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PROGRAMA DE DOCTORADO: Alimentación y nutrición

STRATEGIES TO IMPROVE THE OXIDATIVE STABILITY OF BAKERY PRODUCTS FORTIFIED WITH HEME IRON

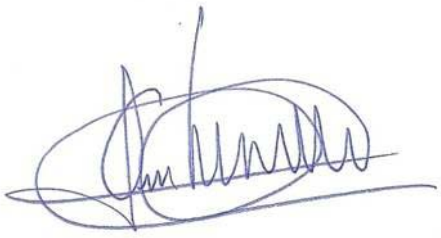
Estrategias para mejorar la estabilidad oxidativa de productos de panadería fortificados con hierro hémico

Memoria presentada por

Mercedes Alemán

Para optar al título de Doctor por la Universidad de Barcelona

Los directores,



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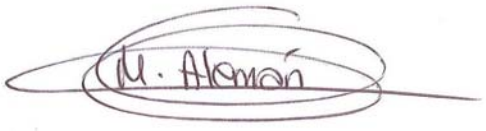

Mercedes Alemán

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“I am among those who think that science has great beauty.”

“Me encuentro entre aquellos que piensan que la ciencia posee una gran belleza.”

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Abstract

Iron deficiency is the most prevalent nutritional issue worldwide. The fortification of food products could help covering the iron requirements, mainly in certain population groups where prevalence of iron deficiency anaemia is high because its amount in the diet is poor and/or presents low bioavailability. In this regard it is worth to mention that the population groups with higher prevalence of iron deficiency are both women at childbearing age and children.

However, iron fortification should take into account several variables that determine its effectiveness being the bioavailability of the iron form used and its interaction with the matrix the most crucial ones.

The aim of this thesis is to obtain a fortified food product with a high bioavailable iron while being accepted by consumers and stable towards oxidation. In this regard, a sandwich-type cookie filled with a chocolate cream has been selected as example of heme iron fortification in bakery products. The presence of chocolate, apart from making the food product to be more appealing to children, may help disguise the intense colour of the heme iron ingredient, which is intended to be used for its high bioavailability.

Since palm oil is widely used in the manufacture of biscuits, cakes and pastries, it is planned to previously study those strategies that minimize oxidation in a model based on palm oil fortified with heme iron before these are employed in the final food products. By means of this model, we assessed the efficacy of the following strategies: the addition of antioxidants; the heme iron ingredient encapsulation and the combination of both strategies. The strategies that were shown to be efficient in this fortified palm oil model were further assessed on sandwich-type chocolate cookies fortified with heme iron. In these cookies and over a period of one year of storage in the dark at room temperature, the oxidative stability and consumers' overall acceptability has been studied

By combining the two strategies, the addition of an antioxidant (ascorbyl palmitate) and the encapsulation of heme iron by co-spray-drying it with calcium caseinate, the cookies were oxidative stable and accepted by consumers over one year of storage at room temperature in the dark.

Resumen

La deficiencia en hierro es la deficiencia nutricional con más prevalencia a nivel mundial. El fortalecimiento de productos alimenticios con hierro puede contribuir a cubrir las necesidades diarias de este elemento, sobre todo en determinadas poblaciones donde la prevalencia de esta deficiencia es elevada debido a que su cantidad en las dietas es pobre y/o presenta una baja biodisponibilidad. Cabe destacar que los grupos con mayor prevalencia de deficiencia en hierro son las mujeres en edad fértil y niños.

Sin embargo, el fortalecimientos de alimentos con hierro debe tener en cuenta diversas variables que condicionan su efectividad, siendo la biodisponibilidad del hierro y su interacción con la matriz alimentaria las más cruciales.

El objetivo que se plantea en esta tesis es conseguir un producto rico en hierro altamente biodisponible que presente una buena aceptabilidad y estabilidad oxidativa. Dentro de los productos de galletería/ bollería, se escoge una galleta rellena de chocolate como ejemplo de fortalecimiento en hierro hémico por su difusión y simplicidad. El chocolate, aparte de hacer el producto más apetecible a la población infantil y juvenil, permite enmascarar el color del ingrediente de hierro hémico que se pretende utilizar por su elevada biodisponibilidad.

Dado que la manteca de palma es ampliamente utilizada en la elaboración de productos de galletería y bollería, se plantea, previamente, estudiar aquellas estrategias que permitan minimizar la oxidación en un modelo basado en manteca de palma fortalecida con hierro hémico. Mediante este modelo se han contemplado las siguientes estrategias: la eficacia de la adición de antioxidantes, la encapsulación del ingrediente de hierro hémico y la combinación de ambas. Las estrategias que resultaron ser más eficaces en este modelo fueron posteriormente estudiadas en galletas tipo sándwich rellenas de chocolate fortalecidas con hierro hémico. En el producto desarrollado a lo largo de un año de almacenamiento en la oscuridad a temperatura ambiente, se ha estudiado su estabilidad oxidativa y su grado de aceptación por parte de los consumidores.

Gracias a la combinación de ambas estrategias, la adición de un antioxidante (palmitato de ascorbilo) y la encapsulación del hierro hémico por co-atomización de éste con caseinato de calcio, se ha obtenido un producto de galletería fortalecido con hierro altamente biodisponible estable frente a la oxidación y que a su vez ha sido aceptado por los consumidores a lo largo de un año de almacenamiento.

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1. INTRODUCTION

In the last decades, people's awareness on the importance of following an equilibrated and healthy diet is increasing exponentially. Consumers are concerned about the potential benefits of eating healthier foods. However, despite of all this, nutritional deficiencies are still present in our daily life compromising our health. There are two main reasons why nutritional deficiencies are still an important issue; on one side, nutrient intakes may be lower than required and on the other, the intake may be appropriate but the bioavailability of the compounds may be low because they are not being absorbed in sufficient amounts.

In general, nutritional deficiencies could be addressed by two different approaches; (i.) a food-based approach, such as diet diversification and food fortification, and (ii.) a medical-based approach in which pills, injections and so on are used as supplements. Both strategies are deeply studied and extensively reviewed in the literature. Food fortification has been pointed out as the most effective one due to its great cost-effect ratio and its long-term effectiveness. Indeed, in 2008 the Copenhagen Consensus estimated that correcting iodine deficiency has a 30:1 benefit/cost ratio and correcting iron deficiency a benefit/cost ratio of 8:1 (Horton et al., 2009).

Nutritional or medical outcomes following the strategy of food fortification are not new. For example, especially in Europe it has been reported the use of biscuits as micronutrient vehicles already in the XIX century. Although the process of biscuits fabrication has no relationship with pharmaceutical issues, different formulations called "medicinal breads or biscuits" appeared in that era. These new products were formulated with the addition of inorganic salts (iron, iodide and mercury salts) or herbal medicines, especially vermifuges, such as *Ipomoea purga* (or Jalap root) and *Convolvulus scammonia* (known as scammony).

In the United States, salt is awarded as being the first food product fortificated by the addition of iodide. Through this, it was aimed to decrease the incidence of goitre in 1924. It is recognized that the actual term food fortification was employed for the first time to describe this event. Therefore, food fortification of food products with micronutrients it's a known and extended practice.

At present, fortified food products are generally well accepted by consumers. At least one third of American consumers believe that the fortification of food products with micronutrients has a moderate-to-high positive effect on health (IFIC foundation, 2013). Frequently, consumers of fortified foods present better nutritional status than consumers of non-fortified foods. This seems to be related with the fact that consumers of fortified foods are more aware of their diets than non-fortified food consumers. In this sense, it is important to clearly label fortified food products in order to build a confident relationship between producers and consumers.

Despite all these facts, the consumption of fortificated food products in Europe is low. Food fortification supposes just 3% of Europeans' diet in base of the per capital rent (Godfrey et al., 2004). Nevertheless, it is expected that the consumption of fortified food products increases in the near future due to several facts, including (i.) increment in life expectancy, (ii.) a higher

prevalence of non-communicable diseases, (iii.) increase in health expenses and (iv.) consumer's acceptation of the strong link between health and diet (Kearney, 2010). It is important to keep in mind that food fortification could give solutions to nutritional problems; however, it should never substitute a healthy and equilibrated diet.

Among the different nutritional deficiencies, the most widespread nutritional disorder worldwide is iron deficiency. The *World Health Organization* (WHO) estimated that at least 20% of the world's population suffer this disorder. Besides, it is considered one of the 10 leading health risk factors all over the world (WHO, 2001). In the *World Summit* for children, in New York 1990, was settled the objective of reducing by one third the incidence of anaemia in women in the next ten years. The strategy followed was iron-folate supplementation to pregnant women in developing countries and after that period (2000), the prevalence was not reduced at all. The insufficient quantities of iron supplies and the lack of women compliance had been pointed out as the main causes for its ineffectiveness (Annan, 2001). Thus, the same objective was again settled to be reached before 2010.

Taking into account all these considerations, iron food fortification would mean a clear benefit for decreasing the incidence of this nutritional issue. Different programs institutionally implemented had aimed to fortify with iron staple foods, such as flour or salt, with different iron compounds. Nevertheless, due to the iron pro-oxidant properties, the incorporation of the more bioavailable iron forms into several food matrixes is a challenge.

The main drawback raised from iron addition to foods is the modification of the organoleptic properties. The appearance, flavour and taste of the fortified product can be affected, not only decreasing its shelf life but also its nutritional properties. Therefore, iron food fortification with any iron compound, especially with the more bioavailable iron forms (heme iron and soluble non-heme iron compounds), is still a challenge for the food industry.

In some cases, the iron intake through the staple food will not be enough to meet the requirements of some populations groups with special needs, such as school age children and women at their reproductive years. These populations groups have increased iron needs, due to growth period or menstruation among others, but their total food intake is lower than other populations groups. Therefore the intake of iron fortified staple foods is not enough to reach their iron requirements. Indeed, iron deficiency prevalence in these groups is higher than that of any other population group. Thus, iron fortification of food products especially addressed to these populations groups would be a good strategy for overcoming their iron deficiency. It is important that the selected food product for iron fortification as being part of the population's group common diet in order to ensure its intake in sufficient amounts. It is also crucial that the iron fortified food product have similar organoleptic properties as the non-fortified one because otherwise consumers will not accept it.

This challenge for the food industry could be addressed by several strategies, such as antioxidant addition to counteract the pro-oxidant properties of iron or the encapsulation of the iron forms to avoid their contact with the food matrix. Summing up, in order to implement an effective iron fortification strategy it is crucial to select the population group for

addressing the fortified food product, the iron compound selected for fortifying (the more bioavailable the better) and the food vehicle selected to be fortificated. Also, it is critical to combine these decisions with the best strategy to produce a stable iron fortified food product.

2. REVIEW OF LITERATURE

2.1. Iron deficiency

Iron deficiency is the result of a long-term negative iron balance. The World Health Organization (WHO) identified in 2002 iron deficiency as being amongst the ten most serious health risk factors in countries resulting in high infant mortality coupled with high adult mortality. In total, 0.8 million deaths are attributable to iron deficiency in the world, 1.3% of all male deaths and 1.8% of all female deaths (WHO, 2002). This micronutrient deficiency is regarded as the most common and widespread nutritional disorder in the world (WHO, 2001). Moreover, it is the only highly frequent nutritional deficiency in both developing and developed countries (Benoist et al., 2008). Thus, iron deficiency is one of the leading risk factors for disability and death worldwide, affecting an estimated population of 1620 million people (Benoist et al., 2008).

2.1.1. STAGES AND CONSEQUENCES OF IRON DEFICIENCY

The development of iron deficiency starts when the iron stores in the form of hemosiderin and ferritin are progressively depleted and no longer meet the needs of normal iron turnover (Allen et al., 2006). From this point on, the supply of iron to the transport protein apotransferrin is compromised.

Iron deficiency represents a spectrum ranging from iron depletion to iron deficiency anaemia. Three different sequential stages of iron deficiency can be defined: (I) iron depletion; (II) iron deficient erythropoiesis; and (III) iron deficiency anaemia.

The first stage, iron depletion, is characterized by the absence of measurable iron stores. As iron stores decline with a negative iron balance, serum ferritin, the iron storage protein, continues to drop. In this initial stage the amount of transport and functional iron may not be affected; however, those with iron depletion have no iron stores to mobilize if the body requires additional iron. There are few known functional consequences until stores are completely depleted.

Once the iron stores fall, transferrin saturation is further reduced and the amount of iron absorbed is not sufficient to replace the amount lost. In this stage, the shortage of iron limits red blood cell production and results in increased erythrocyte protoporphyrin concentration and thus iron deficient erythropoiesis appears. In this stage, tissues begin to have insufficient iron but there is still absence of anaemia (WHO, 2001).

The last and more critical stage of iron deficiency is iron deficiency anaemia (IDA). In this stage there is shortage of iron stores, transport and functional iron thus resulting in reduced haemoglobin in addition to low serum ferritin, low transferrin saturation and increased erythrocyte protoporphyrin concentration.

Anaemia, of any aetiology, is considered to be present when the haemoglobin concentration of an individual is below the 95th percentile of the distribution of haemoglobin concentration in plasma of the healthy matched population group. Therefore, the prevalence of anaemia in a population is a statistical concept, more than a physiological concept (WHO, 2001).

The terms anaemia, iron deficiency and IDA are used interchangeably. However, anaemia aetiology could be different from iron deficiency (such as vitamins deficiency, chronic diseases, autoimmune or acquired haemolysis, etc.). Therefore it is important to use each term properly.

In order to clarify this issue, in Figure 2.1 is represented the relationship between iron deficiency and anaemia in a population. It is important to note that the extent of overlap between iron deficiency and anaemia considerably changes from one population to another according to gender and age group. In fact, the overlap between anaemia and iron deficiency also varies with the population observed, being greatest in populations in which dietary iron absorbability is low or blood losses are common (because of menstruation, nasal blood losses, haemorrhoids or gastric bleeding due to ulcers or hookworm infestation).

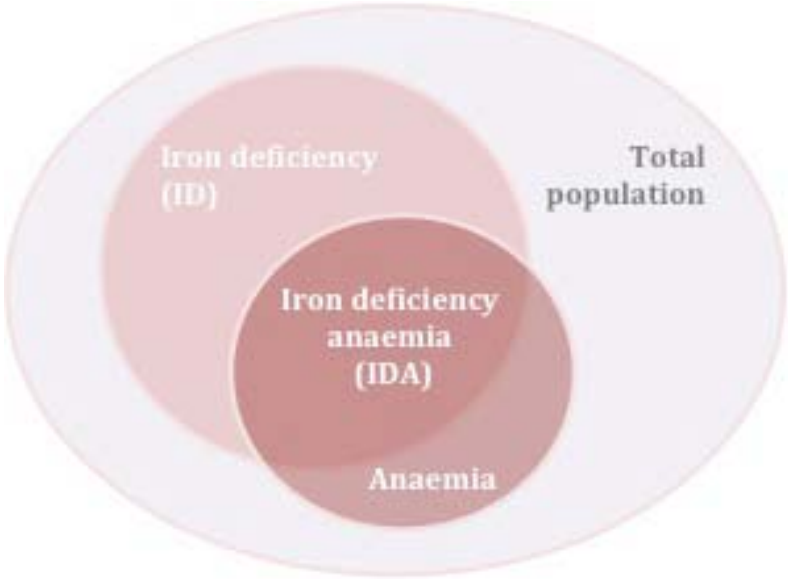

Figure 2.1: Relationship between iron deficiency and anaemia in a population. Source: WHO, 2001.

There is an extensive concern on iron deficiency diagnosis. Although haemoglobin concentration is used as reference indicator in anaemia, there is no sole reliable biochemical indicator that consistently diagnoses iron deficiency with the exception of the bone marrow aspirates, which is not a common test as it is expensive and invasive (Clark, 2009). In the Merck manual for medical information (Beers, 2008) is stated that once anaemia is diagnosed, serum ferritin is regarded as the most reliable parameter for assessing iron deficiency as the cause of anaemia. If the serum ferritin level is lower than the threshold settled for the population group of the individual, iron deficiency is present. However, in chronic diseases (e.g. infections, inflammations and cancer) serum ferritin levels may be increased thus masking iron deficiency. In such case, the transferrin receptor concentration should be also determined. Bone marrow aspirates should be considered the last choice.

Regarding the iron quantity in individuals, body iron content is approximately 3–4 g, which corresponds to a concentration of 40–50 mg of iron per kilogram of body weight. Approximately 60% is present in the form of haemoglobin in circulating red blood cells; however, the body incorporates iron into hundreds of different enzymatic and non-enzymatic proteins that are crucial to a wide range of physiological functions. Some examples of the biological uses of iron are shown in Table 2.1. As it can be observed, iron plays a pivotal role in the transport and storage of oxygen, the oxidative metabolism and in the cellular growth and proliferation.

Table 2.1: Biological functions of iron

Function	*Iron containing forms*
Oxygen transport and storage	Haemoglobin in red blood cells transports oxygen in the blood and myoglobin stores oxygen in muscles
Oxygen homeostasis	An iron-dependent prolyl-hydroxylase plays a critical role in the physiological response to hypoxia
Electron transport and energy production	Cytochromes and dehydrogenases are essential components of mitochondrial electron transport for ATP synthesis
Metabolism and detoxification	Cytochromes are also involved in the metabolism of biological molecules, drugs and pollutants
Enzymatic antioxidant activity	Catalases and peroxidases act in the metabolism of hydrogen peroxide to reduce the risk of oxidative cellular damage
Beneficial pro-oxidant activity	Myeloperoxidase synthesizes reactive oxygen species within neutrophils to aid bacterial cell killing
DNA synthesis	Ribonucleotide reductase is required for DNA synthesis

Due to the various functions of the iron in the body, it is generally accepted that, even without developing IDA, mild to moderate iron deficiency has adverse functional consequences (Allen et al., 2006). It is likely that key proteins and enzymes that either depend on or use iron as co-factor account for some, if not most, of the clinical findings observed in patients.

Indeed, clinical manifestations of iron deficiency were described since 1500 BC and were known in 16th and 17th century Europe as part of the disease “chlorosis”. However, it was not till 1800s that the decreased content of iron in the blood was clearly associated with chlorosis (Costa & Drews, 2010).

In this context, here are described some of the most common consequences of iron deficiency.

- Cognitive performance

Several areas of the brain contain iron and this micronutrient plays a key role in brain function. The effect of IDA on the delay in brain and psychomotor development has been extensively reviewed in children (Lozoff & Georgieff, 2006; Pala et al., 2010; Thomas et al.,

2009). Some authors point out that the early iron deficiency can have a profound long-term effect on brain function, with possible irreversible brain damage at cellular and neuronal level (Youdim et al., 2010). Nevertheless, other authors believe that the impairment on cognitive development due to IDA may be equivocal because studies of children with iron deficiency are usually performed in developing countries which limit the interpretation of the data (Zimmermann & Hurrell, 2007).

