	35.68	0.61^{a}	8.49^{a}	0.27^{a}	0.34^{a}	0.12^{a}	0.01	0.04^{a}	0.00	0.03^{a}
p values[3]		0.433	0.029	0.001	0.000	0.000	0.001	0.001	0.008	0.079	0.594	0.000	0.173	0.000	0.000	0.000	0.000	0.000	0.388	0.000	0.477	0.000

Abbreviations: SCP, blends of AO from seed oils, cocoa butter and palm oil; SP, blends of AO from seed and palm oils; O, AO from olive pomace oil and blends of AO from olive pomace and olive oils; BS, blends of AO from seed oils; SU, AO from sunflower oil; SU-SO, blends of AO from sunflower and soybean oils; SO, AO from soybean oil; LFAD, FAD from coconut oil and blends of FAD from coconut and palm kernel oils; PFAD, FAD from palm oil; OFAD, FAD from olive pomace and olive oils. [1] *p* values were obtained from Kruskal-Wallis U test. Stepwise Multiple Comparisons for independent samples were used to compare medians between botanical origin groups within acid oils (AO, n = 79). AO groups bearing different letters (a-d) were significantly different ($p \leq 0.05$). [2] *p* values were obtained from Kruskal-Wallis U test. Stepwise Multiple Comparisons for independent samples were used to compare medians between botanical origin groups within fatty acid distillates (FAD, n = 13). FAD groups bearing different letters (a-c) were significantly different ($p \leq 0.05$). [3] *p* values were obtained from Mann Whitney U test for independent samples to compare medians between AO and FAD (AO, n = 79; FAD, n = 13). Groups bearing different letters (a-b) were significantly different ($p \leq 0.05$).

Table S2.

Mean, standard deviation, median, minimum and maximum values for the different sums of fatty acids identified and quantified by peak area normalization (%) according to the botanical origin of the acid oils (AO).

		SFA (%)	*trans*-C18:1	*cis*-MUFA (%)	n-6 PUFA (%)	n-3 PUFA (%)	UFA/SFA ratio
SCP	Mean ± SD	46.4 ± 3.04	0.6 ± 0.32	39.2 ± 1.59	12.9 ± 1.40	0.9 ± 0.16	2.7 ± 1.72
	Median	47.2^{d}	0.5^{ab}	38.8^{c}	12.7^{b}	0.9^{a}	2.7
	Min	39.1	0.2	37.6	11.2	0.6	0.9
	Max	49.1	1.4	43.3	15.5	1.0	4.5
SP	Mean ± SD	29.8 ± 6.36	0.6 ± 0.61	43.8 ± 3.27	24 ± 4.79	1.9 ± 1.02	2.5 ± 1.31
	Median	27.3^{c}	0.3^{ab}	45.5^{c}	24.9^{c}	1.8^{b}	2.4
	Min	22.8	0.1	39.1	18.4	0.7	1.0
	Max	39.2	1.4	46.7	30.8	3.3	4.1
O	Mean ± SD	18.8 ± 0.66	1.6 ± 0.89	67.3 ± 1.13	11.3 ± 1.00	1 ± 0.09	4.3 ± 1.31
	Median	18.6^{b}	1.7^{c}	67.4^{d}	10.9^{a}	1.1^{ab}	4.7
	Min	18.1	0.3	65.5	10.2	0.8	0.9
	Max	20.2	2.9	69.5	14.2	1.2	5.5
BS	Mean ± SD	17.7 ± 2.68	0.9 ± 0.54	39.3 ± 10.36	41.1 ± 11.83	1 ± 0.42	3.3 ± 1.88
	Median	18.2^{ab}	0.7^{abc}	34.9^{bc}	45.8^{d}	0.9^{ab}	4.1
	Min	14.2	0.2	30.1	18.9	0.4	1.1
	Max	22.5	1.6	59.5	53.2	1.6	6.1
SU	Mean ± SD	17.2 ± 1.23	1.2 ± 0.98	35 ± 6.98	45 ± 7.12	1.6 ± 1.05	3.9 ± 1.61
	Median	17.1^{a}	1^{bc}	33.4^{b}	46.2^{d}	1.3^{b}	4.4
	Min	15.0	0.1	26.7	24.7	0.4	0.2
	Max	19.2	4.5	55.8	54.5	3.7	5.5
SU-SO	Mean ± SD	17.9 ± 1.39	0.4 ± 0.36	27.7 ± 5.45	51.9 ± 5.27	2.2 ± 1.31	3 ± 2.27
	Median	17.6^{ab}	0.4^{a}	23.4^{a}	52.7^{e}	2.0^{b}	3.6
	Min	16.0	0.1	22.7	41.6	0.2	0.1
	Max	20.9	1.1	37.6	59.2	4.0	5.9
SO	Mean ± SD	18.1 ± 2.56	0.6 ± 0.73	26.7 ± 5.21	51.8 ± 0.12	2.8 ± 3.51	5 ± 0.00
	Median	18.1^{ab}	0.6^{ab}	26.7^{a}	51.8^{e}	2.8^{ab}	5.00
	Min	16.3	0.0	23.1	51.7	0.4	5.00
	Max	19.9	1.1	30.4	51.9	5.3	5.00
p values[1]		<0.001	0.001	<0.001	<0.001	0.021	0.057

Abbreviations: SCP, blends of AO from seed oils, cocoa butter and palm oil; SP, blends of AO from seed and palm oils; O, AO from olive pomace oil and blends of AO from olive pomace and olive oils; BS, blends of AO from seed oils; SU, AO from sunflower oil; SU-SO, blends of AO from sunflower and soybean oils; SO, AO from soybean oil; SFA, saturated fatty acids; cis-MUFA, cis-monounsaturated fatty acids; cis-PUFA, cis-polyunsaturated fatty acids; UFA/SFA ratio, unsaturated/saturated ratio. [1] *p* values were obtained from Kruskal-Wallis U test. Stepwise Multiple Comparisons for independent samples were used to compare medians between botanical origin groups within acid oils (AO, n = 79). AO groups bearing different letters (a-d) were significantly different ($p \leq 0.05$).

Table S3.

Mean, standard deviation, median, minimum and maximum values for the different sums of fatty acids identified and quantified by peak area normalization (%) according to the botanical origin of the fatty acid distillates (FAD).

		SFA (%)	*trans*-C18:1	*cis*-MUFA (%)	n-6 PUFA (%)	n-3 PUFA (%)	UFA/SFA ratio
LFAD	Mean ± SD	86.3 ± 1.4	0.2 ± 0.16	10.8 ± 1.57	2.6 ± 0.24	0.1 ± 0.02	3.9 ± 3.40
	Median	87.1[c]	0.1	9.9[a]	2.5[a]	0.1[a]	4.7
	Min	84.3	0.1	9.6	2.3	0.0	0.2
	Max	87.6	0.5	13.2	2.9	0.1	6.8
PFAD	Mean ± SD	53 ± 1.78	0.2 ± 0.07	37.6 ± 1.84	8.9 ± 0.31	0.3 ± 0.02	4.1 ± 0.55
	Median	53.4[b]	0.2	37[b]	8.9[b]	0.3[b]	4.2
	Min	50.0	0.2	35.9	8.5	0.3	3.4
	Max	55.1	0.3	41.0	9.3	0.4	4.6
OFAD	Mean ± SD	14.3 ± 2.14	0.2 ± 0.01	74.3 ± 3.44	10.2 ± 0.89	0.9 ± 0.39	2.6 ± 2.42
	Median	14.3[a]	0.2	74.3[c]	10.2[c]	0.9[c]	2.6
	Min	12.8	0.2	71.9	9.6	0.7	0.9
	Max	15.8	0.2	76.8	10.9	1.2	4.3
p values[1]		0.006	0.493	0.006	0.006	0.006	0.607

Abbreviations: LFAD, FAD from coconut oil and blends of FAD from coconut and palm kernel oils; PFAD, FAD from palm oil; OFAD, FAD from olive pomace and olive oils; SFA, saturated fatty acids; cis-MUFA, cis-monounsaturated fatty acids; cis-PUFA, cis-polyunsaturated fatty acids. [1] *p* values were obtained from Kruskal-Wallis U test. Stepwise Multiple Comparisons for independent samples were used to compare medians between botanical origin groups within fatty acid distillates (FAD, n = 13). AO groups bearing different letters (a-c) were significantly different ($p \leq 0.05$).

8.4. Información suplementaria (supplementary material) de la publicación "Effect of processing and storage conditions on lipid hydrolysis and oxidative stability of feeds containing various fats and fat by-products"

Varona, E., Tres, A., Vidal, V.A., Rafecas, M., Vichi, S., Solà-Oriol, D., Guardiola, F. *Anim. Feed Sci. Tech*. En preparación.

Table S1

Effect of the fat source and feed processing on the volatile compound contents of the freshly prepared feeds (0 days of storage, n = 18).

	Fat source[1]								Feed processing				
	CS	AS	RS	CP	PFAD	RP	SE[2]	*P*[3]	Mash	Pelleted	Extruded	SE	*P*
Pentanal (mg/kg)	0.519	0.591	0.333	0.314	0.468	0.254	0.113	0.318	0.107^{a}	0.692^{b}	0.441^{b}	0.080	**0.001**
Hexanal (mg/kg)	2.548	2.956	2.256	1.521	1.599	1.436	0.688	0.556	1.523	3.011	1.624	0.487	0.100
Heptanal (mg/kg)	0.055	0.070	0.064	0.046	0.062	0.050	0.017	0.906	0.053	0.076	0.045	0.012	0.216
Octanal (mg/kg)	0.007^{a}	0.010^{a}	0.003^{a}	0.006^{a}	0.025^{b}	0.005^{a}	0.002	**0.001**	0.007^{a}	0.014^{b}	0.008^{ab}	0.002	**0.035**
Nonanal (mg/kg)	0.006^{a}	0.011^{a}	0.005^{a}	0.009^{a}	0.034^{b}	0.006^{a}	0.002	**0.000**	0.009^{a}	0.017^{b}	0.009^{a}	0.002	**0.013**
Decanal (mg/kg)	0.014	0.009	0.025	0.007	0.013	0.006	0.007	0.484	0.020	0.014	0.003	0.005	0.110
2-Heptenal (mg/kg)	0.086	0.108	0.072	0.030	0.029	0.033	0.024	0.159	0.085	0.056	0.038	0.017	0.186
2-Decenal (mg/kg)	0.003	0.002	0.003	0.002	0.007	0.002	0.001	0.198	0.005	0.002	0.002	0.001	0.053
2,4-Heptadienal (mg/kg)	0.023	0.045	0.002	0.002	0.003	0.001	0.012	0.144	0.002	0.022	0.014	0.009	0.293
2,4-Nonadienal (mg/kg)	0.005^{ab}	0.011^{ab}	0.004^{ab}	0.002^{a}	0.013^{b}	0.003^{a}	0.002	**0.013**	0.005	0.008	0.006	0.001	0.333
2,4-Decadienal (mg/kg)	0.154	0.462	0.086	0.083	0.107	0.042	0.113	0.187	0.091	0.131	0.245	0.080	0.401
Hexane (mg/kg)	0.199	0.194	0.264	0.271	0.252	0.288	0.037	0.407	0.452^{b}	0.151^{a}	0.131^{a}	0.026	**0.000**
Decane (mg/kg)	0.007	0.011	0.008	0.003	0.003	0.003	0.002	0.140	0.010^{b}	0.005^{ab}	0.003^{a}	0.002	**0.036**
1-Penten-3-one (mg/kg)	0.194	0.113	0.195	0.052	0.010	0.081	0.071	0.406	0.286^{b}	0.014^{a}	0.022^{a}	0.050	**0.005**
2-Heptanone (mg/kg)	0.058	0.113	0.194	0.037	0.113	0.034	0.080	0.706	0.099	0.052	0.123	0.056	0.675
2-Octanone (mg/kg)	0.022^{ab}	0.059^{b}	0.013^{a}	0.016^{ab}	0.033^{ab}	0.009^{a}	0.009	**0.022**	0.059^{b}	0.010^{a}	0.006^{a}	0.006	**0.000**
3-Octanone (mg/kg)	0.046	0.343	0.189	0.240	0.227	0.103	0.069	0.117	0.257	0.182	0.135	0.049	0.248
1-Octen-3-one (mg/kg)	0.066	0.059	0.049	0.039	0.051	0.043	0.008	0.209	0.128^{b}	0.012^{a}	0.014^{a}	0.005	**0.000**
1-Pentanol (mg/kg)	0.256	0.076	0.079	0.082	0.243	0.180	0.070	0.280	0.310^{b}	0.081^{a}	0.066^{a}	0.049	**0.009**
1-Butanol (mg/kg)	0.010^{a}	0.010^{a}	0.009^{a}	0.011^{a}	0.043^{b}	0.007^{a}	0.005	**0.002**	0.017	0.014	0.014	0.003	0.687
2-Pentenol (mg/kg)	0.046^{ab}	0.063^{b}	0.032^{a}	0.024^{a}	0.026^{a}	0.022^{a}	0.006	**0.007**	0.067^{b}	0.023^{a}	0.016^{a}	0.005	**0.000**
1-Hexanol (mg/kg)	0.484	0.940	0.455	0.890	1.447	0.467	0.332	0.302	1.970^{b}	0.242^{a}	0.129^{a}	0.235	**0.000**
1-Octen-3-ol (mg/kg)	3.490	3.023	2.728	3.023	3.045	3.130	0.306	0.670	8.713^{b}	0.318^{a}	0.189^{a}	0.216	**0.000**
1-Nonanol (mg/kg)	0.004	0.005	0.003	0.005	0.011	0.001	0.003	0.261	0.009	0.003	0.003	0.002	0.117
2-Pentylfuran (mg/kg)	0.315	0.857	0.276	0.389	0.843	0.299	0.218	0.243	0.723	0.517	0.250	0.154	0.144
2-Furanmethanol (mg/kg)	0.004	0.004	0.005	0.005	0.007	0.003	0.002	0.647	0.004^{ab}	0.002^{a}	0.008^{b}	0.001	**0.010**
Σ ALD (mg/kg)	3.420	4.275	2.853	2.022	2.359	1.838	0.914	0.453	1.905	4.044	2.435	0.646	0.097
Σ HC (mg/kg)	0.206	0.205	0.273	0.274	0.255	0.291	0.038	0.511	0.462^{b}	0.156^{a}	0.134^{a}	0.027	**0.000**
Σ KET (mg/kg)	0.386	0.688	0.639	0.384	0.434	0.270	0.091	0.057	0.830^{b}	0.271^{a}	0.300^{a}	0.065	**0.000**
Σ ALC (mg/kg)	4.289	4.119	3.306	4.035	4.815	3.807	0.500	0.461	11.087^{b}	0.681^{a}	0.417^{a}	0.354	**0.000**

Σ FUR (mg/kg)	0.319	0.861	0.281	0.394	0.849	0.302	0.218	0.243		0.727	0.519	0.258	0.154	0.149

[1] Values are pooled means from multifactor ANOVA (n = 18). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). [a-c] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: Σ ALD, Σ Aldehydes (sum of pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal and 2,4-nonadienal); Σ HC, Σ Hydrocarbons (sum of hexane and decane); Σ KET, Σ Ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one); Σ ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol, 1-octen-3-ol and 1-nonanol) and Σ FUR, Σ Furans (sum of 2-penthylfuran and 2-furanmethanol).

Table S2

P values for the effects of the interactions between two of the main factors on lipid composition and oxidation parameters, according to multifactorial ANOVA for all the experimental feeds (n = 108).

Parameters	Fat source x Feed processing	Fat source x Storage time	Fat source x Storage Temperature	Feed processing x Storage time	Feed processing x Storage Temperature	Storage Temperature x Storage time
α-T	NS	0.023	NS	0.000	NS	NS
β-T	0.000	0.002	NS	0.000	0.001	NS
Ɣ-T	0.000	0.000	NS	0.000	0.008	NS
δ-T	0.000	0.000	NS	NS	NS	NS
α-T3	0.000	0.014	NS	0.000	0.000	NS
β-T3	0.000	0.035	NS	0.030	0.026	NS
Ɣ-T3	0.000	0.000	NS	0.003	0.025	NS
δ-T3	0.000	0.000	NS	NS	NS	NS
T	0.000	0.000	NS	0.000	0.004	NS
T3	0.000	0.007	NS	0.000	0.001	NS
T+T3	0.000	0.001	NS	0.000	0.002	NS
TAG	0.000	0.035	NS	0.000	NS	NS
DAG	0.000	NS	NS	0.000	0.045	NS
MAG	0.000	NS	NS	NS	NS	NS
FFA	0.001	0.021	NS	0.000	NS	NS
PV	0.000	0.000	NS	0.006	NS	NS
Pentanal	0.005	0.007	NS	0.001	NS	NS
Hexanal	NS	NS	NS	NS	NS	NS
Σ ALD	NS	NS	NS	NS	NS	NS
Σ HC	0.000	NS	NS	0.002	NS	NS
Σ KET	NS	0.028	NS	NS	0.006	NS
Σ ALC	0.002	0.014	NS	0.000	NS	NS
Σ FUR	0.000	0.000	NS	0.000	NS	NS

Abbreviations: T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; PV, peroxide value; Σ ALD, Σ Aldehydes (sum of pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal and 2,4-nonadienal); Σ HC, Σ Hydrocarbons (sum of hexane and decane); Σ KET, Σ Ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one); Σ ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol, 1-octen-3-ol and 1-nonanol) and Σ FUR, Σ Furans (sum of 2-penthylfuran and 2-furanmethanol); NS, non-significant ($P \leq 0.05$). .