- Susceptibility to infection

The incidence of infectious diseases is increased in iron-deficient populations because of the adverse effect of iron deficiency on the immune system (Allen et al., 2006). It has been reported that infections on upper respiratory tract happen more often and have a longer duration in anaemic than in healthy children (Zimmermann & Hurrell, 2007). However, it seems that changes in iron status can affect the immune response in multiple ways due to the influence of iron on the functioning of the immune system. It has been described that the bactericidal activity of macrophages is attenuated and that the number and activity of T-lymphocytes are decreased in iron deficient persons (Beard, 2001). Nevertheless, it has also been reported that depriving pathogens from iron is an important anti-microbial defence. This latter point could be important when supplementing iron to malaria endemic zones as the pathogen could benefit from the iron supply. It is also important to note that chronic immune activation can lead to alterations in iron homeostasis impairing erythropoiesis and contributing to immunopathology (Cherayil, 2010).

- Work capacity and productivity

The causal relationship between iron deficiency and physical work capacity may be related to a reduced oxygen transport associated with anaemia. Tissue iron deficiency may also play a role through reduced cellular oxidative capacity, thus impairing energetic efficiency (Haas & Brownlie IV, 2001). For example, several studies have reported a linear relationship between iron deficiency and work capacity for agricultural workers in Colombia, Guatemala and Indonesia (Allen et al., 2006). However, the work capacity returned rapidly to normal with iron supplementation. In addition, iron deficiency has been linked to a decreased aerobic performance in female military personnel (McClung et al., 2009) and athletes (Reinke et al., 2012).

Therefore, the high frequency of IDA in the developing world has a substantial economic cost. An analysis of ten developing countries indicated that the median value of physical productivity losses per year due to iron deficiency was about US$2.32 per head, or 0.57% of the gross domestic product (Horton & Ross, 2003).

- Pregnancy

Iron deficiency in childbearing women may contribute to maternal morbidity due to an increased susceptibility or severity of infections (Pavord et al., 2012). In fact, 40% of all maternal perinatal deaths are linked to anaemia (WHO, 2001). Nonetheless, the foetus is

relatively protected from the effects of iron deficiency by up-regulation of placental iron transport proteins; which at the same time increases the risk of maternal iron depletion and the risk of iron deficiency in the first three months of life. Infants of iron deficient mothers require more iron at an earlier age than it is supplied by breast milk. Besides, it seems that there is an association between maternal iron deficiency and preterm delivery (Pavord et al., 2012). Moreover, if pregnancy induced iron deficiency is not corrected, women and their infants suffer all the previously and later described consequences.

- Other consequences

Due to the crucial role of iron in several enzymes, iron-deficient persons have impaired gastrointestinal functions and, altered metabolism and patterns of hormone production. The latter include those for neurotransmitters and thyroidal hormones, which are associated with neurological, muscular, and temperature-regulatory alterations that limit the capacity of individuals exposed to the cold to maintain their body temperature. In addition, DNA replication and repair involve iron-dependent enzymes.

Moreover, it seems that there is also an increased risk of heavy-metal poisoning in children due to higher absorption capability of unspecific divalent metal ions (WHO, 2001). In addition, iron deficient individuals had impaired iodine and vitamin A metabolism (Allen et al., 2006).

Besides all the above mentioned consequences, iron deficiency has also been related with fatigue, lassitude and general tiredness sensation (Rattehalli et al., 2013), depressive symptoms and anxiety in women of reproductive age (Murray-Kolb, 2011), and pica which is characterized by an appetite for largely non-nutritive substances (Bryant et al., 2013). Moreover, some authors have related anaemia with heart failure and osteoporosis (Pasricha et al., 2010).

In another order of things, the global prevalence of IDA is compromising the blood transfusion services in many countries. Indeed, in some industrialized countries the thresholds of haemoglobin for being a donor had to be decreased in order to ensure the donations. However, in many developing countries even these reduced criteria would exclude many potential donors (Lewis & Emmanuel, 2010).

2.1.2. PREVALENCE AND GROUPS AT RISK

As it has been stated before, iron deficiency is known to be the most common and widespread nutritional disorder in the world. The WHO considered the IDA among the most important contributing factors to the global burden of disease in 2002. The burden of disease is a measurement of the gap between the current health status of a population and an ideal standard life expectancy where every person in the population would live into old age, free of disease and disability (Lewis & Emmanuel, 2010). There are almost no countries where

anaemia is not at least a mild public health problem in pre-school age children, women at their reproductive years and pregnant women (Benoist et al., 2008).

In industrialized countries, estimates of iron deficiency prevalence are derived from representative nationally samples with specific indicators of iron status. However, since haemoglobin concentration is relatively easy to determine, the prevalence of anaemia has often been used as a proxy for IDA in developing countries (Zimmermann & Hurrell, 2007). Although this approach may be useful, mostly in regions where IDA is the principal cause of anaemia, it has to be taken carefully since the aetiology of anaemia is more complex (chronic diseases, inherited anaemia, autoimmune or acquired haemolysis for instance in endemic malaria zones, etc.).

Most preschool children and pregnant women in non-industrialized countries, and at least 30-40% in industrialized world, are iron deficient (WHO, 2001). In addition, it has been reported that around 600-700 million people worldwide have marked IDA, living the bulk of these people in developing countries. In developed countries, the prevalence of IDA is much lower and usually varies between 2% and 8%. The global percentages of IDA prevalence are shown on Table 2.2.

IDA has been estimated to be responsible for around 50% of all anaemia cases. Also, it was estimated that the same amount of population had iron deficiency without developing anaemia worldwide (Allen et al., 2006). The last data available from the WHO point out that there are 1620 million people with anaemia (Table 2.2), meaning that 810 million people have IDA and a further 810 million people iron deficiency.

Table 2.2: Estimated global prevalence of anaemia and number of individuals affected (1995-2005)

Population group	*Prevalence of anaemia (%)*	*Population affected (million)*
Pre-school age children	47.4	293
School-age children	25.4	305
Pregnant women	41.8	56
Non-pregnant women	30.2	468
Men	12.7	260
Elderly	23.9	164
Total population	**24.8**	**1620**

Source: Benoist 2008

The main risk factors for developing IDA include low iron intake, poor absorption of iron from diet and periods of higher iron requirements, such as growth and pregnancy. The intake of iron is directly related to energy intake, thus the risk of developing iron deficiency is higher when iron requirements are greater than energy requirements (Zimmermann et al., 2007). The risk of iron deficiency may occur as a consequence of different situations, the most common ones are detailed as follows:

- Pathological and dietary

Iron deficiency can be consequence of pathological and/or environmental factors. The pathological causes described for IDA are blood losses from different causes such as regular nasal bleeding, menstruation, haemorrhoids, gastrointestinal ulcers and hookworm infections among others. Obesity has also been related with iron deficiency provided that subjects with high body max index (BMI) had higher iron deficiency prevalence (Zimmermann et al., 2008).

Regarding environmental factors, a given diet may be low in iron or contain adequate amounts of iron, but with low bioavailability. For example, diets rich in phytate or phenolic compounds impair iron absorption from the diet (Benoist et al., 2008).

- Population groups (children, women, women at their reproductive years and pregnant women)

On the subject of groups at risk of developing iron deficiency, women at their reproductive years are at risk of developing iron deficiency due to physiological losses (i.e. menstruation). Girls usually have their growth spurt before menarche, but growth is not finished at that time (WHO/FAO, 2004), which enhances the prevalence of iron deficiency in this population group. Therefore, there is usually a peak of prevalence of iron deficiency on adolescent females (WHO, 2001). It is important to point out that menstrual blood losses are very constant from month to month for an individual woman, but vary markedly from one woman to another.

Another population group with high iron deficiency prevalence is pregnant women. Nearly half of the pregnant women in the world are estimated to be anaemic: 52% in non-industrialized and 23% in industrialized countries (WHO, 2001). Moreover, most pregnant women are thought to suffer some degree of iron deficiency in industrialized countries. For example, a study made at *Hospital Universitari Sant Joan de Reus (Catalonia, Spain)* 45.7% of pregnant women presented iron depletion and 13.5% IDA at delivery (Arija et al., 2013).

During pregnancy, substantial amounts of iron are deposited in the placenta and foetus, which results in an increased need of about 700-850 mg in body iron over whole pregnancy, taking into account that the iron content on a body is 3-4 g; the increased need is really high. Additional iron is needed to supply the mother's expanding blood volume (>20%) and to support the needs of the growing foetus and placenta. Although menstruations stop during pregnancy and iron absorption is increased, still pregnant woman do not absorb sufficient iron and the risk of developing iron deficiency increases. Besides, lactation results in loss of iron via breast milk, which may perpetuate women's iron deficiency during lactation. However, the lactational amenorrhea usually compensates the iron losses through breast milk.

New-borns are normally born with adequate iron stores. The delayed cord clamping also improves iron status up to 6 months of age without adverse effect to the new-born (Moy, 2010). In addition, the iron content in breast milk is relatively low but much better absorbed

than in cow's milk. In consequence, iron deficiency usually develops after six months of age if complementary foods do not provide sufficient bio-available iron (WHO, 2001). The inappropriate early introduction of cow's milk and the low iron content of common weaning diets are the key factors for the development of iron deficiency in infants (Moy, 2010). Because of the marked supply of iron to the foetus during the last trimester of pregnancy, the iron situation is much less favourable in the premature and low-birth-weight infant than in the healthy term infant. An extra supply of iron is therefore needed in these infants during the first 6 months of life (WHO/FAO, 2004).

Iron requirements on a body weight basis are proportional to growth velocity. Thus, besides to women at their reproductive years, iron deficiency is more common in the preschool years and during puberty. The prevalence rate among preschool children is usually similar to, or higher than, the rate among pregnant women (WHO, 2001). It is estimated that worldwide, 600 million preschool children and school-age children are anaemic, and it is assumed that at least half of these cases are attributable to iron deficiency (Benoist et al., 2008). Even European children and adolescents are regarded to suffer from inadequate iron intakes (Serra-Majem, 2001).

- Socioeconomic

Iron deficiency is most commonly observed among groups of low socioeconomic status. It seems that the main source of variation in iron status in different populations is not related to a variation in iron requirements but to a variation in the absorption of iron from diets (disregarding infestations with parasites) (Allen et al., 2006). In this context, the incidence of iron deficiency is higher in Asia, which can be related with their food consumption model. In this continent, diets are usually poor in meat (high bio-available iron content) and rich in rice and tea and, therefore, rich in food ligands such as phytates and phenolic compounds that impair the bioavailability of dietary iron.

2.1.3. STRATEGIES TO PREVENT AND OVERCOME IRON DEFICIENCY

The high prevalence of iron deficiency, especially in developing countries, arise the need of developing strategies to restore iron normal levels of populations. There are no easy solutions to overcome iron deficiency and the interventions have their drawbacks. However, efforts should be addressed to overcome iron deficiency because of the extensive damages to health and wellbeing caused by micronutrient malnutrition, and more concretely iron deficiency. The elimination of this deficiency by whatever means should be regarded as a moral duty of governments, donors and international and national agencies.

Low dietary intake of bioavailable iron is an important factor in developing iron deficiency. Targeted interventions to provide iron to vulnerable population groups, in particular pregnant women and children are being implemented worldwide. Iron deficiency, like most deficiencies of public health concern, is mainly a consequence of poverty. However, even in

developed countries, it affects a significant proportion of people that are particularly vulnerable (WHO, 2001).

There are three main intervention strategies to prevent and/or overcome iron deficiency, namely dietary modification, administration of iron supplements and food fortification. These strategies are employed worldwide to overcome any micronutrient deficiency. Supplementation with pharmacological preparations (capsules, injections or parenteral routes) is a medically based approach whereas both diet modification and food fortification are food-based approaches. A food-based approach usually represents the most desirable and sustainable method of preventing not only iron deficiency but also micronutrient malnutrition (Lotfi et al., 1996). It is important to note that these strategies could be implemented alone or in combination (Zimmermann & Hurrell, 2007).

2.1.3.1. Dietary modification

The primary goal of dietary modification is to improve and maintain the iron status of a population. To do so, nutrition education programs seek for a dietary diversification and improvement of the target population. On this strategy, the recommendations given to the population should be directed to change dietary components, alter food processing or preparation, and modify meal patterns. These changes should focus on improving the bioavailability as well as the dietary amount of iron (WHO, 2001).

Efforts should be addressed to promote the availability and access to iron-rich foods, such as meat and organs from cattle, fowl, fish and poultry, legumes and green leafy vegetables. There are two kinds of dietary iron: heme iron and non-heme iron. In the human diet, non-heme iron is the main form and is obtained from cereals, legumes, fruits and vegetables. Its absorption ranks from 5-10% (WHO/FAO, 2004). Conversely, the primary sources of heme iron are the haemoglobin and myoglobin, which are more bioavailable forms (15-35%) that are obtained through consumption of meat, poultry, and fish. Heme iron can be degraded and converted to non-heme iron if foods are cooked at high temperature for too long.

Non-heme iron bioavailability is strongly affected by enhancers and inhibitors in the diet. Thus, these diets should include foods with components that enhance the absorption of iron and avoid those with inhibitors. Nutrition education may be needed to increase the demand and consumption of such foods to guarantee the effectiveness of these programs.

Enhancers of iron absorption include ascorbic acid, which is present in certain food groups such as fruits, tubers and vegetables. Ascorbic acid (vitamin C) is the most potent enhancer of non-heme iron absorption, even so that this effect is considered as one of vitamin C's physiological roles (WHO/FAO, 2004). There are two reasons that explain this role: ascorbic acid prevents the formation of insoluble and un-absorbable iron compounds and it has the ability of reducing ferric to ferrous iron, which is more easily absorbed by mucosal cells (Hallberg et al., 1989). Meat, fish and seafood also enhance non-heme iron absorption. The

promoting effect of meat, fish and seafood on its absorption has not been fully clarified but, besides providing heme iron, it stimulates non-heme iron absorption.

Common inhibitors of iron absorption include phytates, foods with high inositol content, iron-binding phenolic compounds (e.g. tannins) and calcium. Phytates are found in all kinds of grains, seeds, nuts, vegetables, roots and fruits (WHO/FAO, 2004). Chemically, phytates are inositol hexaphosphate salts serving as a source of storage of phosphates and minerals. Phytates strongly inhibit iron absorption in a dose-dependent manner and even small amounts of phytates have a marked effect. However, heme iron absorption is not affected by phytates.

Almost all plants contain phenolic compounds as part of their defence systems against insects and animals. Only some phenolic compounds (mainly those with galloyl groups) seem to be responsible for the inhibition of iron absorption (WHO/FAO, 2004). Tea, coffee, cocoa, green leafy vegetables, herbs and spices contain appreciable amounts of galloyl groups. As for the phytates, phenolic compounds do not affect heme iron availability.

Calcium, consumed as salt or in dairy products, interferes in the absorption of both heme and non-heme iron. However, as calcium is an essential nutrient, it cannot be considered to be an inhibitor of iron absorption in the same way as phytates or phenolic compounds. Besides, this interference has not been observed in dietary or long-time clinical intervention studies, suggesting an adaptive response (Bendich, 2001).

To sum up, the absorption of non-heme iron from a certain meal not only depends on its iron content but also, and to a marked degree, on the composition of the meal. The bioavailability can vary by more than 10-fold in meals with similar contents of iron, energy, protein and fat (WHO/FAO, 2004).

Regarding the cooking techniques, methods of food preparation and processing also influence on the bioavailability of iron (WHO/FAO, 2004). For example, food processing and culinary techniques such as germination and fermentation (e.g. sauerkraut and soy sauce) promote enzymatic hydrolysis of phytic acid in whole grain cereals and legumes thus reducing its content. Moreover, the use of non-enzymatic methods such as thermal processing, soaking and milling for reducing phytic acid content in plant-based staples have also shown to be successful (Huma et al., 2007).

With respect to alterations in meal patterns, there are simple but effective examples including:

- Separate tea drink from meal time (at least two hours)
- Include in meals fruit juices such as orange juice (source of ascorbic acid)
- Consume dairy products as a between-meal snack
- Avoid consuming foods containing inhibitors (such as fibre) at the same time with meals with high iron content

To conclude, the strength of this strategy is its potential to result in multiple nutritional benefits and achieve a long term sustainability (WHO, 2001). However, these recommendations should be adapted to dietary regional and local variations, the age of group concerned and seasonal availability. Although dietary modification and diversification is the most sustainable approach, changes in dietary practices and in preferences are difficult. Besides, foods that provide highly bio-available iron (such as meat) are expensive (Zimmermann & Hurrell, 2007). In addition, this strategy requires a relatively long time to achieve concrete results. Probably, this strategy to overcome iron deficiency is more suitable in industrialized countries, where it is easier to find all kinds of foods and the economy of the society is better than in developing countries.

2.1.3.2. Administration of iron supplements

Supplementation is the daily or periodic provision of relatively large doses of micronutrients, usually in the form of pharmacological preparations (pills, injections, capsules, syrups and tablets).

According to the literature, the most common iron forms employed in iron supplementation are ferrous salts, such as ferrous sulphate and ferrous gluconate, because of their low cost and high bioavailability. Standard therapy in adults consists in 300 mg per tablet of ferrous sulphate (60 mg of iron) three or four times per day (Zimmermann & Hurrell, 2007). Absorption of iron is enhanced when given on an empty stomach but nausea and epigastric pain might develop. Although food reduces absorption of medicinal iron by about two-thirds, this supplement should be provided with meals in case that those side effects arise or else lower doses between meals should be attempted.

In general, iron supplementation through daily or periodic administration of pharmacological preparations (injections, capsules and tablets) is an effective strategy because produces rapid changes in iron status. Besides, directs the iron to those segments of the population in greatest needs and can be cost-effective (Baltussen et al., 2004; Zimmermann & Hurrell, 2007). In situations when there is clinical urgency, iron supplementation is of immense value to saving sight and life. In these cases the iron is administered by parenteral route. However, oral administration of the supplements is more widespread to prevent and overcome iron deficiency.

In 2011, the WHO recommended the intermittent use of iron supplements as a public health intervention in settings where the prevalence of anaemia in preschool or school age children is 20% or higher. The aim of this intervention is to improve iron status and reduce the risk of anaemia among children (WHO, 2011).

The reason for intermittent supplementation is that intestinal cells turn over every 5-6 days and have limited iron absorptive capacity. Thus, the intermittent strategy would expose only the new epithelial cells to iron, which should improve the efficiency of absorption. In addition,

it would minimize blockage of absorption of other minerals due to high iron levels in the gut lumen and in the intestinal epithelium (WHO, 2011).

The experience in different populations has shown that intermittent regimens reduce the frequency of other side effects associated with daily iron supplementation and are also more acceptable to recipients, thus increasing supplementation programs compliance. It has been reported that untargeted iron supplementation in children from tropical countries, mainly in areas of high transmission of malaria, is associated with increased risk of serious infections (Zimmermann & Hurrell, 2007). By means of intermittent iron supplementation, the iron availability for the parasites growth is reduced and thus the risk of infections (WHO, 2011).

In pregnant women, the intermittent iron supplementation together with folic acid caused similar maternal and infant outcomes at birth as daily supplementation but the former was associated with fewer side effects (Peña-Rosas et al., 2012). Intermittent iron supplementation has been proved to improve haemoglobin concentrations and reduce the risk of having anaemia or iron deficiency in various groups, for example children younger than 12 years (De-Regil et al., 2011) and Mexican women (Fernández-Gaxiola & De-Regil, 2011). However, the intermittent supplementation on those groups was found to be less effective than daily supplementation to prevent or control anaemia. On the other hand and in comparison with a placebo or no intervention, it seems that the intermittent supplementation is more effective as more severe is the iron deficiency. Thus, this strategy has been shown to be quite effective for IDA, less effective for improving haemoglobin and ferritin levels and almost no effective for iron deficiency (WHO, 2011).

In industrialized countries, universal iron supplementation of pregnant women is widely advocated even though so far little evidence exists on the improvement of maternal or foetal outcomes. For instance, Cogswell et al. (2003) and Siega-Riz et al. (2006) performed controlled trials of prenatal iron supplementation in iron-replete, non-anaemic low-income pregnant women in the USA. The authors reported that the iron supplementation increased birth weight and/or reduced incidence of preterm delivery, but did not affect prevalence of IDA during the third trimester.

However, the major limitation of iron supplementation programs is the logistic distribution (Zimmermann & Hurrell, 2007) and the difficulty in maintaining compliance (Hurrell & Cook, 1990). Besides, iron supplementation requires an effective system of health delivery that is elaborate and costly to maintain (Cook & Reusser, 1983). In addition, iron supplementation just reaches concrete segments of populations, which may be helpful in some ways but also a limitation.

2.1.3.3. Food iron fortification

Food fortification refers to the addition of micronutrients to processed foods and is one of the main strategies that can be used to improve micronutrient status (Allen et al., 2006).

Fortification should be viewed as part of a range of measures that influence the quality of food including improved agricultural practices (also comprising bio-fortification of corps), improved food processing and storage, and improved consumer education to adopt good food preparation practices. This strategy is acknowledged for reducing the severity of certain nutritional problems from a public health perspective in which iron, among others, is present (Serra-Majem, 2001).

Iron fortification is probably the most practical, sustainable, and cost-effective long-term solution to control iron deficiency at national level (Hurrell & Cook, 1990). The initial cost is modest and the maintenance expenses is far less than that of supplementation (Cook & Reusser, 1983). Cost-effectiveness for iron fortification is estimated to be $66-70 per disability-adjusted life years (DALY) (Zimmermann & Hurrell, 2007). Being the DALY a measure of the number of years lost by disability or early death due to ill. Table 2.3 summarizes some of the advantages of fortification over high-dose supplementation.

Table 2.3: Advantages of food fortification over high-dose supplementation.

	Supplementation	*Food Fortification*
Effectiveness and timeframe	Effective strategy usually for short term	Effective medium to long term measure
Delivery requirements	An effective and health delivery system	A suitable food vehicle and already instituted manufacturing and distribution facilities
Coverage	Reaches only populations receiving the service	Reaches all segments of target population
Compliance	Requires sustainable motivation of participants	Does not require intensive cooperation or compliance of individuals
Cost of maintenance	Relatively high financial resources needed	Low cost compared to supplementation; to maintain the system self-financing in the end
External resources	Foreign currency or external support required for obtaining supplements	Adequate technology that is locally available or can be easily transferred
Sustainability	Relies to compliance and existing resources	Fortificant compounds may need to be imported

Source: Lotfi et al. 1996

Food iron fortification can reach all segments of the population and does not require the cooperation of the individual (Cook & Reusser, 1983). Universal iron fortification is generally recommended for those developing countries where the risk of developing iron deficiency is high for all groups other than adult men and postmenopausal women. However, infants and young children in developing countries are at high risk of iron deficiency and might not be reached by universal fortification programs (Uauy et al., 2002; Zimmermann & Hurrell, 2007).

Thus, targeted fortification of food products seems to be a good approach on overcoming iron deficiency in these population groups.

Regarding to this matter, food fortification programs can be classified as follows: (i) mass fortification, (ii) targeted fortification and (iii) market-driven fortification.

The (i) mass fortification is the fortification of foods widely consumed by the general population. Mass fortification is generally selected when the majority of the population is at risk of becoming iron deficient. The basic commodities (flour, sugar and salt) are more suited to mass fortification. In general it requires a strong collaboration between academia/researchers, governments and food producers to succeed (Harvey & Dary, 2012; Mehansho, 2002).

(ii) Targeted fortification refers to the act of fortify foods designed for specific population sub-groups. Therefore, the iron intake of that particular group will be increased. Examples include complementary foods for infants and foods developed for school feeding. Commonly, the fortified food should reach a substantial proportion of the daily iron requirement of the targeted group.

Finally, the (iii) market-driven fortification is the voluntary fortification of food by manufacturers and usually is a business-oriented initiative. Although it is voluntary, governments set regulatory limits.

Some examples of the effectiveness of iron fortification are the mass-fortification of refined flour with non-heme iron in Sweden and the USA which reduced the iron deficiency anaemia prevalence in these countries (Lotfi et al., 1996). For instance, the iron deficiency rate in Sweden has been reduced from about 25-30% to 7% as a result of the iron fortification of wheat flour. About 40% of the iron consumed in Sweden and 20% of that in North America comes from fortified wheat flour and bakery products. In the US, besides of the flour iron fortification, infant formulas are fortified (i.e. 80% of all infant formulas sold in 1985) resulting in the reduction of the prevalence of anaemia in infants of both low-income and middle-class populations. Other foods that had been fortified with iron are milk and milk powders, biscuits, rice flour, salt, fish sauce and curry powder (Lotfi et al., 1996; Zimmermann & Hurrell, 2007).

The main problem on assessing the efficacy of iron fortification programs is related to various factors, such as:

- Use of iron compounds with low bioavailability or without proper enhancement of iron from inhibitory diets
- Inadequate iron fortification
- Consumption of fortified food too low to deliver adequate iron amounts
- High frequency of parasitic infections

Besides, there might have been failures in the detection of effectiveness by failure in the definition of iron status, failure to recognize other causes of anaemia not related with iron

deficiency, as haemolysis (for instance in malaria endemic zones), or poor program control and enforcement (Zimmermann & Hurrell, 2007).

The effectiveness of a fortification program can be assessed by means of efficacy trials and/or reports of program effectiveness. The efficacy trials are conducted controlling feeding situations. They are relatively numerous and have useful documented impact. Evidence of program effectiveness is obtained by assessing changes in nutritional status and other outcomes once a program has been implemented; however, the information about these programs is less widely available (Allen et al., 2006).

We can find in the literature several efficacy trials on non-heme iron fortification. For instance, in Vietnam the iron fortification of fish sauce improved the iron status and reduced anaemia and iron deficiency on non-pregnant anaemic female workers (Van Thuy et al., 2003). Iron fortified rice (Ultra Rice®) reduced the prevalence of anaemia of women in Mexico (Hotz et al., 2008). In India, the wheat flour fortification with iron reduced the prevalence of both iron deficiency anaemia and iron deficiency in children after 7 months of intervention (Muthayya et al., 2012). In addition, positive results have been reported in the assessment of fortification programs of infant formulas in the USA (Fomon, 2001) and wheat and maize flours in Venezuela (Layrisse et al., 1996).

It is important to note that iron fortification is more difficult than any other micronutrient fortification, such as iodine or vitamin A, because the most bioavailable iron compounds are soluble in water and often react with other food components causing off-flavours, colour changes and lipid oxidation. Since the population will seldom accept the fortified vehicle if the added iron changes the organoleptic characteristics, inert iron compounds are commonly used. However, these iron sources are poorly absorbed and have little effect on iron status (Cook & Reusser, 1983).

In order to improve the acceptability of iron fortified products and without compromising the bioavailability of the iron compound employed, strategies to disguise the iron in the food matrix are needed. Thus, there are many technical difficulties in fortifying the diet with iron, being the most important the identification of a form of iron that is adequately absorbed and yet does not alter the appearance or taste of the food vehicle.

Important facts to have into account when planning an iron food fortification program are the selection of the iron compound and the type of fortification (general or targeted). These two facts should be selected on the basis of the fortification vehicle, iron requirements of the target population and iron bioavailability of the local diet. These facts will be extensively reviewed in the following section.

In order to guarantee the success of food fortification programs it is often required implementing public health measures of a more general nature to help prevent and correct micronutrients deficiencies. Such measures include infection control and improvement of water and sanitation. Other factors, such the quality of child care and maternal education also need to be taken into consideration (Allen et al., 2006). Because consumer perception of iron

fortified foods is associated with nutrition knowledge, this suggests that nutrition education may be crucial on consumer choices regarding fortified food products (Pounis et al., 2011).

2.2. Food fortification with iron

The process of addition nutrients to foods has been named with several terms such as fortification, enrichment or nutrification. Usually, these terms had been used interchangeably; however, the joint FAO and WHO expert committee on nutrition considers the term fortification to be the most appropriate to describe the process whereby macro- or micro-nutrients are added to foods commonly eaten to maintain or improve the nutritional quality of individual foods in the total diet of a group, community or population. Thus, fortification is the term used in this text.

Food fortification has a primary role in the prevention of the iron deficiency. In their *Guidelines for food fortification with micronutrients*, the WHO/FAO state the following public health benefits of iron fortification (Allen et al., 2006):

- Prevention or minimization of the risk of occurrence of iron deficiency in a population or specific population groups
- Contribution to correction of a demonstrated iron deficiency in a population or specific population groups
- A potential for an improvement in nutritional status and dietary intakes that may be, or may become, suboptimal as a result of changes in dietary habits/lifestyles
- Plausible beneficial effects of iron consistent with maintaining or improving health.

It is important to bear in mind that according to the European Comunities Commission (2008), in order to be able to qualify a food product as fortified, there is need to add at least 15 % (per serve or 100 g of product) of the daily recommended intake (DRI) that has been set for iron at 14 mg/day by the same European Commission thus representing the amount of 2.1 mg.

The success of any iron-fortified food intervention depends on several factors such as the consumption pattern of the fortified food, the effect of added fortificants on the taste and appearance of the food vehicle, the shelf-life of the fortified food, the bioavailability of the iron fortificant and the baseline iron status of the population, among others (Gera et al., 2012).

Technically speaking, the food that carries the nutrient is called the vehicle, whereas the nutrient (iron compound) is called the fortificant. The selection of a suitable combination of food vehicle and fortificant, concretely the chemical form of the micronutrient, is fundamental to any food fortification program. The appropriate combination food-vehicle/fortificant will minimize the need of encouraging individual compliance or changes in customary diets.

Apart from the three main strategies of food fortification described previously, there are other particular strategies for iron fortification that are being lately used, named: household and community fortification, and bio-fortification of staple foods.

The household and community fortification are considered a combination between supplementation and fortification, and are based on the addition of a supplement rich in iron

to either food cooked at home or produced in a small community. With respect to household fortification, the products that can be fortified with iron are multiple. To do so, we can find soluble or crushable tablets or micronutrient-based powders, usually called "sprinkles". The use of crushable tablets and fortified powders are expensive ways of increasing micronutrient content in foods when compared with mass fortification. However, their use may be especially useful for improving local foods fed to infants where universal fortification is not possible.

Fortification of foods at the community level is still an experimental technique. Nowadays, it is under research the addition of micronutrient premixes at adequate doses to small batches of flour during the milling process (Allen et al., 2006). This community level fortification is being supported by initiatives such as the "Flour Fortification Initiative" where different partners (public, private, and civic) are collaborating.

Regarding their effectiveness, a systematic review pointed out that home fortification of foods is an effective intervention to reduce anaemia and iron deficiency in children from six months to 23 months of age (De-Regil et al., 2013). Similarly to iron supplementation programs, there is need to achieve good distribution network for the micronutrient supplement that is not easy to accomplish.

Bio-fortification of staple foods via plant breeding or genetic engineering is another food fortification technique. The variation in the iron content of cultivars of wheat, bean, cassava, etc. suggested that selective breeding might increase iron content of staple foods.

There are three main bio-fortification strategies that can be applied to crops, to favour iron absorption from the human diet: (i) reduce the concentration in food ligands (such as phytic acid and polyphenols) that impair the absorption of dietary iron; (ii) increase the concentration in compounds that may favour iron absorption; and (iii) direct increase of the iron concentration (Murgia et al., 2012).

By genetic engineering the amount of absorbable iron in plant foods could be incremented by introduction of ferritin gene from soybean, and phytic acid content could be lowered by introduction of phytase gene from *Aspergillus fumigatus* (Zimmermann et al., 2007). There is some evidence of stimulatory effects of inulin on dietary iron absorption in rats and weaning piglets; thus increasing the concentration of inulin and ascorbic acid in crops may favour the iron absorption (Murgia et al., 2012).

Breeding of nutrient-rich staple food crops is indeed the main goal of different international consortia, such as HarvestPlus (http://www.harvestplus.org/), who aim to reduce micronutrient malnutrition (provitamin A, zinc and iron) through different bio-fortification programs including the dissemination of iron-rich bean varieties in Rwanda and Congo and iron-rich pearl millet varieties in India. AgroSalud (www.agrosalud.org) is another international consortium supporting the production and dissemination of iron- and zinc-rich bean and rice varieties in Latin America and in the Caribbean.

The huge range of opportunities for fortifying a food product with iron will be reviewed in the following sections. Concretely, they will be focused on the addition of a certain source of iron (fortificant) to a food product (vehicle), which is the common procedure in mass, targeted and market-driven fortification strategies.

2.2.1. IRON SOURCE SELECTION

The selection of the iron source for food fortification has to be addressed carefully. There are general criteria that should be taken into account when selecting an iron compound for a fortification program (Lotfi et al., 1996). The iron form selected should present good bioavailability during normal shelf life of the fortified product. It is important that the amount of bioavailable iron remains constant during the storage time in order to ensure the correct consumption amount, regardless of the time lapse. Besides, the selected iron compound should not interact with the flavour or colour of the vehicle. The organoleptic characteristics of the product must not change because it will not be consumed otherwise.

The form of iron and/or technology used to fortify foods should have an affordable cost. The final price of the fortified food should be low because the price of the fortified food should be not higher than that of the non-fortified to avoid any bias in the consumption pattern.

However, the main problems associated with the selection of an iron form for the fortification of a food product are its bioavailability and its interaction with the food matrix. Heme iron is regarded as more bioavailable (absorption, 15-35%) than non-heme iron (5-10%), which also is influenced by the ligands that may be present in the meals impairing its absorption. In this context, the selection of an iron compound that is both unobtrusive and well absorbed is a critical step. Besides, it is important to ensure the stability of the iron compound during the storage or processing of the fortified product, as ferrous sources are susceptible of being oxidized (mainly depending on the pH but also on temperature and moisture content) leading to ferric forms that may form complexes that diminish their bioavailability and produce brownish discoloration (Martínez-Navarrete et al., 2002).

The inorganic (non-heme) iron compounds that can be employed in food fortification usually are sub-divided into their different solubility in water (Table 2.4). This is so because iron bioavailability is directly related with its solubility. Thus, those non-heme iron compounds soluble in water are more bioavailable than those that are insoluble. Therefore, the iron compounds must be dissolved in the gastric fluids in order to be absorbed by the enterocytes for being bioavailable. Nevertheless, those compounds with higher water solubility are also more reactive. Therefore, its addition to a food product may develop off-flavours and rancidity and thus reduce its self-life.

Table 2.4: Major non-heme iron compounds used in food fortification and their relative bioavailability and cost

Iron compound	*Relative bioavailability in men*	*Relative cost (per mg iron)*
Water soluble		
Ferrous sulphate. $7H_2O$	100	1
Ferrous sulphate, dried	100	1
Ferrous gluconate	89	6.7
Ferric ammonium citrate	51	4.4
Ferrous ammonium sulphate	NA*	2
Ferrous lactate	67	7.5
Ferrous bisglycinate	>100	17.6
Poorly water-soluble, soluble in dilute acid		
Ferrous succinate	92	9.7
Ferrous fumarate	100	2.2
Ferric saccharate	75	8.1
Water-insoluble, poorly soluble in dilute acid		
Ferric orthophosphate	25-31	4
Sodium ferric pyrophosphate	NA	17
Ferric pyrophosphate	21-74	4.7
Elemental iron	13-90	1.5
H-Reduced	13-148	0.5
CO-Reduced	12-32	<1
Atomized	24	0.4
Electrolytic	75	0.8
Carbonyl	5-20	2.2
Encapsulated forms		
Ferrous sulphate	100	10.8
Ferrous fumarate	100	17.4
Complexes		
NaFeEDTA	>100	16.7

Source: Allen et al. 2006 and Lotfi et al. 1996

In this table the relative bioavailability is calculated considering 100 to that of the ferrous sulphate. Relative cost is calculated considering 1 to that of ferrous sulphate.

* NA: Not available

2.2.1.1. Non-heme iron compounds used in food fortification

The addition of inorganic iron compounds to food products is authorized around the world. In the European Union, its addition is regulated by the directive 1925/2006 (European Parliament and Council, 2006) and amended by the commission regulation number 1161/2011 (European Commission, 2011). On these regulations, the iron forms authorized

for iron fortification are the following: ferrous carbonate, ferrous and ferric citrate, ferrous gluconate, ferrous fumarate, sodium ferric pyrophosphate, ferrous lactate, ferrous sulphate, ferrous ammonium phosphate, ferric sodium EDTA, ferric pyrophosphate, ferric saccharate and elemental iron (H-Reduced, electrolytic and carbonyl).

Water-soluble iron compounds are quite often the preferred choice. Almost all of the water-soluble iron compounds have high bioavailability and ferrous sulphate is the cheapest one (Cook & Reusser, 1983; R. Hurrell, 2002). This fact explains why this iron form is the most employed source of iron to fortify foods. However, soluble ferrous salts often produce colour changes by forming complexes with sulphur compounds, tannins, polyphenols and other food substances, which make especially difficult the fortification of white food products such as rice, salt or sugar. Moreover, reactive iron compounds catalyse oxidative reactions resulting in undesirable odours and flavours and may impair metallic taste to the fortified food.