Table S3

Effect of the interaction between the fat source and the feed processing on the lipid composition and oxidation parameters of all the experimental feeds (n = 108).

	Fat source x Feed processing[1]																			
	CS			AS			RS			CP			PFAD			RP				
Parameters	Mash	Pelleted	Extruded	Mash	Pelleted	Extruded	Mash	Pelleted	Extruded	Mash	Pelleted	Extruded	Mash	Pelleted	Extruded	Mash	Pelleted	Extruded	SE[2]	*P*[3]
α-T (mg/ kg)	106.3	85.5	88.4	101.3	85.8	84.2	99.1	87.4	84.3	101.5	82.2	83.0	83.6	67.3	66.0	101.0	86.4	83.0	1.844	0.523
β-T (mg/ kg)	6.6	4.5	4.9	6.4	1.5	1.7	5.0	3.7	3.5	4.1	3.1	3.1	3.8	1.3	1.1	4.2	3.3	3.1	0.098	**0.000**
Υ-T (mg/ kg)	89.2	49.8	54.2	71.8	9.2	10.5	48.4	31.4	30.0	9.2	6.6	6.4	8.7	2.8	2.5	9.7	7.3	7.1	1.919	**0.000**
δ-T (mg/ kg)	23.1	20.4	21.4	38.9	20.8	22.5	9.9	9.5	9.2	2.4	2.3	2.2	2.6	1.4	1.5	2.6	2.3	2.3	0.375	**0.000**
α-T3 (mg/ kg)	10.8	6.5	6.7	9.8	5.9	6.5	10.1	7.3	7.1	29.3	11.1	11.1	12.2	7.9	7.6	24.1	11.1	10.8	0.367	**0.000**
β-T3 (mg/ kg)	14.5	10.7	11.1	13.0	7.2	7.8	13.5	11.4	10.8	14.0	12.3	11.5	12.1	8.2	7.6	14.5	12.6	12.1	0.282	**0.000**
Υ-T3 (mg/ kg)	1.4	0.9	1.0	1.3	0.9	0.9	1.3	1.1	1.0	22.3	15.6	14.6	2.7	2.0	1.8	14.9	10.1	9.9	0.191	**0.000**
δ-T3 (mg/ kg)	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	2.5	2.5	2.4	1.6	0.9	1.0	1.2	1.0	1.0	0.073	**0.000**
T (mg/ kg)	225.2	160.3	168.8	218.3	117.2	119.0	162.4	132.0	127.0	117.3	94.2	94.7	98.7	72.8	71.1	117.4	99.4	95.5	3.386	**0.000**
T3 (mg/ kg)	26.6	18.1	18.8	24.0	14.0	15.1	24.9	19.9	18.9	68.1	41.4	39.7	28.6	19.0	18.1	54 .6	34.9	33.8	0.705	**0.000**
T+T3 (mg/ kg)	251.9	178.4	187.6	242.3	131.3	134.1	187.3	151.9	146.0	185.4	135.6	134.4	127.3	91.9	89.2	172.1	134.2	129.3	3.905	**0.000**
TAG (%)	51.1	83.1	83.1	20.0	32.1	33.1	56.5	83.0	83.1	52.5	72.7	72.5	10.9	20.6	19.6	59.7	81.1	81.1	2.184	**0.000**
DAG (%)	13.5	8.8	9.2	12.4	14.3	14.5	12.5	9.0	9.6	14.8	13.8	14.0	5.4	5.9	5.7	13.4	11.2	11.3	0.392	**0.000**
MAG (%)	3.2	1.3	1.2	2.8	2.7	2.8	2.6	1.3	1.2	2.6	2.0	1.9	1.7	1.5	1.4	2.4	1.5	1.4	0.124	**0.000**
FFA (%)	32.1	6.8	6.5	64.0	49.6	48.6	28.4	6.6	6.1	30.2	11.5	11.6	82.1	72.1	73.3	24.5	6.3	6.2	1.742	**0.001**
PV (meq O_2/kg)	7.8	13.5	14.9	12.4	11.0	10.4	11.8	12.6	13.2	4.1	8.6	6.4	3.8	6.9	6.0	5.2	9.9	7.3	0.750	**0.000**
Pentanal (mg/kg)	0.1	0.5	0.2	0.2	0.8	0.8	0.1	0.6	0.3	0.1	0.4	0.3	0.2	0.5	0.4	0.1	0.2	0.2	0.061	**0.005**
Hexanal (mg/kg)	1.8	2.9	0.8	2.5	4.2	3.2	1.9	3.3	1.0	1.1	2.5	1.1	1.2	1.8	1.3	1.5	1.1	0.6	0.446	0.186
Σ ALD (mg/kg)	2.1	3.6	1.2	3.0	5.4	4.7	2.2	4.1	1.4	1.4	3.2	1.5	1.6	2.5	1.8	1.7	1.4	0.9	0.533	0.087
Σ HC (mg/kg)	0.3	0.1	0.1	0.2	0.1	0.1	0.5	0.2	0.1	0.3	0.2	0.1	0.4	0.1	0.1	0.5	0.2	0.1	0.034	**0.000**
Σ KET (mg/kg)	0.8	0.2	0.2	0.8	0.5	0.5	0.8	0.2	0.3	0.6	0.2	0.1	0.8	0.2	0.3	0.5	0.3	0.1	0.066	0.150
Σ ALC (mg/kg)	11.0	0.9	0.7	7.6	1.0	0.9	10.3	0.4	0.2	9.3	0.3	0.3	10.7	0.5	0.6	11.0	0.7	0.2	0.479	**0.002**
Σ FUR (mg/kg)	0.3	0.6	0.1	0.5	0.6	0.5	0.3	0.7	0.1	0.3	0.7	0.2	0.8	0.3	0.1	0.3	0.7	0.1	0.074	**0.000**

[1] Values are pooled means from multifactor ANOVA (n = 108). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; T, sum of α-, β-, γ- and δ-tocopherols; T3, sum of α-, β-, γ- and δ-tocotrienols; T + T3, sum of tocopherols and tocotrienols; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acids; PV, peroxide value; Σ ALD, Σ Aldehydes (sum of pentanal, hexanal, heptanal, octanal, 2-heptenal, nonanal, 2,4-heptadienal, decanal, 2-decenal, 2,4-decadienal and 2,4-nonadienal); Σ HC, Σ Hydrocarbons (sum of hexane and decane); Σ KET, Σ Ketones (sum of 1-penten-3-one, 2-heptanone, 3-octanone, 2-octanone and 1-octen-3-one); Σ ALC, Σ Alcohols (sum of 1-butanol, 1-pentanol, 2-pentenol, 1-hexanol, 1-octen-3-ol and 1-nonanol); Σ FUR, Σ Furans (sum of 2-penthylfuran and 2-furanmethanol); Tr, traces.

Table S4

Effect of the interaction between the fat source and the storage time on the lipid composition and oxidation parameters of all the experimental feeds (n = 108).