Because of ferrous sulphate high reactivity, its use is limited to fortified foods that have a relatively fast turnover such as bread or cereal flours. Water-soluble compounds are also used in dry foods, like pasta, milk powder or milk based infant formulas. Nevertheless, when a vehicle wants to be used there is need to evaluate its suitability previously due to iron compounds metallic taste and catalytic oxidation properties (Allen et al., 2006).

The bioavailability of those iron compounds that are poorly water-soluble but soluble in dilute acid is lower. However, their addition produces less organoleptic changes in the fortified food. Among those forms, both ferrous fumarate and ferric saccharate are the most used because its bioavailability in adults is similar to that of ferrous sulphate and its addition produces less organoleptic modifications in the fortified product. However, their price is higher than that of ferrous sulphate. As these compounds are soluble in dilute acid, like the gastric juice during digestion, its bioavailability will not be very different from those water-soluble iron compounds (R. Hurrell, 2002). Nonetheless, as infants may secret less acid to the gastric juice, there is some concern on its addition to infant formulas or cereals because its bioavailability may be compromised (Allen et al., 2006). However, it has been also described that beta-carotenes increase the solubility of these compounds at neutral pH (Boccio & Iyengar, 2003).

With respect to those compounds that are insoluble in water and poorly soluble in dilute acid, they are poorly absorbed and thus the last choice for iron fortification. Their main drawback is that they dissolve slowly and not completely in the gastric juice during digestion (R. Hurrell, 2002). In spite of the lowest bioavailability, water-insoluble iron compounds are extensively employed by the industry because they cause fewer organoleptic modifications and some are cheaper than the more soluble compounds (especially elemental iron compounds) (Allen et al., 2006; Boccio & Iyengar, 2003). There are two kinds of insoluble iron forms, phosphate compounds and elemental iron. The bioavailability of ferric phosphate could be modified during the processing of the food product (Allen et al., 2006) and that of elemental iron depends on its physical characteristics (particle size, shape and surface area) (R. Hurrell, 2002).

In this context, there are different strategies to increase the amount of non-heme iron absorbed in fortified products. As has been stated in the previous section, the absorption of non-heme iron depends on the proportion of inhibitors of iron absorption in the diet. Therefore, the bioavailability of iron fortified food products would be increased by selecting those food vehicles with low content in phytate and phenolic compounds.

Another strategy commonly employed is the addition of ascorbic acid. This vitamin increases the absorbability of various non-heme iron compounds such as ferrous sulphate, ferric chloride, ferric ammonium citrate, ferrous fumarate, ferric orthophosphate and electrolytic iron (R. Hurrell, 2002). Its addition in 2:1 molar ratio (ascorbic acid: iron) increases iron absorption by 2- to 3-folds. It should be taken into account that its addition may also increase the cost of production and thus the cost of the fortified product. In addition, ascorbic acid is a reactive compound that will be lost during the storage of the fortified food or production (it is easily oxidized and thermo labile). Thus, it should be added to foods that are not processed and have a short self-life (Allen et al., 2006; S. Lynch et al., 2002). Alternatively, the ascorbic acid derivative ascorbyl palmitate (AP), which is resistant to higher temperatures, has also been proven to enhance iron absorption (14.6 % more absorption when AP is added at 2:1 ratio (AP:Fe) whereas at 4:1 ratio this is increased to 20.2%) (Pizarro et al., 2006).

The addition of sodium ethylenediaminetetracetic acid (NaEDTA) has been used also to increase non-heme iron bioavailability. NaEDTA is stable during the storage and at low pH chelates iron thus avoiding its binding to phytates or phenolic compounds. Therefore, the addition of NaEDTA enhances the iron absorption in fortified foods rich on iron inhibitors. As for the ascorbic acid, iron absorption is increased by 2- to 3- folds when NaEDTA is added in a 0.5-1 (molar ratio NaEDTA : iron). Nevertheless, it is important to keep in mind that NaEDTA enhances the absorption of both food iron and soluble iron fortificants, but not that of the relatively insoluble iron compounds such as ferrous fumarate, ferric pyrophosphate or elemental iron (Allen et al., 2006; S. Lynch et al., 2002).

2.2.1.2. Heme iron compounds used in food fortification

Non-heme iron ingredient is currently authorized to be added for the fortification of foods in the European Union. However, the use of heme iron concentrates is only legal as a source of iron for food fortification in countries such as Japan and Mexico (EFSA Panel on Food Additives and Nutrient Sources added to Food, 2010; Polo & COFEPRIS, Federal Commission for the Prevention of Health Risk, 2007).

The Japanese government approved the use of a heme iron concentrate as a FOSHU ingredient. FOSHU is the Japanese term for a functional food or a food with a specific health application. The concentrate is a heme iron polypeptide (HIP) obtained by enzymatic hydrolysis and subsequent concentration from pig haemoglobin. In the approval of this concentrate as FOSHU ingredient it is recognized that heme iron is a highly bioavailable source of iron in human nutrition

(http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html). In addition, a HIP product is currently being marketed by Colorado BioLabs Inc. in the United States under the Proferrin brand (www.coloradobiolabs.com). Besides, the Mexican government, through the Federal Commission for the Prevention of Health Risks (COFEPRIS), recently approved another HIP similarly obtained from pig haemoglobin, named AproFer 1000™, as a safe ingredient for use as a heme iron fortificant in foods. Moreover, other heme iron based products had been used in Europe as supplements. Several examples are: Hemofer in Sweden, HemFerin in Germany and GlobiFer in England and Ireland.

Heme iron compounds employed in fortification of foods are commonly co-products coming from blood obtained in bovine or porcine slaughterhouses. Heme iron polypeptides (HIPs) are heme concentrates obtained from the hydrolysis of haemoglobin with unspecific proteases and further separation of globin peptones from the heme fraction. The heme fraction contains the porphyrin complex bound to amino acid chains that make this iron more available (Polo & Rodriguez, 2013). These heme iron ingredients usually contain low quantities of iron (≈1%). However, the technology has currently improved and the iron content has increased in the last decade (1.45%) (Quintero-Gutiérrez, González-Rosendo et al., 2008). In Figure 2.2 is represented the equivalence of 1 g of HIP (1.4% iron) to other sources of iron.

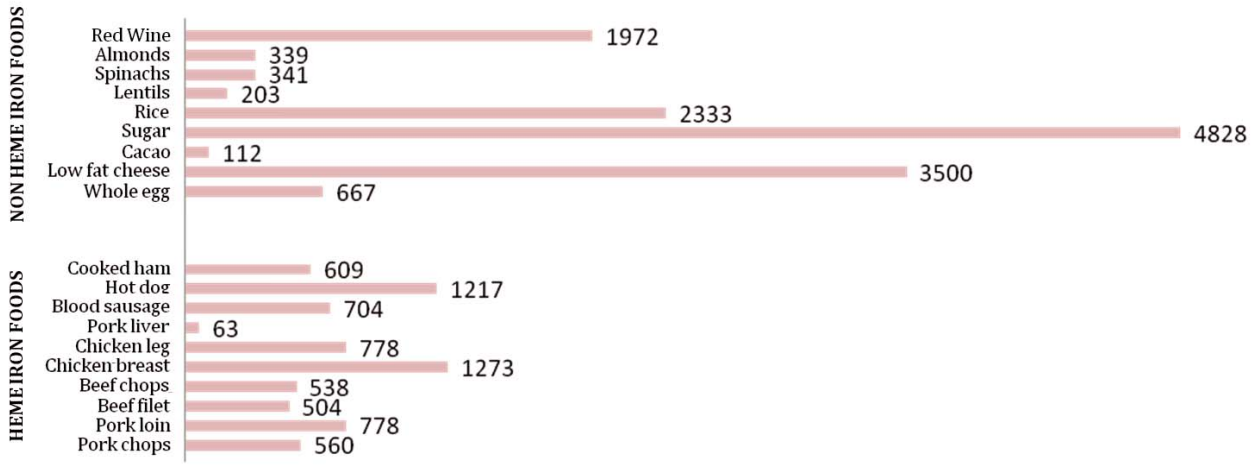

Figure 2.2: Equivalence of 1 g of heme iron polypeptide (1.4% iron) to other sources of iron (g), considering bioavailability. Source: Polo & Rodriguez, 2013.

There are few studies in the literature reporting the bioavailability of heme iron fortified products in either humans or animals. However, heme iron is a very attractive fortificant due to its predictable high bioavailability. It has been reported that in iron-replete individuals, 15% of the total heme iron consumed is absorbed whereas in iron-deficient individuals the absorption is increased up to 35% (R. Hurrell, 2002). In 2004, the European Food Safety Authority (EFSA) stated that heme iron absorption was higher than that of inorganic iron.

The only mineral that may impair the heme iron absorption is calcium; however, as calcium is also an essential mineral and its inhibitory effect is several orders of magnitude higher for non-heme iron, its incidence in heme iron absorption is not considered in the same way as non-heme iron absorption inhibitors (Polo & Rodriguez, 2013). Moreover, an adaptive response is suggested because no interferences have been observed in dietary or long-time

clinical intervention studies (Bendich, 2001). EFSA also stated that heme iron absorption was not affected by other substances in the diet and that absorption was only regulated by levels of iron already present in the body (EFSA, 2004).

Regarding the HIPs, it has been pointed out that the globin peptide residues around the heme group may affect the absorption of heme iron. In fact, on a study with rats, the hydrolysis done with the enzyme subtilisin increased the absorption of iron compared with the absorption of the HIP obtained with pepsin (Vaghefi et al., 2002). In addition, it has been reported that when the hydrolysis of haemoglobin is higher than 8%, iron absorption is enhanced compared with non-hydrolysed haemoglobin (Vaghefi et al., 2000).

It is also known for a long time ago that heme iron is not absorbed by the same mechanism as inorganic iron, and that the intact heme molecule enters the enterocyte before releasing its iron (Hurrell & Cook, 1990). The iron absorption process has been described as the pool concept, which implies that there are two main pools in gastrointestinal lumen: one pool for heme iron and another pool of non-heme iron, and that iron absorption takes place independently from each pool (WHO/FAO, 2004). Nevertheless, the absorption of heme iron cannot up regulate to same extent as non-heme iron during iron deficiency (West & Oates, 2008). In fact, it has been reported that the heme iron absorption is saturable, whereas absolute amount of absorbed non–heme- iron progressively increases with intake (Pizarro et al., 2003).

Although the water solubility of HIPs is usually less than 50%, heme iron modifies the organoleptic properties of the fortified food similarly to the more water-soluble inorganic iron compounds described previously. As other iron forms, HIP catalyses the lipid oxidation of the food matrix, causing off-flavours and reducing the self-life of the fortified product. Besides heme iron has a dark black-red colour that challenges its addition into food products.

In spite of these difficulties, heme iron ingredients had been successfully used in the fortification of wheat cookies in Chile and Brazil (Asenjo et al., 1985; Lamounier et al., 2010; Walter et al., 1993). Indeed, the consumption of these products improved the iron status of school-age children. Another example of heme iron fortification was reported by González-Rosendo et al. (2010), where chocolate filled biscuits were given to adolescent girls in Mexico. Other food products that had been tried to be fortified with heme iron are baby food in jars mixed with cereals, pâtés or meat products (Polo & Rodriguez, 2013).

2.2.2. FOOD VEHICLE SELECTION

The selection of the combination between the fortificant and the vehicle is crucial for the correct iron fortification program. However, various authors consider the selection of the vehicle as the most crucial step. The selection of the food vehicle has to consider the pattern of consumption, the technical feasibility and the marketing of its fortification (Table 2.5).

Table 2.5: Criteria for selecting a food vehicle

Consumption	High proportion of the population covered
	Regular consumption in relatively constant amounts
	Minimal variation in consumption pattern between individuals
	Minimal regional variation in consumption pattern
	Appropriate serving size to meet a significant part of daily dietary requirements of the micronutrient added
	Consumption not related to socioeconomic status
	Low potential for excessive intake (to avoid any probable toxicity)
	No changes in consumer acceptability after fortification
	No change in quality (in broad sense) as a result of micronutrient addition
Processing/storage	Centralized processing
	Simple low-cost technology
	Good masking qualities (dark colour and strong odour of the food help to mask slight changes)
	High stability and bioavailability of added micronutrient in the final product
	Minimal segregation of the fortificant and the vehicle
	Good stability during storage
	No micronutrient interaction
Marketing	Appropriate packaging that will ensure stability
	Labelling according to prescribed standards
	Adequate turnover rate

Source: Lotfi et al., 1996

Overall, the consumption of the food vehicle should be high in the population at risk of developing iron deficiency. If the aim is a mass fortification, staple foods, such as dairy products and flours may be the selected vehicles. However, when targeted fortification is pursued, there is need to select a product that is commonly eaten by the target population group. At the same time, it is necessary that the selected fortified product would have little potential for excessive intake in order to avoid any possible iron overload. Preferably, if the food vehicle is fortified with non-heme iron, it should be able to be eaten along with different meals because percentage of absorption varies between meals.

It is also important that the selected vehicle should be consumed evenly in a region or country. Additionally, if any economic bias may happen during the production, it should favour the low-income groups, because usually they are the most affected by iron deficiency (Cook & Reusser, 1983).

Regarding the technical considerations, the food vehicle should be centrally processed in order to favour the control of the production. The rest of the considerations mentioned in

Table 2.5, namely good masking characteristics, stability and no micronutrient interactions are crucial in order the product to be consumed, accepted and effective.

Finally, the marketing followed by producers should be clear and rigorous in order to establish a confident relationship between producers and consumers. Also, the formulation of the product, for instance addition of antioxidants and its concentration would play a pivotal role on the stability of the product (discussed further in this text). Regarding this matter, consumers concerns about synthetic additives added to the product should be taken into account. Additionally, the use of encapsulated iron forms should be considered when necessary as it may increase also the stability of the fortified product. Finally, the packaging conditions should be the appropriate to ensure the stability of the product till the expiration date labelled.

There are several food products that have been fortified with iron. Following there are some examples of fortified vehicles.

- Wheat and maize flour

Flour, bread and cereals are suitable vehicles for mass iron fortification because they are frequently consumed, especially in Western countries. Bread fortification is of particular interest due to its low risk for organoleptic deterioration because of its low lipid content and its fast turnover.

Regarding wheat flour fortification it is simple, cheap, and a major strategy for preventing anaemia (Huma et al., 2007). The iron content in 70% extraction flour is about 11–12 mg Fe/kg and this content is fortified up to 44 mg/kg, which is the approximate content of whole-wheat grains (Hurrell, 1997). Also as a result of government regulations, the fortification of milling cereals (wheat and maize) has been adopted in several countries in Latin America and the Caribbean (Lotfi et al., 1996). It is important to note that flours contain a high content of phytates thus likely compromising the fortification effectiveness (Lotfi et al., 1996). In spite of this, an efficacy trial in Thailand has recently demonstrated its applicability. In this case, the subjects of study were women, the vehicle was wheat flour cookie and the selected fortificants were electrolytic iron and ferrous sulphate (Allen et al., 2006).

The addition of NaFeEDTA to wheat and maize flour was tried. However, it seems that this iron form in wheat flour interferes with the bread fermentation. Therefore, its use was recommended in maize flour. Nevertheless, this water-soluble iron form rapidly oxidizes the maize flour and, in addition, the iron absorption from corn dough is strongly inhibited (Allen et al., 2006).

In the guidelines for iron fortification of cereal-based staples, the use of ferrous sulphate is recommended as preferred, followed by ferrous fumarate and electrolytic iron (added at twice concentration than the others) (Allen et al., 2006). The maximum ferrous sulphate that can be added to flour for bakery is 40 mg/kg, and the flour should not be stored for more than 3 months (Lotfi et al., 1996). Frequently flours are added with ascorbic acid, more as rising

agent than as an enhancer of the absorption provided that it is degraded during the baking process of the product (Allen et al., 2006).

Apart from non-heme compounds, some research has been performed on flour fortification with heme liposomes in which heme iron coming from blood obtained in slaughterhouses was encapsulated in lecithin : cholesterol liposomes (Albaldawi et al., 2005).

The majority of studies in the literature are focused on the effectiveness of intervention trials (Andang'o et al., 2007; Layrisse et al., 1996; S. R. Lynch et al., 2007; Miglioranza et al., 2008; Muthayya et al., 2012; Nestel et al., 2004; Van Stuijvenberg et al., 2008; Van Stuijvenberg et al., 2007) and the bioavailability of iron compounds that have been employed in fortification of flours (Troesch et al., 2011 and Yameen et al., 2013) (Table 2.6).

Nevertheless, we can also find some studies focused in the technological aspects of flour iron fortification (Kiskini et al., 2011 and Tripathi et al., 2011) (Table 2.7). For instance, in the study of Kiskini et al. (2011), the effects of different iron compounds on the colour, crust firmness and sensory attributes of gluten-free breads are studied. These authors concluded that electrolytic iron was the only compound of the assessed that is suitable for its fortification. In Tripathi et al. (2011) the use of fortified flours on sensory and texture of different kinds of breads (dumpling and roti) was assessed. Both preparations were acceptable for both texture and sensory, however dumpling discoloration was the main drawback.

- Milk and milk powder

There are several countries were milk iron fortification is mandatory. The target group of this fortification is mainly young children (Lotfi et al., 1996).

The addition of iron in this food product is usually done together with ascorbic acid. It is important to note that ferrous sulphate cannot be used in liquid whole fat milk because it causes rancidity. In this case, ferrous ammonium citrate, ferrous glyscinate and micronized ferric pyrophosphate are more suitable. The fortification of liquid milk is recommended after the homogenization process and before pasteurization in order to minimize the lipid oxidation (Allen et al., 2006). With respect to milk powders, these were successfully fortified with ferrous sulphate.

Several studies aimed the development of milk iron fortified beverages. Among them, we can find examples of bioavailability trials (Contreras et al., 2013; Trinidad et al., 2014; Walczyk et al., 2013) (Table 2.6), on additional efficacy of encapsulated iron forms (Abbasi & Azari, 2011; Xia & Xu, 2005; Zubillaga et al., 1996) (Table 2.11) and stability trials (Lee et al., 2012) (Table 2.7). Lee et al. (2012) studied the effect of different iron compounds and packaging conditions on the stability (free fatty acids content, TBA value, browning, water activity and moisture) of milk powder, concluding that the less stable forms were those of iron (III) in aerobic conditions.

- Infant formulas/infant cereals

Iron fortified infant foods are available around the world. Breastfeeding is recommended, but because of the increased demand for energy and iron for growing, it is preferably to supplement the infant diet with other food products apart from breast milk after 6 months of age.

It is important to differentiate between infant formulas and infant cereals. Infant formulas are more liquid and not designed for addition to milk, whereas infant cereals meet the solid food requirement, are usually dry, and most are designed to be added to milk.

Cow's milk is not recommended before the age of one year due to allergies. Thus, the use of soya products is often recommended. Soya flour products have higher iron content; therefore these formulas will contain higher iron contents than other flour-based content infant formulas. In addition, these formulas are usually fortified with ferrous sulphate (commonly hepta-hydrate) to both liquid and powdered forms. However, due to oxidation, encapsulated forms are also used nowadays.

The main drawback of iron fortification of infant formulas is the off-flavour production and colour changes. For a proper fortified product, a balanced composition and appropriate packaging are needed. Adequate packaging is commonly a limiting factor regarding production of fortified formulas in small local industries such as the ones from developing countries.

Infant cereals generally consist mainly of a mixture of cereals (wheat, barley, rye, millet, oat, rice, maize or sorghum). Besides, vitamins and minerals are added for fortification and sugar may be added for flavouring. To fortify them, ferrous sulphate is not compatible (because of is high reactivity) and thus elemental iron, the more bioavailable one (small particle), is commonly used (Lotfi et al., 1996). The addition of ascorbic acid is useful to enhance the absorption of iron in these products (Theuer, 2008). Ferrous fumarate and succinate, which has a brownish colour, is also commonly added due to the fact that it is easily disguised in the yellowish infant cereals.

The addition of heme iron is also a possibility. A bovine haemoglobin concentrate (BHC) was used to fortify an infant cereal (Hertrampf et al., 1990). Moreover, extruded rice cereal fortified with BHC was found to be adequate to prevent iron deficiency anaemia only if consumed in a dose over 30 g/day, which was a high dose for some of the infants during the trial because they had problems to eat all the serving.

Other infant food products, such as weaning foods, have been fortified with heme iron. In the study of Martínez Graciá et al. (2000) the addition of a heme iron concentrate at 0.5% to weaning foods was successfully achieved from technological and sensory point of view as the sensory characteristics of these samples remained unchanged even after 8 months of storage at 37 °C.

Regarding the iron fortification level, the *Codex Alimentarius* sets the minimal amount at 1 mg Fe/100 kcal (7 mg Fe/L formula) and the maximum at 3 mg/100 kcal (20 mg Fe/L) in infant formulas (Lotfi et al., 1996). Similarly to other food vehicles, the addition of vitamin C (100-200 mg/L) is considered to improve its absorption (Hurrell, 1997).

In Table 2.6, there are more examples of products addressed for infants consumption such as porridges or complementary foods (Nogueira et al., 2012; Perez-Exposito et al., 2005; Shamah-Levy et al., 2008).

- Biscuits

The effect of adding NaFeEDTA to biscuits (*Petit Beurre Biscuits)* was assessed by Mohammadi et al. (2011). In their study, biscuits were fortified with different amounts of NaFeEDTA and different parameters, such as composition, sensory analysis and lipid oxidation were assessed. The addition of NaFeEDTA significantly affected the colour, texture and flavour of the fortified biscuits; however, it seems that its addition is acceptable at levels as high as 9 mg/100 g of biscuits.

Apart from that kind of biscuits, wheat biscuits were fortified with inorganic iron. H-reduced iron and an encapsulated ferrous sulphate were assessed as fortificants in the study of Biebinger et al. (2009). These authors reported that both fortificants increased the iron status of Kuwaiti women.

Apart from non-heme iron, these kind of biscuits had also been fortified with a heme iron concentrate in Chile (Asenjo et al., 1985). The target population of this study was school children. The heme iron concentrate was produced from bovine blood from healthy animals being 0.27% the total iron content. Due to its low iron content, it was necessary to add high amounts of heme iron concentrate. Therefore, this resulted in a dark colour, which is acceptable in biscuits but not acceptable in other food products.

This heme-iron fortification model was used in another trial in Chile (Walter et al., 1993) and in Brazil (Lamounier et al., 2010). In the former trial it was reported that there were significant differences in haemoglobin concentrations between children from the fortified vs. the non-fortified group.

A similar model was reported by González-Rosendo et al. (2010), who studied the bioavailability of sandwich cookies filled with a chocolate cream which was indeed fortified with heme iron concentrate (HIC). This was assessed in adolescent girls by comparing cookies fortified with HIC with cookies fortified with iron sulphate. According to the authors, the bioavailability of the HIC was 23.7% higher than that found for iron sulphate. A similar study was carried out using piglets as an animal model (Quintero-Gutiérrez et al., 2008). In this case, the bioavailability of the HIC was 23% greater compared to ferrous sulphate which is in agreement with the latter.

- Rice – Beans

There are different technologies for fortifying whole grains such as rice or beans. Among the employed technologies, we can find grain coating, infusing or use of extruded grain analogues. Thereafter, the fortified grains are mixed (1:100 or 1:200) with normal grains (Hurrell, 1997; Moretti et al., 2005).

Also in the study of Schümann et al. (2005), refried black beans were fortified with either heme iron or ferrous sulphate. The subjects of the study were children aged between 12-36 months with initial haemoglobin values between 100 and 115 g/L. No differences between treatments were observed but, in both cases, the haemoglobin levels increased regarding the initial values. Other examples of rice or beans fortified with iron are shown on Table 2.6 and comprises the studies of Hotz et al. (2008); Li et al. (2008); Nogueira et al. (2012) and Nogueira et al. (2013).

- Condiments

As for an effective food fortification, a large proportion of the population at risk of developing iron deficiency should consume the food vehicle. In this regard, the fortification of condiments that are traditionally used in developing countries seems to be a good approach. Especially it can have a potent effect in countries where central processing of staple food is absent (Uauy et al., 2002). Among different condiments, monosodium glutamate, fish sauce, curry powder and bouillon cubes were tried as vehicles in food fortification programs.

The success of fortified condiments relies on the absence of adverse colour and reactions, and the addition of an iron absorption enhancer such as EDTA. An example of condiment without adverse characteristics is monosodium glutamate which was successfully fortified with ferric orthophosphate and encapsulated ferrous sulphate (Hurrell, 1997).

In Thailand, fish sauce is an important salt substitute. In this type of product, the use of NaFeEDTA was proved to be successful. This is explained by the clear brown liquid of the fish sauce which makes possible to disguise any discoloration caused by the addition of iron fortificant (Lotfi et al., 1996).

In an Indian population of Durban (South Africa), the fortification of a curry powder was also successfully fortified with NaFeEDTA (Ballot et al., 1989). Other examples of condiments fortified with iron are the studies on fish sauce and soy sauce (Huo et al., 2002; Longfils et al., 2008; Van Thuy et al., 2003). See Table 2.6 for more details.

- Salt

Salt is considered as a good food vehicle for mass-fortification because it is consumed by all segments of populations, there is no relation between salt consumption and socioeconomic status and it is daily consumed (Lotfi et al., 1996). Nonetheless, iron fortification of salt present many technical barriers, iron compounds turns the salt yellowish, and for developing countries an efficient production and distribution system must exist (Hurrell, 1997). Indeed,

many studies focus on the stabilization of iron compounds for salt fortification (see Table 2.11 for examples).

- Sugar

Sugar has also been employed as vehicle for mass fortification. Its fortification has mainly been done in regions were sugar is produced, namely Caribbean and Central America. Nonetheless, it should be borne in mind that its consumption in other developing countries is more common in middle and upper socioeconomic classes, where prevalence of iron deficiency is lower. It is also important to remark that the addition of fortified sugar in citrus drinks, that are rich in ascorbic acid, will make iron more bioavailable. However, if the fortified sugar is used to sweeten coffee or tea, its absorption will be compromised due to its high phenolic compounds content (Hurrell, 1997).

- Others

Other usually consumed foods such as coffee, cheese, eggs, etc. had been fortified with iron. Although the high content of phenolic compounds of coffee will strongly inhibit iron absorption, some trials aimed the addition of iron to coffee (Hurrell, 1997). Cereal based foods, as cereal bars or ready-to-eat cereals are commonly fortified with iron (Karl et al., 2010; Naghii & Mofid, 2007). We can also find records on fruit juices fortified with iron (Blanco-Rojo et al., 2011; Nogueira de Almeida et al., 2003).

In Table 2.6, it is reviewed the iron compounds that are commonly used and food vehicles in which they have been added.

Table 2.6: Experimental studies with different iron fortificants and food vehicles in humans.

Author	*Fortificant*	*Food vehicle*	*Population*	*Iron consumption*	*Duration of intervention*	*Results/Comments*
Short-term intervention trials						
Trinidad et al., 2014	NaFeEDTA	Oat beverages without and with vitamin C	Healthy 6-year-old children	3 mg Fe	2 days	The addition of vitamin C improved iron absorption by an additional 1.5%.
Contreras et al., 2013	IV Fe^{1} and $FeSO_4$	Vanilla flavored milk product	Seventeen non-Fe-deficient healthy adults (18-35 years)	39 mg Fe	2 days, 7 days between	Transferrin saturation significantly increased after the intake of both No significant differences were detected
Walczyk et al., 2013	$FeNH_4PO_4$ and $FePO_4$	Full-cream milk powder	Young women	5 mg Fe (two doses separated 3h)	1 time	Replacement of ferric pyrophosphate with ferrous ammonium phosphate increased bioavailability
Yameen et al., 2013	Elemental Fe, $FeSO_4$ and $FeSO_4$ + Na_2EDTA (1:1)	Wheat flour	Pakistani children, 5-10 years old	730 µg elemental Fe, 670 µg $FeSO_4$ and 380 µg $FeSO_4$ + Na_2EDTA	1 day	Higher absorption of $FeSO_4$ (alone or with Na_2EDTA) than elemental Fe
Troesch et al., 2011	$FeSO_4$ + ascorbic acid NaFeEDTA	Maize porridge	Healthy women	6 mg	1 day	Fastest absorbed with $FeSO_4$ + ascorbic acid No increase non transferrin-bound iron
Navas-Carretero et al., 2009	$FeSO_4$ $FePO_4$ encapsulated[2]	Meat paté	Seventeen women with low iron stores (ferritin 30 g/L)	19 mg Fe	1 day	The effect of type of fortificant was not significant
Perez-Exposito et al., 2005	$FeSO_4$, $FeO_4C_4H_2$[3], H-reduced Fe[4] + Na_2EDTA	Milk based weaning food	Children aged 2–4 years	9 mg	15 days	Fe absorption from $FeSO_4$ was greater than from either ferrous fumarate or reduced iron + Na_2EDTA
Long term intervention studies						
Nogueira Arcanjo et al., 2013	Micronized $FePO_4$	Ultrarice	Infants aged 10 to 23 months of age	56.4 mg/day	18 weeks	Reduced IDA 27.8 to 11.1 %
Quintero-Gutiérrez et al., 2012	Heme Fe concentrate (HIC) + $FeSO_4$	Biscuits	Adolescents Mexican girls	9.5 mg Fe/day	13 weeks	Increased Hb[5] levels in HIC group Minimal improvement of Hb level for the $FeSO_4$ group
Nogueira et al., 2012	Micronized $FePO_4$	Ultrarice	Infants 10–23 months	56.4 mg/day	18 week	Reduced IDA 31.25% to 18.75%
Muthayya et al., 2012	NaFeEDTA	Whole Wheat Flour	Fe-depleted Indian children	6 mg Fe/day	7 months	Reduced ID 62 to 21%; IDA 18 to 9%,
Nogueira et al., 2012*b*	$FeSO_4$	Milk-based cornstarch porridge	In 4-year old children	10 mg/day	14 weeks	Reduced IDA from 75% to 20%

González-Rosendo et al., 2010	Heme Fe concentrate (HIC) and $FeSO_4$	Biscuits	Mexican adolescent girls	10.3mg Fe/d.	7weeks	Heme Fe bioavailability 23.7% greater ($P<0.05$) compared to $FeSO_4$
Karl et al., 2010	Encapsulated $FeSO_4$	Cereal bars	Female soldiers during basic combat training	27.9 mg Fe/bar, 2 bars/day	9 weeks	Attenuated declines in iron status of iron deficient soldiers
Biebinger et al., 2009	$FeSO_4$ µcapsules or H-reduced Fe	Wheat-based biscuits	Kuwaiti women with low body iron stores	20 mg H-reduced elemental Fe/day or 10 mg $FeSO_4$/day, 5 days/week	22 weeks	H-reduced Fe was not efficacious $FeSO_4$ µcapsules improved Fe stores
Van Stuijvenberg et al., 2008	NaFeEDTA $FeO_4C_4H_2$ Electrolytic Iron	South African brown bread	Schoolchildren (6-11 years)	2.35 mg NaFeEDTA 4.70 mg $FeO_4C_4H_2$ 8.30 mg Electrolytic Fe	34 weeks	No effect on Hb concentration, transferrin saturation or serum ferritin Fe, Fe, or transferrin receptor
Shamah-Levy et al., 2008	$FeSO_4$ $FeO_4C_{12}H_{22}$[6]	Complementary baby food (Nutrisano)	Healthy toddlers 12 to 30 months	10 mg Fe/day	6 months	$FeO_4C_{12}H_{22}$ beneficial effect on markers of Fe status.
Hotz et al., 2008	$FePO_4$ µencapsulated	Rice	Non-anaemic Mexican women	13 mg Fe/day, 5 days/week.	6 months	Decrease of ID and IDA 10.3 and 15.1% respectively
Miglioranza et al., 2008	H-reduced Fe	Corn-flour derived products (biscuits, cakes and pies)	Brazil school age children from public educational centres	9.8 mg Fe	6 months	Decrease of ID and IDA 18 and 14.9% respectively
Longfils et al., 2008	NaFeEDTA $FeSO_4$+citrate	Fish sauce	Cambodia students aged 6-21 years	10 mg Fe	21 weeks	No difference among treatments, effective on increasing Hb and serum ferritin levels
Andang'o et al., 2007	NaFeEDTA Electrolytic Fe	Whole maize flour porridge	Kenyan Children (3-8 years)	3-5 years old: 5.6 or 2.8 mg Fe/day 6-8 years old: 8.4 or 4.2 mg Fe/day 2 doses of NaFeEDTA (high and low) 1 dose of electrolytic iron (high)	5 months	Electrolytic Fe not affects children status. NaFeEDTA improved children iron status in a dose depend manner
van Stuijvenberg et al., 2007	Ferrous bisglycinate Electrolytic Fe	Brown bread	School age children (6-11 years) with serum ferritin <20microg/L	3.66 mg elemental iron/day	7.5 months	Positive effect of ferrous bisglycinate but not electrolytic Fe on serum Fe, Hb, and transferrin saturation
Naghii & Mofid, 2007	$FeSO_4$	Ready-to-eat cereal and pumpkin seed kernels	Women at reproductive ages (aged 20-37)	7.1 mg iron/day from cereal + 4.0 mg iron/day from seeds	4 weeks	Significant difference between pre and post consumption: higher serum Fe and higher transferrin saturation
S. R. Lynch et al., 2007	Electrolytic and H-reduced Fe	Wheat-based snacks	Thai women	12 mg Fe/day, 6 days/week	35 weeks	Efficacies compared with ferrous sulphate monohydrate of 77% and 49%
Schümann et al., 2005	Heme Fe from bovine and $FeSO_4$	Beans	Children 12-36 months with Hb levels 100-115 g/L	32.5 mg $FeSO_4$ /day, 34 mg heme Fe/day, 5 times/week.	10 weeks	Hb concentrations increased; no differences between treatments. Ferritin concentrations unaffected
Nestel et al., 2004	Electrolytic and reduced Fe	Wheat flour	Sri Lanka pre-schoolers (9 and 71 months old), primary	66 mg/kg	2 years	Not beneficial in reducing anaemia in this population: low prevalence and

			scholars (6 to 11 years old) and non pregnant women			bioavailability
Van Thuy et al., 2003	NaFeEDTA	Fish Sauce	Anaemic Vietnamese women	10 mg Fe/day, 6 days/week	6 months	Reduced prevalence of IDA
Huo et al., 2002	NaFeEDTA	Soy sauce	Fe-deficient anaemic school children (11-17 years)	5 mg Fe/day or 20 mg Fe/day	3 months	Effective, no differences between low and high doses groups after 3 months
Nogueira de Almeida et al., 2003	$FeSO_4 \cdot 7H_2O$	Orange juice	Preschool children	4 mg / day, 5 days a week	4 months	Mean Hb increased Prevalence IDA decreased from 60 to 20%
Layrisse et al., 1996	$FeO_4C_4H_2$	Precooked yellow and white maize and wheat flours	Venezuelan children aged 7, 11, and 15	20 and 50 mg Fe/kg flour, respectively[7]	2 years	Reduced ID 37 to 15% ; IDA 19 to 10%
Walter et al., 1993	Bovine Hb concentrate	Biscuits	Chilean children	1mg Fe/day	3 years	Significant Hb concentrations fortified vs. the non-fortified
Hertrampf et al., 1990	Bovine Hb concentrate	Extruded rice flour	Healthy, term breast-fed infants.	4.2 mg Fe/day	1 year	IDA = 17 % controls, 10% intervention group
Ballot et al., 1989	NaFeEDTA	Curry powder	Indian iron deficient population	55 mg Fe/day	2 years	Reduced IDA 22 to 5%

[1] Ferric saccharate
[2] Encapsulated in liposomes Lipofer and an added pigment
[3] Ferrous fumarate
[4] Hydrogen reduced iron
[5] Hb: Haemoglobin
[6] Ferrous gluconate
[7] Ingested supposed 45% of the total energy consumed daily

Table 2.7: Studies on technological and quality parameters of food products fortified by different iron compounds.