	Fat source x Storage time[1]																			
	CS			AS			RS			CP			PFAD			RP				
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	SE[2]	*P*[3]
α-T (mg/ kg)	102.7	95.6	81.9	100.2	90.1	81.0	98.8	89.1	82.9	92.9	93.7	80.2	77.7	72.9	66.3	100.7	90.0	79.8	1.844	**0.023**
β-T (mg/ kg)	5.8	5.4	4.8	3.5	3.2	2.8	4.5	4.0	3.7	3.5	3.5	3.2	2.2	2.0	2.0	3.7	3.6	3.3	0.098	**0.002**
Υ-T (mg/ kg)	73.8	65.1	54.4	36.7	31.2	23.5	42.9	36.3	30.5	8.0	7.5	6.7	5.2	4.6	4.2	8.9	7.9	7.2	1.919	**0.000**
δ-T (mg/ kg)	22.7	21.5	20.7	30.5	26.9	24.8	10.1	9.2	9.3	2.3	2.1	2.6	1.9	1.7	1.8	2.4	2.3	2.5	0.375	**0.000**
α-T3 (mg/ kg)	8.1	8.5	7.4	7.9	7.8	6.4	8.9	8.3	7.4	17.1	18.4	16.1	8.7	9.7	9.3	16.3	15.6	14.0	0.367	**0.014**
β-T3 (mg/ kg)	12.1	12.6	11.6	9.8	9.5	8.6	12.5	11.9	11.4	12.2	13.4	12.2	9.0	9.8	9.2	13.3	13.0	12.8	0.282	**0.035**
Υ-T3 (mg/ kg)	1.1	1.1	1.1	1.1	1.0	1.0	1.2	1.1	1.2	17.9	18.2	16.4	2.1	2.2	2.1	12.6	11.7	10.6	0.191	**0.000**
δ-T3 (mg/ kg)	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	2.1	2.4	2.9	1.2	1.1	1.2	0.9	1.1	1.3	0.073	**0.000**
T (mg/ kg)	205.0	187.5	161.8	170.9	151.5	132.1	156.3	138.6	126.5	106.8	106.7	92.6	87.0	81.3	74.3	115.7	103.8	92.8	3.386	**0.000**
T3 (mg/ kg)	21.4	22.1	20.1	18.8	18.3	16.0	22.6	21.2	19.9	49.3	52.3	47.6	21.0	22.8	21.9	43.1	41.5	38.7	0.705	**0.007**
T+T3 (mg/ kg)	226.4	209.6	181.9	189.7	169.8	148.1	178.9	159.9	146.4	156.1	159.0	140.2	108.1	104.1	96.2	158.8	145.3	131.5	3.905	**0.001**
TAG (%)	81.3	69.3	66.7	30.2	27.0	28.0	80.9	75.4	66.3	72.1	63.6	61.9	18.0	16.8	16.3	80.2	71.7	70.0	2.184	**0.035**
DAG (%)	9.2	11.2	11.2	13.7	13.6	13.9	9.6	10.3	11.2	13.3	14.6	14.6	5.4	5.8	5.8	10.9	12.5	12.5	0.392	0.210
MAG (%)	1.7	2.2	1.9	2.9	2.8	2.5	1.7	1.6	1.8	2.1	2.3	2.0	1.5	1.5	1.5	1.8	1.9	1.6	0.124	0.142
FFA (%)	7.9	17.4	20.0	52.2	55.5	54.5	7.8	12.7	20.6	12.5	19.4	21.4	75.1	75.9	76.4	7.1	13.9	15.9	1.742	**0.021**
PV (meq O_2/kg)	18.8	8.2	9.2	12.7	9.7	11.5	14.7	10.5	12.4	7.6	5.5	6.1	5.8	5.2	5.6	7.9	7.0	7.5	0.750	**0.000**
Pentanal (mg/kg)	0.5	0.1	0.2	0.6	0.5	0.6	0.3	0.3	0.3	0.3	0.2	0.2	0.5	0.4	0.2	0.3	0.1	0.1	0.061	**0.007**
Hexanal (mg/kg)	2.5	0.6	2.3	3.0	3.2	3.8	2.3	2.0	1.9	1.5	1.5	1.7	1.6	2.0	0.7	1.4	1.3	0.5	0.446	0.069
Σ ALD (mg/kg)	3.4	0.9	2.7	4.3	4.1	4.7	2.9	2.5	2.4	2.0	1.9	2.1	2.4	2.6	1.0	1.8	1.5	0.7	0.533	0.095
Σ HC (mg/kg)	0.2	0.1	0.3	0.2	0.1	0.1	0.3	0.2	0.3	0.3	0.1	0.2	0.3	0.2	0.2	0.3	0.2	0.3	0.034	0.374
Σ KET (mg/kg)	0.4	0.3	0.5	0.7	0.5	0.6	0.6	0.4	0.3	0.4	0.3	0.3	0.4	0.4	0.4	0.3	0.3	0.4	0.066	**0.028**
Σ ALC (mg/kg)	4.3	3.3	5.0	4.1	3.0	2.4	3.3	3.2	4.4	4.0	3.1	2.8	4.8	2.5	4.4	3.8	3.1	5.0	0.479	**0.014**
Σ FUR(mg/kg)	0.3	0.3	0.4	0.9	0.4	0.3	0.3	0.4	0.4	0.4	0.5	0.4	0.8	0.3	0.1	0.3	0.2	0.5	0.074	**0.000**

[1] Values are pooled means from multifactor ANOVA (n = 108). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). See Table S3 for abbreviations.

Table S5

Effect of the interaction between the feed processing and the storage time on the lipid composition and oxidation parameters of all the experimental feeds (n = 108).

	Feed processing x Storage time[1]										
	Mash			Pelleted			Extruded				
	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	SE[2]	*P*[3]
α-T (mg/ kg)	107.9	102.3	86.1	91.1	81.1	75.2	87.5	82.3	74.8	1.304	**0.000**
β-T (mg/ kg)	5.4	5.2	4.5	3.1	2.8	2.7	3.1	2.9	2.8	0.069	**0.000**
Υ-T (mg/ kg)	46.7	40.7	31.1	20.7	17.2	15.7	20.4	18.4	16.5	1.357	**0.000**
δ-T (mg/ kg)	14.2	13.2	12.4	10.3	9.2	8.8	10.5	9.5	9.6	0.265	0.330
α-T3 (mg/ kg)	17.1	17.2	13.9	8.1	8.5	8.3	8.3	8.5	8.1	0.260	**0.000**
β-T3 (mg/ kg)	13.8	14.1	12.8	10.7	10.5	10.1	9.9	10.4	10.0	0.200	**0.030**
Υ-T3 (mg/ kg)	7.7	7.6	6.6	5.3	5.1	4.9	5.0	4.9	4.7	0.135	**0.003**
δ-T3 (mg/ kg)	0.8	0.8	1.0	0.6	0.8	0.8	0.7	0.7	0.8	0.051	**0**.493
T (mg/ kg)	174.3	161.4	134.0	125.2	110.3	102.4	121.4	113.0	103.7	2.394	**0.000**
T3 (mg/ kg)	39.4	39.8	34.3	24.7	24.8	24.1	24.0	24.6	23.6	0.498	**0.000**
T+T3 (mg/ kg)	213.7	201.1	168.3	150.0	135.1	126.5	145.4	137.6	127.3	2.761	**0.000**
TAG (%)	57.3	38.2	29.7	62.1	62.0	62.2	61.9	61.7	62.7	1.544	**0.000**
DAG (%)	10.3	12.6	13.1	10.3	10.6	10.6	10.4	10.8	10.9	0.277	**0.000**
MAG (%)	2.4	2.7	2.5	1.8	1.7	1.6	1.6	1.7	1.6	0.088	0.487
FFA (%)	29.8	46.3	54.5	25.6	25.5	25.4	25.9	25.6	24.6	1.232	**0.000**
PV (meq O_2/kg)	8.2	6.7	7.6	13.6	8.4	9.3	11.9	8.0	9.2	0.531	**0.006**
Pentanal (mg/kg)	0.1	0.2	0.1	0.7	0.4	0.5	0.4	0.3	0.3	0.043	**0.001**
Hexanal (mg/kg)	1.5	2.1	1.4	3.0	2.0	2.9	1.6	1.2	1.2	0.316	0.074
Σ ALD (mg/kg)	1.9	2.5	1.7	4.0	2.5	3.5	2.4	1.7	1.6	0.377	0.065
Σ HC (mg/kg)	0.5	0.2	0.4	0.2	0.1	0.2	0.1	0.1	0.1	0.024	**0.002**
Σ KET (mg/kg)	0.8	0.6	0.7	0.3	0.2	0.3	0.3	0.2	0.3	0.047	0.234
Σ ALC (mg/kg)	11.1	8.1	10.7	0.7	0.6	0.7	0.4	0.4	0.6	0.338	**0.000**
Σ FUR (mg/kg)	0.7	0.3	0.2	0.5	0.6	0.7	0.3	0.2	0.2	0.052	**0.000**

[1] Values are pooled means from multifactor ANOVA (n = 108). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). See Table S3 for abbreviations.