Author	*Fortificant*	*Food vehicle*	*Iron amount*	*Storage time*	*Aim*	*Results/Comments*
Lee, Ho, Khoo, & Chow, 2012	$FeSO_4$ / $FeCl_3$[1]	Milk-powder	11.4mg Fe/100g sample	9 months	Measured FA, TBA, colour, moisture, water activity, browning and microstructure.	Fe(III)-fortified formula has the lowest stability in aerobic condition but its stability improved significantly with vacuum/anaerobic packaging
Mohammadi et al., 2011	NaFeEDTA	Petit Beurre Biscuits	-	-	Effect of Fe quantity on the physicochemical and sensory characteristics	No differences pH, ash, moisture and breaking strength. 60 day storage PV increased. Fe modified colour, texture and flavour
Kiskini et al., 2011	$FePO_4$, NaFeEDTA, $FeSO_4$ μencapsulated, electrolytic Fe	Gluten-free breads	40 mg Fe/kg	-	Differences on colour, crust firmness, and sensory attributes (pore number, smell, "moisture", metallic taste and stickiness)	Elemental iron was more stable Ferric pyrophosphate and elemental iron caused undesirable changes
Tripathi et al., 2011	$FeO_4C_4H_2$	Roti and dumplings [2]	60 mg iron/kg flour	60 days	Sensory quality attributes using quantitative descriptive analysis, and their texture	No effect of the fortificant on the texture and aroma Discolouration of dumplings Sensory panellists accepted roti
Martínez Graciá et al., 2000	Porcin heme concentrate	Weaning foods	-	Storage for 8 months at 37ºC	Technological feasibility: stability of iron and organoleptic characteristics.	Stable organoleptic attributes, heme content decresed, colour changed
Asenjo et al., 1985	Heme iron concentrate (HIC)	Biscuits	-	7 months	Shelf life study at 40°C. Measured protein content and lipid peroxidation.	Biscuits could be satisfactorily stored up

[1] Different formulations tested: comercial powder (CP) / CP + vit E + EPA / CP + iron. Packed either in aerobic or anaerobic atmosphere
[2] Products prepared with finger millet and sorghum flour

2.2.3. RISK OF IRON CONSUMPTION AND IRON OVERLOAD

2.2.3.1. Risk of iron consumption

Several studies have reported associations between iron intake red meat consumption, or iron stores, with different illness such as cardiovascular disease (CVD) (Lapice et al., 2013; Muñoz-Bravoet al., 2013), diabetes mellitus (Bao et al., 2012; Montonen et al., 2012; Zhao et al., 2012) and cancer of the gastrointestinal tract (especially lower bowel) (Demeyer et al., 2008; Ferguson, 2010; Weinberg, 1996). The theory beyond this association is related to the oxidant properties of iron which may cause cellular changes leading to atherosclerosis, by catalysing the oxidation of low-density lipoprotein (LDL) cholesterol (Sempos et al., 2010), or tumour formation.

In spite of this, it seems that regarding CVD disease the vast majority of the epidemiological data does not support the hypothesis that body iron stores are directly related to the risk of developing coronary heart disease (Sempos et al., 2010). In various recent reviews, the iron lack of effect on CVD disease has been reported (Lapice et al., 2013; Muñoz-Bravo et al., 2013).

With respect to diabetes mellitus and body iron stores, some reviews support the hypothesis that elevated iron stores and heme iron consumption are associated with risk of type II diabetes (Bao et al., 2012; Montonen et al., 2012; Zhao et al., 2012). In fact two epidemiological studies have reported an association between indicators of body iron intake or stores and the incidence of diabetes mellitus. However, according to the EFSA opinion (EFSA, 2004, 2010), these epidemiological studies do not provide convincing evidence of a causal relationship between iron intake or stores and type II diabetes. Hence, the official opinion of EFSA regarding both diabetes mellitus and CVD is that the associations between iron status and increased risks of CVD are contradictory and unconvincing at the present time.

However, there are several studies that indicate the possibility of a role of luminal exposure to excessive iron in the development of colon carcinoma. In this sense on the report of the World Cancer Research Fund released at the end of 2007 is advised to “limit intake of red meat and avoid processed meat”, as a result of the “convincing evidence” for an association with an increased risk of colorectal cancer development. The presence of heme iron and endogenous formation of N-nitrous compounds has been pointed out to be the most likely potential factors in the contribution to processed meats colorectal cancer (Demeyer et al. 2008). Nevertheless, the EFSA (EFSA, 2004) also considers that the reported associations between cancer and iron intake (or iron stores) do not provide a consistent body of evidence, and do not demonstrate causality between high iron intakes and cancer development.

2.2.3.2. Risk of iron overload

Considering the estimates of current iron intakes in European countries, the risk of adverse effects derived from high iron intake from food sources, including fortified foods, but excluding supplements, is considered to be low for the population as a whole (EFSA, 2004). The exception is, of course, those homozygous individuals with hereditary haemochromatosis (up to 0.5% of the population).

Some groups at special risk for poor iron status, such as menstruating women or children, could benefit from additional iron intake and/or improved availability of dietary iron. However, according to EFSA opinion about the iron intake from supplements in men and postmenopausal women, there might be an increase in the proportion of the population likely to develop biochemical indicators of high iron stores.

Especial concern has been raised according to the iron fortification safety. For addressing this matter, we should take into account the upper intake levels (UIL) for iron. The UIL is defined as the highest level of daily consumption that current data have shown to cause no side effects in humans when used indefinitely without medical supervision. In this case, the UIL set by the institute of medicine of the national academy (USA) for iron consumption are much higher than the levels used in iron fortification (Table 2.8). Nevertheless, the European Food Safety Authority has not set an UIL for iron as considered the available data insufficient (EFSA, 2004).

Table 2.8: Upper intake levels settled by the Institute of Medicine of the National Academy of Sciences for iron for healthy people

Age	*Males (mg/day)*	*Females (mg/day)*	*Pregnancy (mg/day)*	*Lactation (mg/day)*
7 to 12 months	40	40	N/A	N/A
1 to 13 years	40	40	N/A	N/A
14 to 18 years	45	45	45	45
19+ years	45	45	45	45

Source: Trumbo et al., (2001).
N/A: data not applicable

As stated previously, in order to be able to qualify a food product as fortified in the European Union, there is need to add at least 15 % of the daily recommended intake (DRI, 14 mg) per serve or 100 g of food product (European Comunities Commission, 2008). For iron compounds with high bioavailability, it is recommended that the fortification level should not be higher than one third of the DRI (4.7 mg of iron). In both cases, the iron levels allowed for fortification of foods are far away from the marked UIL.

When iron fortification of staple foods is recommended quite often there are concerns regarding the risk of iron overload among persons susceptible to this condition. However, several research reports and national policy documents establish that the amount of iron likely to be ingested through fortification of foods would be enough to improve iron deficiency at the population level and represents a small risk, even in persons homozygous for

haemochromatosis. For instance, in the report of Flynn et al. (2009), where it is studied the intake of different nutrients from foods, fortification and supplements in Europe, it is stated that the risk of adverse effects from high iron intake from food sources, including fortified foods, is considered to be low.

The possibility that certain individuals may develop iron overload at some time is a risk benefit question for the MI/UNICEF (Gillespie, 1998). In this report, various considerations are taken into account, such as:

- ***First:*** *Iron fortification will not lead to the development of iron overload in normal individuals. This is because there is a very efficient system of down-regulation of dietary iron absorption and an actual blocking, at certain iron-store thresholds. This applies even to diets with high iron bioavailability, high heme-iron content, and to iron fortified diets.*

- ***Second:*** *Several genetic conditions predispose risk of iron overload. The risk has been found to be related to a defective gene when it is homozygous. This risk occurs among a small minority, mainly Europeans, and largely concentrated in genetic "hot spots," not evenly distributed throughout the population. Heterozygotes, who are more numerous, are not at risk of iron overload.*

- ***Third:*** *The amount of iron added to the diet through fortification can make a significant rightward shift in the distribution of iron status of an iron-deficient population and fewer people become anaemic. However, such amounts would make little or no difference to the outcome for those with various haemoglobinopathies.*

The overall idea of the report of the MI/UNICEF, "Major Issues in the control of Iron Deficiency", supports the fact that even if in the future it is found an association between high iron stores and an illness, it would not be a contra-indication for programs to prevent or control iron-deficiency anaemia in developing-country populations where iron status is a major issue (Gillespie, 1998). Again, the potential benefit of an iron intervention to a predominantly iron-deficient population is considered to outweigh any risk that this may cause for a few individuals.

2.3. Lipid oxidation

Lipids are important constituents of foods and a source of energy. They may also provide essential nutrients, e.g. linoleic and linolenic acid, and lipid-soluble vitamins (A, D, E and K). Apart from the previous mentioned nutritional contribution, lipids also have structural and functional properties in foods. Lipids contribute in the characteristic flavour, odour, colour and texture of the product as well as providing a feeling of satiety and palatability to foods. Therefore, even when lipids are present in a small proportion, they have a significant effect on the quality of the food product.

Nowadays, lipid oxidation is the major form of deterioration in most foods. Lipid oxidation not only determines the development of rancid flavours but also decreases the nutritional quality and safety of foods. Therefore, lipid oxidation supposes a problem for the food industry.

On top of that, consumers demand food products with added nutritional value. This includes the use of lipid sources rich in polyunsaturated fatty acids, which are more prone to oxidize, or containing other nutrients of interest such as iron. This latter element catalyses oxidation reactions and, consequently, the susceptibility of the fortified food to oxidize is increased. Given that consumers' demands are also accompanied by the request of clean labels where synthetic additives are not well perceived by consumers, the utilization of synthetic antioxidants is limited thus making the prevention of lipid oxidation in foods more challenging.

2.3.1. LIPID OXIDATION MECHANISM

Lipid oxidation mechanisms are extensively described in the literature (Belitz et al., 2008; Frankel, 2005; Kamal-Eldin et al., 2003; D. B. Min & Boff, 2002a; D. Min & Boff, 2002b). Basically, lipid oxidation is a process where lipids react with oxygen leading to the formation of lipid hydroperoxides also known as primary oxidation compounds. These compounds further decompose (with time, temperature or action of enzymes) to secondary oxidation compounds, which are mainly carbonyl compounds. These secondary oxidation products are responsible of the appearance off-flavours and rancidity. Apart from carbonyl compounds, dimers and polymers and other compounds are also produced. There are two main mechanisms of lipid oxidation namely non-enzymatic and enzymatic.

2.3.1.1. Non-enzymatic oxidation

2.3.1.1.1. Auto-oxidation

In bulk oils and fats, the non-enzymatic oxidation of lipids usually takes place by the so-called auto-oxidation process. This mechanism involves the reaction of molecular oxygen with

organic compounds under mild conditions. The initiation reaction cannot take part directly by reaction of lipids and triplet oxygen as it is thermodynamically forbidden. In the ground state, oxygen is at the triplet state ($^3O_2$) and is characterized by having two un-paired electrons. However, the spin direction of both electrons is in the opposite direction of those of the lipids. This impediment is overcome in the presence of initiators of the radical chain. Initiators, such as transition metals and energy (radiations and temperature) are capable of removing hydrogen from a lipid molecule. Thereafter, lipid oxidation takes place by a free radical chain mechanism, which involves three stages that can be schematized as follows:

1) Initiation:	$LH \xrightarrow{I} L^{\cdot} + H^{\cdot}$	[1]
2) Propagation:	$L^{\cdot} + {}^3O_2 \rightarrow LOO^{\cdot}$	[2]
	$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$	[3]
	$LO^{\cdot} + LOOH \rightarrow LOO^{\cdot} + {\cdot}OH$	[4]
	$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot}$	[5]
	$LH + {\cdot}OH \rightarrow L^{\cdot} + H_2O$	[6]
3) Termination:	$2\ L^{\cdot} \rightarrow L\text{-}L$	[7]
	$LO^{\cdot} + L^{\cdot} \rightarrow LOL$	[8]
	$2\ LOO^{\cdot} \rightarrow LOOL + {}^1O_2$	[9]
	$2\ LO^{\cdot} \rightarrow LOOL$	[10]
	$LOO^{\cdot} + L^{\cdot} \rightarrow LOOL$	[11]

Commonly, these stages consist of a complex series of reactions that are both sequential and overlapping.

Once alkyl radicals are generated, the triplet state of oxygen can react directly with them [1]. The reaction between the alkyl radical of lipids ($L^{\cdot}$) and molecular oxygen causes the formation of lipid peroxyl radicals ($LOO\cdot$), which are consecutively converted into lipid hydroperoxides (LOOH) by abstraction of a hydrogen atom from another molecule (reactions [2] and [3], respectively).

These two reactions describe the first oxidation stages of lipids, making lipid hydroperoxides the fundamental primary oxidation products. This step is slow and rate determining. Therefore, the weakest hydrogen bound is lost and thus the susceptibility to auto-oxidation of a lipid depends on the availability of the allylic hydrogen of the radical and the easiness to react with peroxyl radicals to form lipid hydroperoxides.

Lipid hydroperoxides are relatively unstable and, in the presence of heat, radiation or metal ions, they dissociate giving peroxyl ($LOO^{\cdot}$), alkoxyl ($LO^{\cdot}$) or hydroxyl (${\cdot}OH$) radicals as it is shown in reactions [12] and [13]. These reactions help feeding the free radical chain.

$LOOH \rightarrow LO^{\cdot} + {\cdot}OH$	[12]
$2\ LOOH \rightarrow LO^{\cdot} + LOO^{\cdot} + H_2O$	[13]

Reaction [12] is important at high temperatures or in the presence of heme iron. With respect to reaction [13], this reaction is not important in food products because, for this reaction to happen, high ROOH concentrations are needed and when those levels are reached the food product is no longer eatable. At the same time, these radicals have the ability to react with other lipid molecules resulting in more lipid hydroperoxides, alcohols and new radicals (reactions [3] to [6]).

In the presence of metals, the lipid oxidation is initiated as shown in reaction [14] whereas the decomposition of lipid hydroperoxide is catalysed by metal oxygen transition complexes or metal hydroperoxides complexes (reactions [15] and [16]). It is important to note that reaction [15] takes part in a faster way than reaction [16]. For reactions [15] and [16] to take part, the involved metal fulfils two conditions: the first one is that it needs to have two oxidation states, and the second one is that the redox potential of those should be among concrete values. In the presence of this kind of metals, these reactions initiate new radical chains that accelerate the lipid oxidation reactions.

$$M^{(n+1)+} + LH \rightarrow M^{n+} + L^{\cdot} + H^{+} \quad [14]$$

$$M^{n+} + LOOH \rightarrow M^{(n+1)+} + LO^{\cdot} + OH^{-} \quad [15]$$

$$M^{(n+1)+} + LOOH \rightarrow M^{n+} + LOO^{\cdot} + H^{+} \quad [16]$$

The reaction [15] takes part *in vivo* and when specifically iron ions get in contact with hydrogen peroxide, the well-known Fenton reaction [17] takes place.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^{-} \quad [17]$$

The most likely free radical chain feeding process for lipid oxidation in any food product is the metal catalysed decomposition of preformed LOOH. The thermal oxidation of unsaturated lipids, which is usually autocatalytic and involves initiation by lipid decomposition, is also generally considered to be metal-catalysed due to the difficulties of removing trace metals from the raw materials. Therefore, if even trace amounts of metals act as potent catalysts of lipid oxidation in any lipid matrix, it is easy to notice that the fortification of food products with iron will suppose a technological challenge for the food industry.

A crucial factor that influences the pro-oxidant behaviour of metals is pH, e.g. for iron and copper the optimum pH range is 5.5-6.0. Besides, the presence of ascorbic acid, which maintains the reduced state of transition metals, seems to accelerate lipid hydroperoxide decomposition. The water activity of the food matrix also influences the pro-oxidant activity of metals, being higher in dehydrated foods than in foods with high water activity (Figure 2.3).

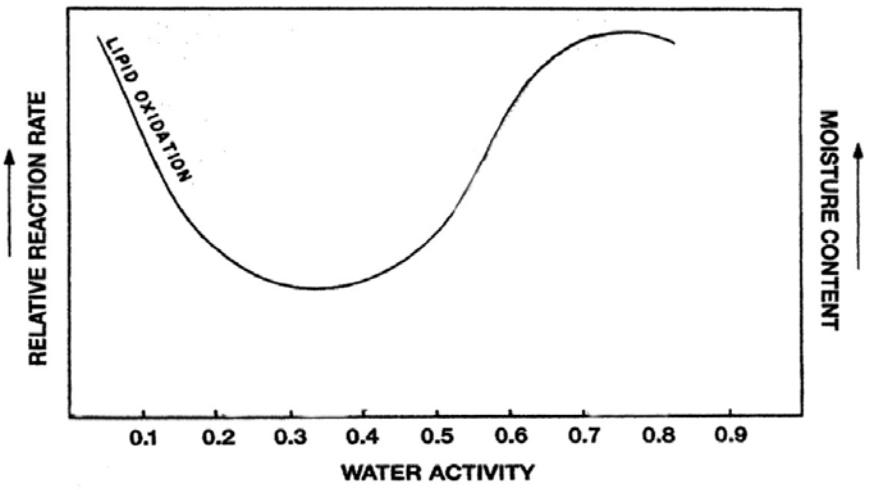

Figure 2.3. Lipid oxidation evolution as a function of water activity. Source: Belitz et al., 2008.

It is important to note that lipid hydroperoxides from highly unsaturated fatty acids accumulate in lower ratios. This is because they are really reactive and thus tend to decompose fast, especially in the presence of metals. Indeed, the kinetics of the lipid oxidation gets complicated in the presence of metal, e.g. in fortified iron foods, as the rate of radical production increases with the matrix metal concentration and lipid hydroperoxides content.

The last step of the free radical chain mediated oxidation is termination, which takes part when the amount of fatty acids or oxygen in the matrix is decreased. In this stage, the quantity of peroxyl, alkoxyl and alkyl radicals is high and therefore they react with each other to form more stable non-radical structures (reactions [7]-[11]). At low oxygen pressure and high temperatures, alkoxyl and alkyl radicals produce either carbon-linked dimers [7] or ethers [11]. Additionally, at low temperatures peroxyl radicals condense forming peroxyl-linked dimers with the formation of oxygen [10].

2.3.1.1.2. Photo-oxidation

Lipid oxidation can be initiated by another non-enzymatic mechanism that is the photo-oxidation of fats. The main difference with the auto-oxidation is that in this case the reaction takes place between lipids and singlet oxygen instead of triplet oxygen. The presence of a sensitizer, light and triplet oxygen determines the formation of singlet oxygen in foods.

The photosensitizer absorbs the ultraviolet or visible radiation energy fast and becomes into an unstable, excited, singlet molecule (^{1}Sen*). Thereafter, the excited singlet photosensitizer loses its energy by (i) internal conversion, (ii) emission of light or (iii) intersystem crossing. In (i) internal conversion, the high-energy photosensitizer transforms itself to a less energy state by releasing energy as heat. In (ii) the emission of fluorescence converts the excited singlet sensitizer to ground state sensitizer. Finally, the sensitizer can undergo (iii) intersystem crossing from the excited singlet state molecule to an excited triplet state molecule (^{3}Sen*), which by emission of phosphorescence converts into ground state singlet sensitizer. The lifetime of ^{3}Sen* is higher than that of ^{1}Sen* and has the ability of convert triplet oxygen ($^3O_2$)

to singlet oxygen ($^1O_2$) and singlet sensitizer (^{1}Sen) in ground state (Figure 2.4). Sensitizers may generate 10^3 to 10^5 molecules of singlet oxygen before becoming inactive. Examples of photosensitizers in foods are chlorophyll, pheophytins, porphyrins, riboflavin and myoglobin, which absorb energy from light and form singlet oxygen.