Table S6

Effect of the interaction between the feed processing and the storage temperature on the lipid composition and oxidation parameters of all the experimental feeds (n = 108).

	Feed processing x Storage temperature[1]							
	Mash		Pelleted		Extruded			
	22 °C	35 °C	22 °C	35 °C	22 °C	35 °C	SE[2]	*P*[3]
α-T (mg/ kg)	101.0	96.6	82.9	82.0	81.2	81.8	1.065	0.066
β-T (mg/ kg)	5.2	4.8	2.9	2.9	2.9	2.9	0.056	**0.001**
ϒ-T (mg/ kg)	42.9	36.1	18.2	17.5	18.6	18.2	1.108	**0.008**
δ-T (mg/ kg)	13.7	12.8	9.6	9.3	9.8	9.9	0.216	0.101
α-T3 (mg/ kg)	16.6	15.5	8.1	8.5	8.0	8.6	0.212	**0.000**
β-T3 (mg/ kg)	13.9	13.3	10.4	10.4	10.0	10.2	0.163	**0.026**
ϒ-T3 (mg/ kg)	7.5	7.1	5.1	5.1	4.8	5.0	0.110	**0.025**
δ-T3 (mg/ kg)	0.9	0.9	0.7	0.7	0.7	0.8	0.042	0.782
T (mg/ kg)	162.8	150.3	113.6	111.7	112.6	112.8	1.955	**0.004**
T3 (mg/ kg)	38.9	36.7	24.3	24.8	23.6	24.5	0.407	**0.001**
T+T3 (mg/ kg)	201.7	187.1	138.0	136.5	136.2	137.3	2.254	**0.002**
TAG (%)	44.0	39.5	62.1	62.1	62.3	61.9	1.261	0.150
DAG (%)	11.5	12.5	10.4	10.6	10.8	10.7	0.226	**0.045**
MAG (%)	2.6	2.5	1.7	1.7	1.7	1.6	0.072	0.898
FFA (%)	41.7	45.3	25.5	25.5	25.1	25.6	1.006	0.157
PV (meq O_2/kg)	7.2	7.8	10.1	10.8	9.6	9.9	0.433	0.888
Pentanal (mg/kg)	0.1	0.1	0.5	0.5	0.4	0.3	0.035	0.688
Hexanal (mg/kg)	2.0	1.4	2.7	2.6	1.6	1.0	0.258	0.606
Σ ALD (mg/kg)	2.4	1.6	3.5	3.3	2.3	1.5	0.308	0.606
Σ HC (mg/kg)	0.4	0.4	0.2	0.1	0.1	0.1	0.020	0.709
Σ KET (mg/kg)	0.8	0.6	0.3	0.3	0.3	0.2	0.038	**0.006**
Σ ALC (mg/kg)	10.0	9.9	0.8	0.5	0.5	0.4	0.276	0.973
Σ FUR (mg/kg)	0.4	0.4	0.6	0.6	0.3	0.2	0.043	0.341

[1] Values are pooled means from multifactor ANOVA (n = 108). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). See Table S3 for abbreviations.

Table S7

Effect of the fat source, feed processing, storage time and storage temperature on the volatile compound contents of all the experimental feeds (n = 108).