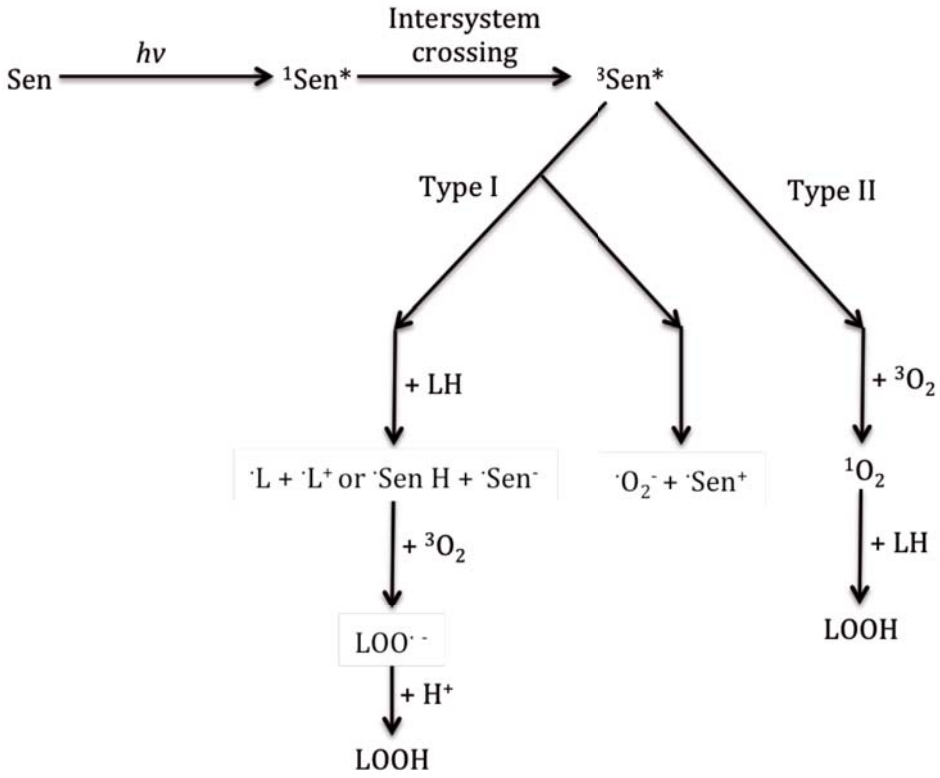

Figure 2.4. Formation of excited triplet sensitizer (^{3}Sen*) and its reaction with substrate via Type I and Type II reactions. Source: Min et al. 2002.

There are two types of photosensitizers. The type I photosensitizers are those photo-chemically activated (^{3}Sen*), converting themselves into free radical initiators. In their triplet state sensitizers react with lipid substrates (LH) by donating and accepting hydrogen or electron and producing free-radical ions. Thus, ^{3}Sen* acts as a photo-chemically activated free-radical initiator for L· formation, which further abstract hydrogen for other compounds to initiate the free-radical chain reaction (Figure 2.4). Also, ^{3}Sen* can react with $^3O_2$ to form superoxide anion; however less than 1% of the reaction between ^{3}Sen* and $^3O_2$ produces superoxide anion. The rate of Type I pathway is mostly dependant on the type and concentration of sensitizers and the substrate compound.

With respect to the type II sensitizers, the excited triplet sensitizer (^{3}Sen*) reacts with $^3O_2$ to form $^1O_2$ and singlet sensitizer. Therefore, there is an energy transfer from high-energy excited triplet sensitizer to low-energy triplet oxygen by forming high-energy singlet oxygen and low-energy ground state singlet sensitizer (^{1}Sen) (Figure 2.4). The rate of Type II sensitizers mainly depends on solubility and concentration of oxygen in the food system. Here it is important to remember that oxygen is more soluble in lipid matrixes than in water

(Battino, 1981). In a system where the oxygen is depleted, the shift from type II to type I mechanism is favoured.

Irrespective of the mechanism, oxidation is enhanced by either the formation of reactive radical compound species or the production of singlet oxygen. This is because singlet oxygen has the property of reacting directly with the double bounds in fatty acids by an "ene" addition mechanism. Thus, the oxidation rate of highly unsaturated lipids in the presence of this oxygen state is directly proportional to the number of double bounds in the lipid matrix. This photo-oxidation initiation reaction mediated by singlet oxygen does not involve radical formation from lipids.

However, this photosensitized oxidation may be inhibited by the presence of carotenoids that interfere in the activation of the triplet oxygen to singlet oxygen. The rate of singlet oxygen quenching by carotene is highly dependent on the number of conjugate double bonds in the carotenoid. Also, the type and number of functional groups on the ring portion of the molecule are important factors. Therefore, effectiveness on quenching singlet oxygen of carotenoids is increased with the number of double bounds in the carotenoid and the concentration of carotenoid.

Another compound that also has the ability of quenching the singlet state of oxygen is α-tocopherol by forming stable addition products. This reaction involves an electron donation from tocopherol to singlet oxygen, forming a charge transfer complex. The transfer complex undergoes an intersystem crossing to ultimately form triplet oxygen and the starting tocopherol (D. Min & Boff, 2002a).

It is important to borne in mind that the photochemical oxidation is usually of little concern to the food industry. This is due to the fact that lipid-rich foods normally have low photosensitizer concentrations and are not directly exposed to sunlight or fluorescence light as opaque containers protect them. However, it has also been pointed out that even lower energy radiations may play a role on lipid oxidation. Besides, singlet oxygen could be also generated by non-photosensitive reactions, especially by the decomposition of hydroperoxides by heme-proteins (further discussed on the next section). Therefore, the occurrence of these reactions supposes a problem in food products fortified with heme iron.

2.3.1.2. Enzymatic oxidation

The lipid oxidation can also be catalysed by enzymes, such as lipoxygenase that catalyse the oxidation of polyunsaturated lipids to hydroperoxides. As other enzymatic reactions, these reactions are substrate specific, form hydroperoxides selectively and have optimum pH and temperature for the enzyme to be active. For instance, lipoxygenase only peroxides 1-cis, 4-cis-pentanoic fatty acids. Other enzymes such as NADPH oxydase, xantine oxydase, nitric oxide oxydase or mieloperoxydase are also able to initiate lipid oxidation.

2.3.1.3. Lipid hydroperoxides decomposition into secondary oxidation products

Lipid hydroperoxides, the primary oxidation products, easily decompose into secondary oxidation products due to their instability. As lipid oxidation progresses, the hydroperoxides start to decompose giving a mixture of monomeric, polymeric and small molecular weight volatile compounds (Frankel, 2005). This process is faster when the lipid matrix is rich in polyunsaturated lipids or when transition metals are present. The secondary oxidation products generated from lipid hydroperoxides breakdown are mainly volatile compounds that contribute to rancid flavour. Indeed, the impact of volatile compounds on flavours takes part even at low concentrations, as low as ppb. In complex food matrixes, lipid hydroperoxides may also interact with other food components, both major and minor components, such as proteins or secondary oxidation products. Apart from the well-known implications in the development of off-flavours, off-odours and overall rancidity, secondary oxidation products are able to damage proteins and enzymes as well as participate on a variety of addition reactions leading to adducts and browning (Schaich, 2003; Zamora & Hidalgo, 2005). All these reactions have an important impact on product sensory characteristics.

With respect to the volatile compounds, the formation paths of these compounds are complicated. The most likely decomposition pathway of hydroperoxides is the cleavage between the oxygen and oxygen of the L-O-O-H, that is L-O-O-H → $LO^{\cdot}$ + $^{\cdot}O$-H instead of L-O-O-H → L-O-$O^{\cdot}$ + $^{\cdot}H$. This is so because the activation energy of the cleavage of –O-O- is 44 kcal/mol, whereas the activation energy of the cleavage -O-H is 90 kcal/mol. Therefore, the hydroperoxide groups are cleaved by homolysis to yield an alkoxy and a hydroxyl radical.

The radicals generated are from aldehydic, alkyl or olefinic nature. By reaction of these radicals with hydrogen or hydroxi radicals, hydrocarbons, alcohols, olefins and aldehydes are formed. Unsaturated aldehydes and ketones produced as secondary oxidation products may undergo further auto-oxidation, providing additional sources of volatile compounds. Additionally, some non-volatile secondary oxidation products may undergo also further reactions, leading to dimers, oligomers, hydroperoxide epoxydes, hydroperoxide epidioxides and di-hydroperoxides. In advanced oxidation states, saturated aldehydes accumulate and un-saturated aldehydes are further oxidized to lower aldehydes and di-aldehydes. However, the main compounds derived from lipid hydroperoxide decomposition are carbonyl compounds, alcohols and hydrocarbons.

Overall, it is known that both high temperatures and the presence of metals (reactions [12] and [15-16]) increase lipid hydroperoxides breakdown. Therefore, lipid hydroperoxide decomposition occurs faster when there is an increased amount of iron as it happens in iron fortified foods and thus the formation of secondary oxidation compounds is higher. Considering this, diverse methods to control and evaluate flavour deterioration could be implemented.

The lipid oxidation pathways mentioned in this section are summed up in Figure 2.5.

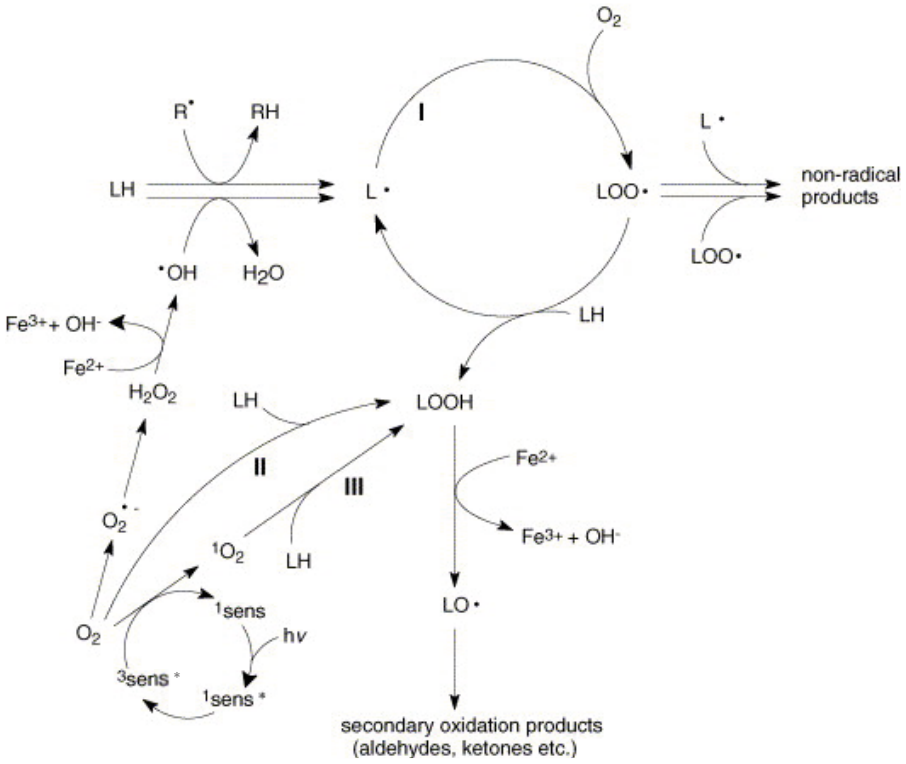

Figure 2.5: Main lipid oxidation pathways: (I) free radical chain reaction, (II) enzymatic formation of lipid hydroperoxides (lipoxigenase activity) and (III) photo-oxidation (Carlsen et al., 2005)**.**

2.3.2. HEME IRON AS PRO-OXIDANT

The catalytic effect on lipid oxidation of enzymes and proteins that contain iron is well known. In fact, the heme catalysis is of significance *in vivo* and in muscle foods such as meat, poultry and frozen fish (Baron & Andersen, 2002). Therefore, the majority of the heme iron pro-oxidant behaviour in the literature is focused on muscle foods, *in vivo* assays or model systems. However, scarce information can be found related to heme iron in other food matrixes such as oils or fats.

Some authors defined heme compounds as much more active than free metal ions in catalysing the lipid hydroperoxides decomposition (Reeder, 2010; Richards et al., 2005). This is so because heme proteins are known to act similarly to redox enzymes. This redox activity of heme-proteins is inherent to the globin molecule (Reeder, 2010). Therefore, the fortification of foods with heme iron and irrespective of the matrix is expected to increase its susceptibility to oxidation when compared with those without heme iron added.

The primary function of heme proteins is to bind oxygen in a reversible way. Therefore, most proteins containing heme are oxygen storage or transporters. Chemically, the ferrous iron of the molecule is coordinated to four nitrogen atoms of a tetrapyrrole ring (making the heme moiety) and to a nitrogen of an imidazole ring, linking the heme to the protein (via the

proximal histidine). This leaves one coordinating site free to bind reversible gaseous molecules such as oxygen (Reeder, 2010).

The capability of heme proteins to bind oxygen depends on their spatial conformation. When the iron is out of the plane, the affinity of the heme-protein for oxygen is low (known as the T state). Conversely, when the iron is in the plane (R state) the affinity for oxygen is really high. The equilibrium between both states is important because the redox reactions take place with the deoxygenated form in a faster rate than with the oxygenated form (Reeder, 2010). This is so because when the oxygen is linked to the heme protein, the iron is in a low spin state and thus it is resistant to oxidation, the opposite happens when no oxygen is linked, the iron atom is in a high spin state and therefore susceptible to oxidation (Richards et al., 2005).

The mechanism of lipid oxidation proposed for haemoglobin and myoglobin involves activation of the iron catalyst by the formation of ferryl heme. This activation takes part in the presence of either hydrogen peroxide or lipid hydroperoxides. However, the interaction between the heme species and lipid hydroperoxides has been addressed in the literature into a much lesser extent than the reaction with hydrogen peroxide (Baron & Andersen, 2002). Heme iron complexes have the ability of decomposing lipid hydroperoxides, in a similar manner as free iron ions do, and thus the development of secondary oxidation products would be also catalysed (Min & Ahn, 2005).

Regardless of the nature of the peroxide, ferryl heme iron is formed from either ferrous or ferric oxidation states.

$$\text{Heme-Fe(II)} + H_2O_2 \rightarrow \text{Heme-Fe(IV)=O} + H_2O \quad [18]$$

$$\text{Heme-Fe(III)} + H_2O_2 \rightarrow \text{Heme}^{\cdot}\text{-Fe(IV)=O} + H_2O \quad [19]$$

Reaction [18] involves the direct oxidation of two-electron of the Heme-Fe(II) species. Opposite, in reaction [19] it is supposed that one oxidation equivalent is located at the iron centre resulting in a oxoferryl complex (Fe(IV)=O), whereas the other equivalent is transferred to an amino acid of the globin providing a protein radical (Baron & Andersen, 2002). These reactions are known as the pseudo-peroxidase mechanism of heme-proteins. Although both reactions are described in the literature, reaction [18] is difficult to achieve due to a reaction of comproportionation (commonly known as synproportionation) leads directly to heme-Fe(III) (Reeder, 2010).

$$\text{Heme-Fe(II)} + \text{Heme-Fe(IV)-OH}^{-} \rightarrow 2\ \text{Heme-Fe(III)} + OH^{-} \quad [20]$$

Therefore, the reaction between both ferrous and ferric heme proteins and peroxides tends to yield ferryl heme plus a protein radical. Once these reactions take place, both high redox potentials of the protein based radical and the ferryl heme can induce a wide range of oxidation reactions. The initiation of lipid oxidation arises from abstraction of a hydrogen form a lipid to create and alkyl radical (Baron & Andersen, 2002; Reeder, 2010).

$$\text{Heme}^{\cdot}\text{-Fe(IV)=O} + LH \rightarrow \text{Heme-H-Fe(IV)=O} + L^{\cdot} \quad [21]$$

$$\text{Heme-Fe(IV)=O + LH} \rightarrow \text{Heme-Fe(III)-OH + L}^{\cdot} \quad [22]$$

Thus, this alkyl radical reacts by the free radical chain mechanism that has been previously described giving peroxides, which in turn are further decomposed giving secondary oxidation products.

Finally, the oxidation of ferrous heme to ferric heme can also take place, liberating superoxide, which is well known as a potent pro-oxidant. The rate of oxidation of ferrous to ferric heme iron increases with increasing the partial pressure of oxygen.

$$\text{Heme –Fe(II)} + O_2 \leftrightarrow \text{Heme-Fe(III)} + O_2^{-\cdot} \quad [23]$$
$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [24]$$

Reaction [23] is pH dependent; at lower pH the rate of oxidation of ferrous heme to ferric heme is higher. It has also been described that the rate of this reaction depends on the subunits, smaller subunits, monomers and dimers, auto-oxidize faster than tetramers (Grunwald & Richards, 2006). The superoxide formed under these conditions may proceed to the formation of hydrogen peroxide and oxygen (triplet state) in presence of superoxide dismutase. The hydrogen peroxide generated will activate the heme forms to the ferryl state thus catalysing the oxidation through reaction [19].

If reaction [23] takes part in a matrix where there is no superoxide dismutase, the rate of generating hydrogen peroxide is much lower. However, under these conditions the superoxide anion leads to singlet oxygen, which has also been described previously as a potent pro-oxidant that catalyses oxidation reactions, instead of triplet oxygen. Besides, it has been described that the ferric heme group is much more reactive than the ferrous one because the heme group is much more loosely bound to the globin, thus favouring lipid oxidation (Richards et al., 2005). Other oxidation reactions can also take place by means of a Fenton like mechanism as schematized in [25] and [26] (Carlsen et al., 2005).

$$\text{Heme-Fe(II)} + H_2O_2 \rightarrow \text{Heme-Fe(III)} + OH^{\cdot} + OH^- \quad [25]$$
$$\text{Heme-Fe(III)} + H_2O_2 \rightarrow \text{Heme-Fe(II)} + HOO^{\cdot} + H^+ \quad [26]$$

These reactions may also occur with lipid hydroperoxides as substrate instead of hydrogen peroxide. Reaction [25] is thermodynamically favourable and a hydroxyl radical is formed. However, the second reaction [26], where a peroxyl radical is formed, is a slower reaction. Commonly, the heme-Fe(II) is linked to oxygen molecules, therefore this reaction is also influenced by the oxygen dissociation rate constant of the heme-Fe(II)O_2 protein at the actual oxygen pressure.