	Fat source[1]								Feed processing					Storage time					Storage temperature			
	CS	AS	RS	CP	PFAD	RP	SE[2]	*P*[3]	Mash	Pelleted	Extruded	SE	*P*	Day 0	Day 30	Day 60	SE	*P*	22 °C	35 °C	SE	*P*
Pentanal (mg/kg)	0.291^{ab}	0.588^{c}	0.319^{ab}	0.260^{ab}	0.352^{b}	0.157^{a}	0.044	**0.000**	0.133^{a}	0.501^{c}	0.350^{b}	0.031	**0.000**	0.413^{b}	0.279^{a}	0.291^{a}	0.031	**0.005**	0.352	0.304	0.025	0.180
Hexanal (mg/kg)	1.833^{a}	3.309^{b}	2.064^{a}	1.598^{a}	1.422^{a}	1.073^{a}	0.278	**0.000**	1.674^{a}	2.641^{b}	1.335^{a}	0.196	**0.000**	2.053	1.773	1.824	0.196	0.565	2.114^{b}	1.652^{a}	0.160	**0.044**
Heptanal (mg/kg)	0.033^{ab}	0.062^{c}	0.043^{abc}	0.039^{ab}	0.054^{bc}	0.030^{a}	0.005	**0.000**	0.040^{a}	0.059^{b}	0.031^{a}	0.004	**0.000**	0.058^{b}	0.040^{a}	0.032^{a}	0.004	**0.000**	0.049^{b}	0.038^{a}	0.003	**0.006**
Octanal (mg/kg)	0.005^{a}	0.010^{b}	0.005^{a}	0.007^{ab}	0.017^{c}	0.004^{a}	0.001	**0.000**	0.007^{a}	0.011^{a}	0.006^{a}	0.001	**0.000**	0.010^{b}	0.008^{ab}	0.006^{a}	0.001	**0.020**	0.009	0.007	0.001	0.084
Nonanal (mg/kg)	0.005^{a}	0.010^{a}	0.006^{a}	0.009^{a}	0.021^{b}	0.005^{a}	0.001	**0.000**	0.008^{a}	0.013^{b}	0.006^{a}	0.001	**0.000**	0.012^{b}	0.009^{ab}	0.007^{a}	0.001	**0.002**	0.010^{b}	0.008^{a}	0.001	**0.027**
Decanal (mg/kg)	0.012^{abc}	0.019^{c}	0.016^{bc}	0.006^{ab}	0.007^{ab}	0.003^{a}	0.002	**0.000**	0.016^{b}	0.011^{b}	0.005^{a}	0.002	**0.000**	0.012	0.011	0.009	0.002	0.462	0.013^{b}	0.008^{a}	0.001	**0.023**
2-Heptenal (mg/kg)	0.049^{a}	0.097^{b}	0.048^{a}	0.028^{a}	0.034^{a}	0.024^{a}	0.007	**0.000**	0.072^{b}	0.042^{a}	0.026^{a}	0.005	**0.000**	0.060^{b}	0.045^{ab}	0.035^{a}	0.005	**0.005**	0.053^{b}	0.040^{a}	0.004	**0.036**
2-Decenal (mg/kg)	0.002	0.003	0.002	0.002	0.004	0.002	0.001	**0.051**	0.004^{b}	0.002^{a}	0.002^{a}	0.000	**0.001**	0.003	0.003	0.002	0.000	0.381	0.003	0.003	0.000	0.278
2,4-Heptadienal (mg/kg)	0.009^{ab}	0.017^{b}	0.001^{a}	0.001^{a}	0.002^{a}	0.001^{a}	0.003	**0.002**	0.002	0.009	0.006	0.002	0.079	0.013^{b}	0.001^{a}	0.002^{a}	0.002	**0.001**	0.005	0.005	0.002	0.971
2,4-Nonadienal (mg/kg)	0.003^{a}	0.008^{b}	0.003^{a}	0.003^{a}	0.007^{b}	0.002^{a}	0.001	**0.000**	0.004	0.005	0.004	0.001	0.195	0.006^{b}	0.004^{a}	0.003^{a}	0.001	**0.000**	0.005^{b}	0.004^{a}	0.000	**0.017**
2,4-Decadienal (mg/kg)	0.076^{a}	0.241^{b}	0.070^{a}	0.062^{a}	0.054^{a}	0.027^{a}	0.030	**0.000**	0.048^{a}	0.074^{ab}	0.143^{b}	0.021	**0.006**	0.156^{b}	0.075^{a}	0.034^{a}	0.021	**0.000**	0.104	0.073	0.017	0.206
Hexane (mg/kg)	0.185^{ab}	0.133^{a}	0.238^{bc}	0.202^{abc}	0.215^{abc}	0.281^{c}	0.023	**0.001**	0.364^{b}	0.147^{a}	0.115^{a}	0.016	**0.000**	0.245^{b}	0.145^{a}	0.237^{b}	0.016	**0.000**	0.217	0.201	0.013	0.412
Decane (mg/kg)	0.005	0.006	0.006	0.004	0.004	0.006	0.001	0.466	0.006^{b}	0.006^{ab}	0.003^{a}	0.001	**0.024**	0.006	0.005	0.005	0.001	0.453	0.005	0.006	0.001	0.295
2-Heptanone (mg/kg)	0.071	0.100	0.115	0.056	0.098	0.050	0.018	0.058	0.078	0.083	0.083	0.013	0.943	0.091	0.064	0.089	0.013	0.243	0.081	0.082	0.010	0.996
2-Octanone (mg/kg)	0.023^{ab}	0.037^{b}	0.019^{a}	0.015^{a}	0.024^{ab}	0.016^{a}	0.004	**0.002**	0.047^{b}	0.012^{a}	0.008^{a}	0.003	**0.000**	0.025	0.016	0.026	0.003	**0.035**	0.023	0.022	0.002	0.608
3-Octanone (mg/kg)	0.133^{a}	0.279^{b}	0.159^{a}	0.161^{ab}	0.206^{ab}	0.164^{ab}	0.029	**0.009**	0.253^{b}	0.156^{a}	0.142^{a}	0.021	**0.000**	0.191	0.168	0.191	0.021	0.657	0.210^{b}	0.157^{a}	0.017	**0.027**
1-Penten-3-one (mg/kg)	0.113	0.125	0.098	0.050	0.038	0.047	0.026	0.074	0.211^{b}	0.011^{a}	0.014^{a}	0.019	**0.000**	0.108	0.072	0.057	0.019	0.145	0.097	0.060	0.015	0.093
1-Octen-3-one (mg/kg)	0.056	0.039	0.051	0.032	0.053	0.046	0.007	0.113	0.118^{b}	0.011^{a}	0.009^{a}	0.005	**0.000**	0.051^{b}	0.032^{a}	0.055^{b}	0.005	**0.002**	0.048	0.044	0.004	0.491
1-Butanol (mg/kg)	0.009^{a}	0.012^{a}	0.010^{a}	0.010^{a}	0.037^{b}	0.009^{a}	0.002	**0.000**	0.016	0.013	0.014	0.001	0.438	0.015^{ab}	0.012^{a}	0.017^{b}	0.001	**0.018**	0.016	0.013	0.001	0.085
1-Pentanol (mg/kg)	0.166	0.108	0.129	0.087	0.176	0.123	0.023	0.063	0.231^{b}	0.085^{a}	0.078^{a}	0.016	**0.000**	0.152^{b}	0.093^{a}	0.149^{b}	0.016	**0.018**	0.121	0.142	0.013	0.288
1-Hexanol (mg/kg)	0.810	0.517	0.689	0.588	0.892	0.762	0.146	0.475	1.690^{b}	0.244^{a}	0.195^{a}	0.104	**0.000**	0.780^{ab}	0.468^{a}	0.880^{b}	0.104	**0.016**	0.717	0.702	0.085	0.898
1-Nonanol (mg/kg)	0.004	0.005	0.005	0.004	0.007	0.004	0.001	0.407	0.008^{b}	0.004^{a}	0.004^{c}	0.001	**0.001**	0.005	0.005	0.005	0.001	0.999	0.005	0.005	0.001	0.552
2-Pentenol (mg/kg)	0.036^{bc}	0.047^{c}	0.026^{ab}	0.019^{a}	0.021^{a}	0.018^{a}	0.003	**0.000**	0.052^{b}	0.018^{a}	0.014^{a}	0.002	**0.000**	0.035^{b}	0.023^{a}	0.025^{a}	0.002	**0.000**	0.032^{b}	0.023^{a}	0.002	**0.001**
1-Octen-3-ol (mg/kg)	3.165	2.481	2.789	2.587	2.787	3.048	0.225	0.252	7.970^{b}	0.289^{a}	0.169^{a}	0.159	**0.000**	3.073^{b}	2.440^{a}	2.914^{ab}	0.159	**0.017**	2.879	2.740	0.130	0.450
2-Pentylfuran (mg/kg)	0.343^{ab}	0.520^{b}	0.364^{ab}	0.420^{ab}	0.413^{ab}	0.337^{a}	0.043	**0.035**	0.404^{b}	0.594^{c}	0.201^{a}	0.030	**0.000**	0.496^{b}	0.354^{a}	0.348^{a}	0.030	**0.001**	0.421	0.378	0.025	0.454
2-Furanmethanol (mg/kg)	0.005	0.004	0.005	0.005	0.005	0.003	0.001	0.336	0.005^{b}	0.002^{a}	0.007^{c}	0.001	**0.000**	0.005	0.004	0.005	0.000	0.648	0.005	0.004	0.000	0.133
Σ ALD (mg/kg)	2.318^{a}	4.365^{b}	2.577^{a}	2.018^{a}	1.974^{a}	1.329^{a}	0.336	**0.000**	2.008^{a}	3.369^{b}	1.913^{a}	0.238	**0.000**	2.795	2.248	2.247	0.238	0.176	2.718^{b}	2.141^{a}	0.194	**0.038**
Σ HC (mg/kg)	0.190^{ab}	0.140^{a}	0.244^{bc}	0.206^{abc}	0.219^{abc}	0.286^{c}	0.023	**0.001**	0.371^{b}	0.153^{a}	0.119^{a}	0.017	**0.000**	0.251^{b}	0.150^{a}	0.242^{b}	0.017	**0.000**	0.221	0.207	0.013	0.450
Σ KET (mg/kg)	0.397^{a}	0.580^{b}	0.442^{ab}	0.314^{a}	0.419^{a}	0.322^{a}	0.043	**0.000**	0.708^{b}	0.272^{a}	0.256^{a}	0.031	**0.000**	0.467^{b}	0.353^{a}	0.417^{ab}	0.031	**0.035**	0.460^{b}	0.365^{a}	0.025	**0.008**
Σ ALC (mg/kg)	4.190	3.170	3.649	3.295	3.919	3.964	0.346	0.251	9.966^{b}	0.652^{a}	0.475^{a}	0.245	**0.000**	4.062^{b}	3.041^{a}	3.991^{b}	0.245	**0.006**	3.771	3.624	0.200	0.604
Σ FUR (mg/kg)	0.348^{ab}	0.524^{b}	0.369^{ab}	0.424^{ab}	0.418^{ab}	0.340^{a}	0.043	**0.034**	0.409^{b}	0.595^{c}	0.208^{a}	0.030	**0.000**	0.501^{b}	0.358^{a}	0.353^{a}	0.030	**0.001**	0.426	0.382	0.025	0.204

[1] Values are pooled means from multifactor ANOVA (n = 108). [2] SE, standard error of the pooled mean. [3] *P* values in bold are significant ($P \leq 0.05$). [a-c] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. See Table S3 for abbreviations.

Table S8

P values for the effects of the interactions between two of the main factors on the volatile compound contents, according to multifactorial ANOVA for all the experimental feeds (n = 108)[1].

	Fat source x Feed processing	Fat source x Storage time	Fat source x Storage Temperature	Feed processing x Storage time	Feed processing x Storage Temperature	Storage Temperature x Storage time
Pentanal (mg/kg)	**0.005**	**0.007**	NS	**0.001**	NS	NS
Hexanal (mg/kg)	NS	NS	NS	NS	NS	NS
Heptanal (mg/kg)	**0.002**	**0.016**	NS	NS	NS	**0.030**
Octanal (mg/kg)	**0.002**	**0.000**	NS	**0.010**	NS	NS
Nonanal (mg/kg)	**0.000**	**0.000**	**0.008**	**0.041**	NS	**0.020**
Decanal (mg/kg)	**0.013**	**0.001**	NS	NS	NS	**0.048**
2-Heptenal (mg/kg)	NS	NS	NS	NS	NS	NS
2-Decenal (mg/kg)	**0.003**	NS	NS	NS	NS	NS
2,4-Heptadienal (mg/kg)	**0.003**	**0.000**	NS	**0.004**	NS	NS
2,4-Nonadienal (mg/kg)	**0.002**	**0.000**	NS	NS	NS	NS
2,4-Decadienal (mg/kg)	**0.000**	**0.000**	NS	NS	NS	NS
Hexane (mg/kg)	**0.001**	NS	NS	**0.003**	NS	NS
Decane (mg/kg)	**0.005**	**0.024**	NS	**0.002**	NS	**0.038**
1-Penten-3-one (mg/kg)	**0.002**	NS	NS	**0.035**	NS	NS
2-Heptanone (mg/kg)	NS	**0.000**	NS	**0.001**	NS	NS
2-Octanone (mg/kg)	NS	NS	NS	NS	NS	NS
3-Octanone (mg/kg)	NS	NS	NS	NS	**0.044**	NS
1-Octen-3-one (mg/kg)	**0.000**	**0.015**	NS	**0.000**	NS	NS
1-Pentanol (mg/kg)	**0.000**	NS	NS	NS	NS	NS
1-Butanol (mg/kg)	**0.042**	**0.004**	NS	**0.002**	NS	NS
2-Pentenol (mg/kg)	NS	**0.002**	NS	**0.010**	NS	NS
1-Hexanol (mg/kg)	**0.020**	**0.014**	NS	NS	NS	NS
1-Octen-3-ol (mg/kg)	**0.012**	NS	NS	**0.014**	**0.002**	**0.010**
1-Nonanol (mg/kg)	**0.000**	**0.038**	NS	**0.000**	NS	NS
2-Pentylfuran (mg/kg)	**0.000**	**0.000**	NS	**0.000**	NS	NS
2-Furanmethanol (mg/kg)	**0.000**	NS	NS	NS	NS	NS
Σ ALD (mg/kg)	NS	NS	NS	NS	NS	NS
Σ HC (mg/kg)	**0.000**	NS	NS	**0.002**	NS	NS
Σ KET (mg/kg)	NS	**0.028**	NS	NS	**0.006**	NS
Σ ALC (mg/kg)	**0.002**	**0.014**	NS	**0.000**	NS	NS
Σ FUR (mg/kg)	**0.000**	**0.000**	NS	**0.000**	NS	NS

See Table S3 for abbreviations.

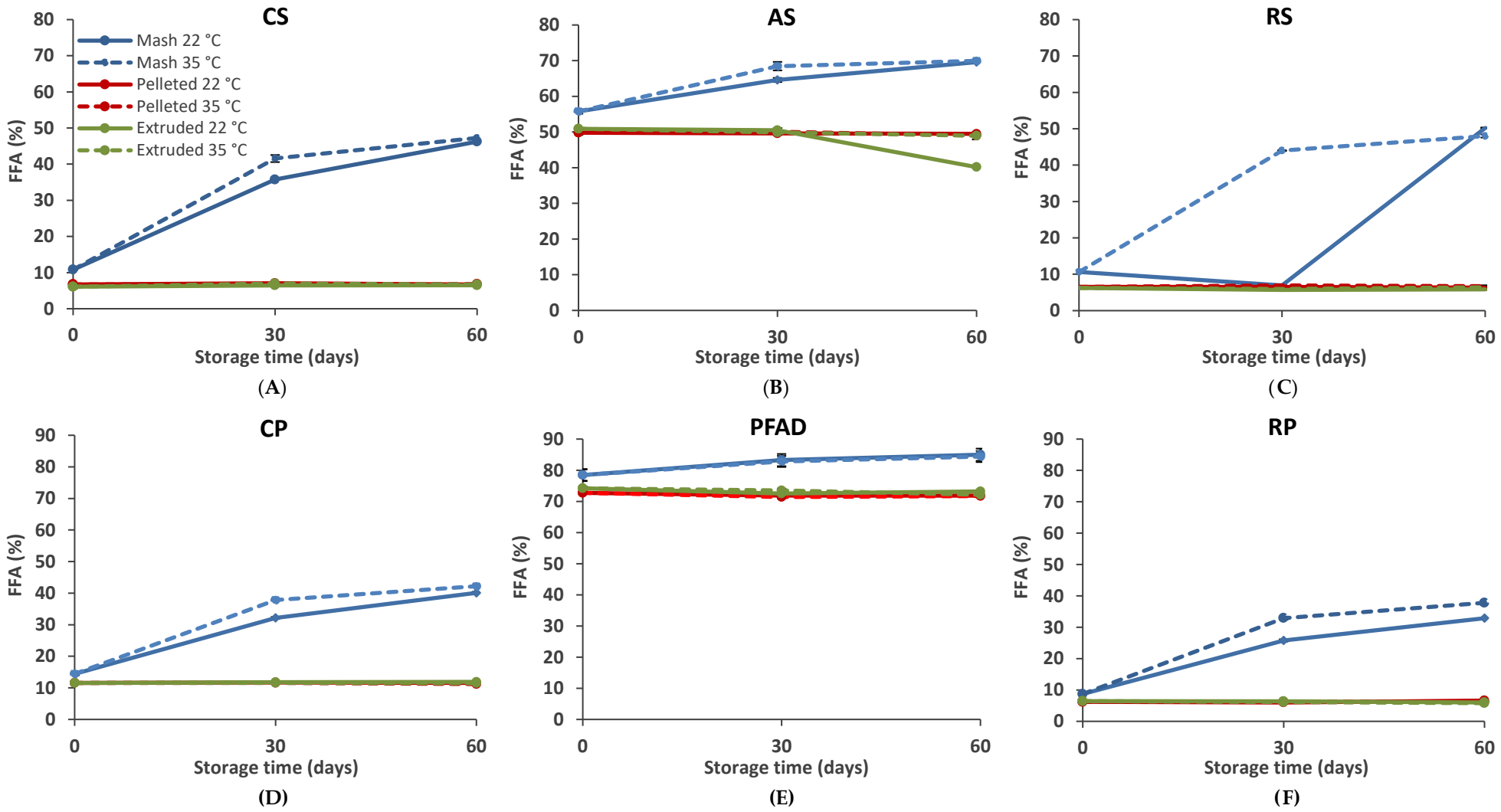


Fig. S1. Evolution of free fatty acid (FFA) percentage throughout storage time (0, 30 and 60 days) in feeds (mash, pelleted and extruded, kept at 22 °C and 35 °C; containing a 6% of (**A**) CS, crude soybean oil feeds; (**B**) AS, soybean acid oil feeds; (**C**) RS, refined soybean oil feeds; (**D**) CP, crude palm oil feeds; (**E**) PFAD, palm fatty acid distillate feeds; or (**F**) RP, refined palm oil. Mean ± SD, of duplicate determinations.

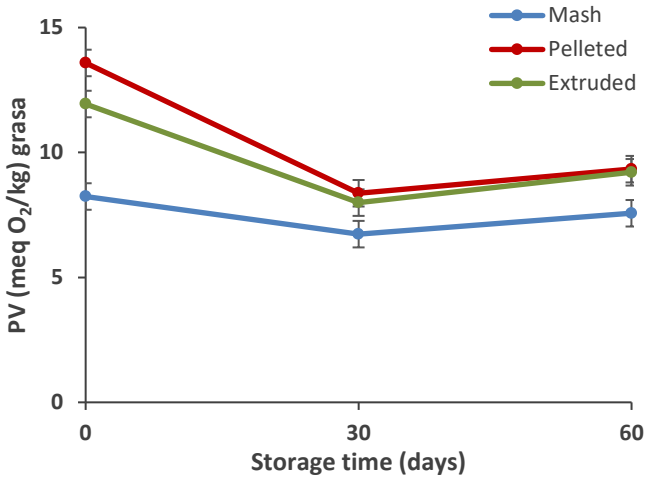


Fig. S2. Effect of the interaction between feed processing (mash, pelleted and extruded feeds) and storage time (0, 30 and 60 days) on the peroxide value (PV) (pooled means ± standard error from multifactor ANOVA with n = 108, $P = 0.003$).

8.5. Información suplementaria (supplementary material) de la publicación "Use of acid oil and palm fatty acid distillate in broiler diets: effects on lipid composition and oxidative stability of meat"

Varona, E., Tres, A., Rafecas, M., Vichi, S., A.C., Barroeta, Guardiola, F. *Poult. Sci*. En preparación.

Table S1. Effect of dietary treatment factor on the individual aldehydes and 2-pentylfuran on fresh meat samples.

	CS	AS	RS	CP	PFAD	RP	*P*
Pentanal (mg/kg)	Tr^{ab}	Tr^{ab}	0.004 ± 0.003^{b}	Tr^{a}	Tr^{a}	Tr^{a}	**0.011**
Hexanal (mg/kg)	0.045 ± 0.03^{ab}	0.025 ± 0.02^{a}	0.095 ± 0.09^{b}	0.008 ± 0.00^{a}	0.006 ± 0.00^{a}	0.007 ± 0.00^{a}	**0.001**
Heptanal (mg/kg)	0.003 ± 0.00^{b}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.001 ± 0.00^{a}	0.002 ± 0.00^{a}	0.001 ± 0.00^{a}	**0.000**
Octanal (mg/kg)	ND	ND	ND	ND	ND	ND	0.495
Nonanal (mg/kg)	0.003 ± 0.00	0.002 ± 0.00	0.002 ± 0.00	0.002 ± 0.00	0.003 ± 0.00	0.002 ± 0.00	0.164
Decanal (mg/kg)	ND	ND	ND	ND	ND	ND	0.996
2-Heptenal (mg/kg)	ND^{a}	ND^{a}	Tr^{b}	ND^{a}	ND^{a}	ND^{a}	**0.007**
2-Decenal (mg/kg)	Tr	Tr	Tr	Tr	Tr	Tr	0.101
2,4-Heptadienal (mg/kg)	0.001 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.001 ± 0.00	0.521
2,4-Decadienal (mg/kg)	0.0004 ± 0.00	0.0007 ± 0.00	0.0009 ± 0.00	0.0006 ± 0.00	0.0007 ± 0.00	0.0005 ± 0.00	0.079
2-Pentylfuran (mg/kg)	0.009 ± 0.01	0.009 ± 0.00	0.013 ± 0.01	0.009 ± 0.01	0.008 ± 0.00	0.008 ± 0.00	0.426

[1] Mean values ± SD from one-way ANOVA (n = 36, resulting from 6 experimental replicates x 6 dietary treatments) [4] *P* values in bold are significant ($P \leq 0.05$). [a-b] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil. ND, not detected; Tr, Traces.

Table S2. Effect of dietary treatment and technological processing factors on the individual aldehydes of fresh (F), cooked (C) and cooked and refrigerated (CR) meat samples.

	Dietary treatment[1]								Technological treatment[2]					Dietary treatment* Technological treatment
	CS	AS	RS	CP	PFAD	RP	SE[3]	*P*[4]	F	C	CR	SE	*P*	*P*
Pentanal (mg/kg)	0.622	0.668	0.728	0.675	0.626	0.613	0.069	0.852	Tr^{a}	0.687^{b}	1.277^{c}	0.049	**0.000**	0.265
Hexanal (mg/kg)	10.425	11.669	12.465	11.127	10.493	9.883	0.987	0.477	0.031^{a}	11.030^{b}	21.970^{c}	0.698	**0.000**	0.679
Heptanal (mg/kg)	0.156^{a}	0.222^{a}	0.191^{a}	0.360^{b}	0.471^{b}	0.395^{b}	0.030	**0.000**	0.002^{a}	0.162^{b}	0.734^{c}	0.021	**0.000**	**0.000**
Octanal (mg/kg)	0.080^{a}	0.113^{a}	0.093^{a}	0.307^{b}	0.380^{b}	0.321^{b}	0.038	**0.000**	ND^{a}	0.096^{b}	0.549^{c}	0.027	**0.000**	**0.000**
Decanal (mg/kg)	Tr^{a}	0.003^{a}	Tr^{a}	0.005^{ab}	0.007^{b}	0.004^{ab}	0.001	**0.000**	ND^{a}	ND^{a}	0.010^{b}	0.001	**0.000**	0.074
2-Heptenal (mg/kg)	0.011	0.011	0.009	0.009	0.013	0.010	0.003	0.822	ND^{a}	0.003^{a}	0.028^{b}	0.002	**0.000**	0.918
Nonanal (mg/kg)	0.035^{a}	0.038^{a}	0.033^{a}	0.143^{b}	0.211^{b}	0.157^{b}	0.023	**0.000**	0.002^{a}	0.041^{a}	0.265^{b}	0.017	**0.000**	**0.000**
2,4-Heptadienal (mg/kg)	0.002	0.002	0.002	0.004	0.004	0.003	0.001	0.288	0.001^{a}	0.003^{b}	0.004^{b}	0.001	**0.000**	0.096
2-Decenal (mg/kg)	0.002^{a}	0.002^{ab}	0.002^{ab}	0.002^{ab}	0.004^{b}	0.002^{ab}	0.001	**0.041**	ND^{a}	0.001^{a}	0.006^{b}	0.000	**0.000**	0.097
2,4-Decadienal (mg/kg)	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.778	0.001^{a}	0.001^{b}	0.002^{c}	0.000	**0.000**	0.162
2-Pentylfuran (mg/kg)	0.188	0.173	0.153	0.180	0.215	0.202	0.024	0.518	0.009^{a}	0.098^{b}	0.448^{c}	0.017	**0.000**	0.623

[1] Values are pooled means from multifactor ANOVA (n = 18 per group, resulting from 6 experimental replicates x 3 technological treatments). [2] Values are pooled means from multifactor ANOVA (n = 36 per group, resulting from 6 experimental replicates x 6 dietary treatments). [3] SE, standard error of the pooled mean. [4] *P* values of the main effects in bold are significant ($P \leq 0.05$). [a-c] Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; F, fresh meat; C, cooked meat; CR, cooked and refrigerate storage meat. ND, not detected; Tr, Traces.

Table S3. Effect of dietary treatment and technological processing factors on the individual hydrocarbons, ketones and alcohols volatile compounds of fresh (F), cooked (C) and cooked and refrigerated (CR) meat samples.

	Dietary treatment[1]								Technological treatment[2]					Dietary treatment* Technological treatment
	CS	AS	RS	CP	PFAD	RP	SE[3]	*P*[4]	F	C	CR	SE	*P*	*P*
Hexane (mg/kg)	0.019	0.015	0.013	0.014	0.013	0.034	0.007	0.223	0.004^{a}	0.037^{b}	0.012^{a}	0.005	**0.000**	0.841
Decane (mg/kg)	0.049^{ab}	0.034^{a}	0.030^{a}	0.096^{c}	0.078^{bc}	0.092^{c}	0.009	**0.000**	0.036^{a}	0.090^{c}	0.064^{b}	0.006	**0.000**	**0.000**
1-Penten-3-one (mg/kg)	0.002	0.002	0.002	0.002	0.003	0.003	0.000	0.301	0.004^{c}	0.002^{b}	0.001^{a}	0.000	**0.000**	**0.016**
1-Octen-3-one (mg/kg)	0.029	0.036	0.029	0.031	0.033	0.030	0.004	0.884	0.0004^{a}	0.031^{b}	0.062^{c}	0.003	**0.000**	0.919
2-Heptanone (mg/kg)	0.115	0.137	0.125	0.121	0.140	0.113	0.015	0.722	0.007^{a}	0.100^{b}	0.269^{c}	0.011	**0.000**	0.886
2-Octanone (mg/kg)	0.044^{a}	0.061^{a}	0.055^{a}	0.239^{b}	0.277^{b}	0.120^{ab}	0.040	**0.000**	0.001^{a}	0.045^{a}	0.352^{b}	0.029	**0.000**	**0.000**
3-Octanone (mg/kg)	0.039^{a}	0.044^{a}	0.042^{a}	0.053^{ab}	0.104^{b}	0.073^{ab}	0.014	**0.008**	ND^{a}	0.086^{b}	0.091^{b}	0.010	**0.000**	0.096
1-Butanol (mg/kg)	0.008	0.007	0.007	0.005	0.008	0.007	0.001	0.837	0.001^{a}	0.014^{c}	0.006^{b}	0.001	**0.000**	0.756
1-Pentanol (mg/kg)	0.048	0.083	0.063	0.061	0.057	0.043	0.015	0.487	Tr^{a}	0.093^{b}	0.084^{b}	0.010	**0.000**	0.931
1-Hexanol (mg/kg)	0.142	0.359	0.163	0.148	0.278	0.112	0.100	0.459	0.008^{a}	0.366^{b}	0.226^{ab}	0.070	**0.002**	0.802
2-Pentenol (mg/kg)	0.011	0.010	0.009	0.008	0.008	0.008	0.001	0.528	ND^{a}	0.010^{b}	0.016^{c}	0.001	**0.000**	0.865
1-Octen-3-ol (mg/kg)	1.552	1.836	1.564	1.625	1.942	1.503	0.207	0.599	0.014^{a}	1.086^{b}	3.911^{c}	0.146	**0.000**	0.651

[1] Values are pooled means from multifactor ANOVA (n = 18 per group, resulting from 6 experimental replicates x 3 technological treatments). [2] Values are pooled means from multifactor ANOVA (n = 36 per group, resulting from 6 experimental replicates x 6 dietary treatments). [3] SE, standard error of the pooled mean. [4] *P* values of the main effects in bold are significant ($P \leq 0.05$). $^{a-c}$ Means bearing different letters are significantly different ($P \leq 0.05$) according to Tukey´s post-hoc test. Abbreviations: CS, crude soybean oil; AS, soybean acid oil; RS, refined soybean oil; CP, crude palm oil; PFAD, palm fatty acid distillate; RP, refined palm oil; F, fresh meat; C, cooked meat; CR, cooked and refrigerate storage meat. ND, not detected; Tr, Traces.