To summarize, ferrous and ferric heme proteins do not react with lipids directly, however, the small amounts of lipid hydroperoxides present at trace levels on the matrix react with the protein to form ferryl heme and lipid radical species as described on reactions [18], [19], [25] and [26]. All these reactions occur at slow rates. Finally, it is worth to mention that it has been reported that at high unsaturated fatty acid/heme protein ratios both oxy– and deoxy-heme

proteins are denaturized. This results in the exposure of the heme group to the matrix, which rises heme-induced peroxidation. Therefore, the peroxidation is catalysed and the reactions are produce in cascade (Baron & Andersen, 2002; Reeder, 2010).

2.3.3. STRATEGIES TO MINIMIZE LIPID OXIDATION

The control of lipid oxidation in food matrixes supposes a challenge for the industry because, in the event of oxidation, nutritional losses and stability problems arise. Therefore, it is worth to minimize oxidation for a number of reasons and including economic issues. To this aim, the safety of foods products will be improved and its content in polyunsaturated fatty acids and lipo-soluble vitamins will be protected during processing and storage.

Among the different methods that the food industry employs to control oxidation we can find different strategies based on the improvement of processing conditions of vegetable oils for decreasing the amount of metal contamination, the exposure to light, temperature and oxygen and thus related with packaging and storage conditions, the addition of antioxidants, the modification of the lipid profile by different means and the encapsulation of either the labile compound that needs to be protected or the reactive compound that initiates oxidation reactions.

However, from all those methods, the most promising ones in order to improve the oxidative stability of iron fortified food product seem to be the addition of metal deactivators and antioxidants to stop radical chain reactions. Among metal deactivators, we can add a chelating agent to the matrix in order to form a complex with the free iron thus avoiding the propagation of the oxidation or we can use an encapsulation technique. In this latter approach, a coating material covers the iron form thus avoiding its contact with the lipid matrix. Following are explained both approaches.

2.3.3.1. Antioxidant addition

The antioxidant addition to food matrixes is the most extended strategy to avoid lipid oxidation. Synthetic antioxidants such as butylated hydroxianisole, butylated hydroxytoluene, propyl gallate, tert-butylhydroquinone had been employed by the food industry in the last decades. These antioxidants are very effective and cheap. However, there is a worldwide trend to avoid the addition of any synthetic additive to food. Related to the use of natural antioxidants, there are different advantages such as being recognized as GRAS (generally recognized as safe) products, the higher concentration allowed to be added to food, the worldwide acceptance of natural antioxidants and, in heated foods, their relatively low volatility.

We can classify antioxidants because of their different mechanism of action against lipid oxidation. The most common used are those considered primary antioxidants that are chain-breaking antioxidants. We can also find secondary antioxidants that can act by different mechanisms.

- Primary antioxidants

Chain-breaking antioxidants retard the radical chain by avoiding the propagation or initiation of radicals by hydrogen-atom transfer to radicals. The reactions followed by chain-breaking antioxidants are:

$$LOO^{\cdot} + AH \leftrightarrow LOOH + A^{\cdot} \quad [27/-27]$$
$$L^{\cdot} + AH \rightarrow LH + A^{\cdot} \quad [28]$$
$$A^{\cdot} + LH \rightarrow AH + L^{\cdot} \quad [29]$$
$$A^{\cdot} + LOO^{\cdot} \rightarrow LOOA \quad [30]$$
$$A^{\cdot} + A^{\cdot} \rightarrow A\text{-}A \quad [31]$$

Phenolic antioxidants with alkyl substituent near the hydroxyl groups are effective chain-breakers because give unreactive radicals that do not propagate the radical chain further and compete with lipid substrate for giving reaction [27] even when the concentration of the antioxidant is lower than the amount of lipid. When the substituent is at the *ortho* position, the hydroxyl bound is labile and the reaction with free radicals is favoured.

The higher stability of the phenolic antioxidants is related with the fact that the phenoxyl radicals generated can delocalize the unpaired electrons around the aromatic structure giving stable radicals due to the high resonance energy. Thus, the antioxidant effectiveness is directly related to the resonance energy of the radical generated.

In general, the radical formed by the antioxidant ($A^{\cdot}$) is relatively stable. However, when the radical formed is not stabilized by resonance, some supposed antioxidants could behave as pro-oxidants and act as chain carriers by generating more radicals (reaction [29]). This reaction is favoured when the antioxidant is a phenol with fewer substituents, and thus the radical formed is less stable, the antioxidant is present at high concentrations in a system at high temperatures (such as frying conditions) or there are metal catalysts or other oxidation promoters (mainly, lipid hydroperoxides and free fatty acids).

Irrespective of their stability, the reaction [29] with the lipid substrate (LH) is slow whereas with peroxyl radical ($LOO^{\cdot}$) as schematized in reaction [30] is fast. Reaction [28] is not important at atmospheric pressure, because in the presence of oxygen the alkyl radical has a really short life because of its fast reaction with O_2 to give peroxyl radical ($LOO^{\cdot}$). However, at low oxygen pressures and high temperatures, reaction [28] becomes significant and thus $A^{\cdot}$ will react with $LOO^{\cdot}$ [30] or this will form a dimer with other $A^{\cdot}$ [31].

With respect to these reactions an important consideration is the antioxidant concentration. When the concentration of the antioxidant is high, the reaction [-27] takes part thus favouring

the development of peroxyl radicals. Generally, the effectiveness of an antioxidant is governed by the balance rate of reactions [27] / [-27] and [29] / [30].

The natural decomposition of hydroperoxides takes place at high temperature [12] and also when reaction [-27] occurs. However, in general, the thermic cleavage of lipid hydroperoxides requires much lower energy than reaction [-27] and thus is more prominent.

From this reaction, the alkoxyl radical can undergo further reaction and give aldehydes, epoxy and keto secondary oxidation compounds and affect the flavour of the product. In the presence of antioxidant, this reaction is stopped by reactions [32] and [33].

$$LO^{\cdot} + AH \rightarrow LOH + A^{\cdot} \quad [32]$$
$$LO^{\cdot} + A^{\cdot} \rightarrow LOA \quad [33]$$

It is important to note that primary antioxidants are not able to neither inhibit photosensitized oxidation nor scavenge singlet oxygen. Examples of primary antioxidants are shown on Table 2.9.

Table 2.9: Primary antioxidants commonly employed in foods

Natural	*Synthetic*
Carotenoids	Butylated hydroxyanisole (BHA)
Flavonoids	Butylated hydroxytoluene (BHT)
Phenolic acids	Ethoxyquin
Tocopherol and tocotrienols	Propyl gallate (PG)
	Tertiary-butylhydroquinone (TBHQ)

- Secondary antioxidants

Secondary antioxidants can act by different mechanisms, such as regenerating primary antioxidants, chelating metals, giving acidic pH to the matrix, thus increasing the primary antioxidant effectiveness, and scavenging oxygen. Some of these secondary antioxidants have different modes of action at the same time. Examples of secondary antioxidants and their mechanisms are presented on Table 2.10.

Metal chelators form stable coordination complexes with metals thus avoiding the metal catalysed initiation of free radical oxidation and the decomposition of lipid hydroperoxides. Examples of metal chelators are citric, ascorbic, tartaric, phytic, phosphoric and etylen-diamintetraacetic (EDTA) acids.

Table 2.10: Secondary antioxidants commonly added to foods and their main mechanisms of action

Mode of activity	*Compounds in use*
Metal chelation	Citric, Malic, Succinic and Tartaric acids,

	Ethylenediaminetetraacetic acid, phosphates
Oxygen scavenging	Ascorbic acid, ascorbyl palmitate, erythorbic acid, sodium erythorbate, sulphites
Singlet oxygen quenching	Carotenoids (β-carotene, lycopene, lutein)

Usually, citric acid, ascorbic acid and EDTA are the most used in the industry. Nonetheless, there have been described some difficulties or drawbacks on their use. For example, EDTA forms preferably complexes with Fe^{3+} than with Fe^{2+}. Therefore, Fe^{2+} catalyses oxidation reactions when EDTA is not added at the proper concentrations as the breakdown of lipid hydroperoxides is more efficient in the presence of this form than with Fe^{3+}. Similar problems can be found when using ascorbic acid as, apart from chelating metal ions, it also reduces Fe^{3+} to Fe^{2+}. This ascorbic acid behaviour depends on both the antioxidant and iron concentration present in the matrix. Therefore, it is commonly recommended that for ensuring the effectiveness of EDTA and ascorbic acid it is needed to add an excess of both, in the case of ascorbic acid this is so to favour its oxygen scavenger properties over its iron reduction activity.

Additionally to the compounds named previously, some proteins have also the ability of chelating and inactivate metal ions, e.g., lactoferrin, transferring, albumin and ceruloplasmin. There is no need to say that the effectiveness of metal chelators on retarding lipid oxidation is higher when the pro-oxidant character of a metal ion is the leading cause of lipid oxidation in a matrix.

Among secondary antioxidants, oxygen scavengers are also commonly used. Sulphites and ascorbic acid and its derivatives, as for example ascorbyl palmitate, are described as oxygen scavengers. The main action of these antioxidants is to sequestrate oxygen from the matrix thus avoiding the substrate for the oxidation reactions. This kind of antioxidant is more effective in those matrixes where the oxygen is the main cause of oxidation. For instance, it has been described that oxygen is more soluble in lipid phases than in water phases (Battino, 1981), thus in complex food matrixes were both phases are together, the oxygen quantity in the lipid phase will be higher and thus it favours the oxidation.

We can also find antioxidants, such as carotenoids, that are able to deactivate ultra-violet light by absorbing it without generating radicals. Carotenoids, which are known to quench the photosensitizers by absorbing the radiation, belong to another type of secondary antioxidants. These also have the ability of transform singlet oxygen to triple oxygen by energy transfer between the singlet oxygen state and the antioxidant.

Finally, when combining primary and secondary antioxidants, as their antioxidant behaviour is based on different mechanism of action, we can find synergisms, which is a cooperative effect between both antioxidant types. In these cases, the antioxidant combination gives more protection against lipid oxidation than the sum of the activities of the antioxidants used separately. Commonly, combinations between metal deactivators and chain-breaking antioxidants are used in the industry because of their known effectiveness.

With respect to foods fortified with iron, there are some studies concerning about the oxidative stability of fortified food (Asenjo et al., 1985; Martínez Graciá et al., 2000; Mohammadi et al., 2011). However, few present the addition of antioxidants as a solution (Bovell-Benjamin et al., 1999) even though the antioxidant addition is a common practice in the food industry.

2.3.3.2. Encapsulating of iron forms

One of the strategies that have been extensively used in iron food fortification is the encapsulation of the iron compounds with different coatings. Encapsulation is defined as entrapping a substance within another one, producing particles with diameters ranging from few nm to few mm.

The substance encapsulated is called the core material, in this case the iron compound, and the capsulation material is called the coating. Regarding this last material, it should be food grade and able to create a barrier for the core material. Indeed, it is important to point out that the regulations for food applications are stricter than those for pharmaceuticals or cosmetics, therefore, several coating materials used for pharmaceutical and cosmetic applications are forbidden in the food industry (Wandrey et al., 2010). The majority of the coating materials used by the food industry are bio-molecules. The most commonly used are carbohydrates (starches and modified starch, cellulose and modified cellulose, plant exudates and extracts, marine extracts, microbial and animal polysaccharides), proteins (gluten, milk proteins, gelatins), lipids (fatty acids and fatty alcohols, glycerides, waxes, phospholipids) and various molecules (polyvinylpyrrolidone, paraffin, etc).

With regard to the encapsulation, there are two main types of encapsulates: reservoir type and matrix type. The complete cover of one particle with the coating characterizes reservoir types, whereas in matrix type several particles are encapsulated together, leading an inner core with several particles and the coating material surrounding it. In this case, there is a chance to find some of the core material in the surface of the encapsulated particles. Finally, the particles could have different geometrical conformations such as spheres, cylinders, ovals or even amorphous shapes (Zuidam & Shimoni, 2010).

The main advantage of iron encapsulation is that it allows the addition of high bio-available iron compounds that otherwise would be difficult to add in order to fortify food due to their reactivity (Zimmermann & Windhab, 2010). By encapsulating the iron compound, the contact between the pro-oxidant iron and the food matrix is avoided and thus the catalysed oxidation of fatty acids and amino acids, among others, is either stopped or delayed. In addition, the encapsulation of the iron may reduce the interaction between iron and other components that may change the colour and may lower the iron bioavailability (tannins, polyphenols and phytates)(Zimmermann & Windhab, 2010).

Several of the most reactive iron compounds are being encapsulated, at least at experimental level, nowadays. Examples of this are: ferrous sulphate with whey protein (Martin & de Jong, 2012), ferrous fumarate with soy stearine (Oshinowo et al., 2012) or with hydrophilic coatings (Romita et al., 2011; Yadava et al., 2012), ferric pyrophosphate with lecithin (Blanco-Rojo et al., 2011) and elemental iron with hydrogenated palm oil (Biebinger et al., 2009). Other examples of iron compounds encapsulated with different coatings are presented in Table 2.11.

Both water-soluble and insoluble coatings are being used to encapsulate iron forms. Water-soluble coatings (e.g. maltodextrin and cellulose) are usually described as not suitable as they do not provide an effective barrier in moist environments. Thus, iron compounds are recommended to be encapsulated with hydrogenated oils that give an effective water barrier (Zimmermann & Windhab, 2010). However, the coating material selected depends on the food vehicle that will be used for the fortification program. For instance, if salt or sugar is selected for a fortification strategy, water-soluble coatings won't be an option, as the moisture in these products will affect the coating protective effect. On the other hand, hydrogenated fat coatings won't be effective in food products that are further heated or processed with heat because the coating will melt and their effectiveness will be compromised. Moreover, it has been described that hydrogenated fat coatings maybe partially removed by abrasion when added to salt (Zimmermann & Windhab, 2010).

Therefore, when selecting the coating material several considerations should be taken into account. Some examples are the selection of the material according to the technological requirement and the final desired properties of the ingredient or this oriented to the final product quality.

Currently, new techniques are being developed where different coatings are applied to the iron compound giving multi-microcapsules. By using this new approach it is expected that each coating will be dissolved in a different part of the gastro intestinal track thus favouring the bioavailability of the compound and avoiding any interaction with the food matrix or side effects in the gastrointestinal track (Zimmermann & Windhab, 2010). Nevertheless, it should be noted that each coating will increase the price of the final product (Wandrey et al., 2010).

The encapsulation of iron forms results in colour changes, making them lighter, and thus favouring its addition to white foods such as sugar or salt. Some examples of food fortification with encapsulated iron are cereals, juices, salt and sugar. A detailed list of iron compounds and encapsulating coatings for food fortification is shown in Table 2.11.

There are several techniques to encapsulate compounds, the most common ones are (Zuidam & Shimoni, 2010):

- Spray-Drying

For encapsulating an iron form by spray-drying, there is need to mix the coating material and the iron compound on a liquid basis forming a solution, an emulsion or dispersion. In the

experimental part of this manuscript co-spray-drying refers to the spray-drying of a dispersion of the core and coating material together. Thereafter, the liquid mixture is sprayed into a chamber, which disperses the mixture into small drops that fall down by gravity. Commonly, in the opposite direction, a hot air current will evaporate the water of the droplets thus drying almost immediately the particles. The size of the droplets will depend on the surface tension and viscosity of the liquid, the pressure during the atomization and the velocity of the spray.

In this specific technique, the coating material usually accomplish if not all, at least some of these requirements: protection of the core material, high solubility in water, high molecular weight, glass transition, crystallinity, diffusibility, good filming properties, good emulsifying properties and low cost. Examples are: natural gums (alginates and carragenates), proteins (dairy proteins, gelatine), carbohydrates (maltodextrin and cellulose derivatives) and lipids (waxes, emulsifiers).

- Vacuum drying

The vacuum drying process is equal to that of spray-drying; however a slight vacuum is present in the chamber where the slurry is sprayed and thus the evaporation process is favoured and the temperature of the air current is reduced.

- Fluid bed coating

A fluidized bed occurs when a powder is moved in such a way that it behaves like a liquid. Then the coating is atomized over the powder covering it homogenously, thus the coating material needs to have an acceptable viscosity to enable the pumping through the powder. In this case, the coating material could be an aqueous solution of cellulose, proteins, etc., but then the water should be further dried by, for instance, the speed of spraying or the airflow. The coating material could also be a lipid, which will be applied directly, and in the case of being hydrogenated fats they are melted before the spraying. Indeed, the use of hydrogenated fats by means of this technique and spray-chilling are one of the preferred strategies to encapsulate iron compounds. Special care should be taken in order that the lipids do not solidify before reaching the powder.

- Spray-cooling or spray-chilling

The theory behind this technique is similar to that of the spray-drying, but the main difference here is that the slurry that enters into the atomizer solidifies when it gets in contact with a cold air current. In this case, a molten coating-core mixture is prepared, atomised as fine droplets into a chilled chamber, solidified and finally fine particles are recovered. A good and common coating for this specific technique is hydrogenated fat, as solidify in contact with the cold current. Concretely, fats with melting points among 34-42 °C are used in spray-chilling whereas materials melting at higher temperature ranges can be used in spray-cooling, where the temperature of the cold air used is lower. The final size of the particles depends on the core material size, melt viscosity and temperature, disk configuration and rotational speed.

This technique is pointed out as one of the cheapest one, and its use is common when iron compounds are encapsulated with hydrogenated fats.

- Melt injection and melt extrusion

In melt injection technique the core material is dispersed into the coating material, that commonly are carbohydrates, at temperatures higher than 100 °C. Following, the mixture is forced to pass through small orifices using a vertical, screwless extruder. On the other side of the holes, there is a cold, dehydrating solvent, commonly isopropanol or liquid nitrogen, that hardens the coating rapidly. With that aim, the coating material should have a transition temperature between 30-70 °C.

Melt extrusion is a very similar technique. In this case, the encapsulation in a carbohydrate melt can also be achieved by using an extruder with one or more screws in a continuous process. The main differences with melt injection are that it uses horizontal screws and the extruded products are not surface washed.

- Emulsification

The emulsification process consists on forming an emulsion where the core material would be situated in the middle of the dispersed droplets. Thereafter, either the emulsion is used as delivery system or the solvent should be eliminated by for instance spray-drying or freeze-drying. If the emulsion is used as delivery system, it should be stable till its use.

- Freeze-drying

Freeze-drying is the process by which a material is frozen and thereafter the pressure of the surroundings is reduced in order to favour the frozen water to sublimate directly from the solid state to the gas state. The principles are the same as those from spray drying, but in this case the water of the slurry is removed by sublimation due to the low pressures and temperatures.

- Other techniques

There are other techniques that have been used to encapsulate various compounds in the food industry comprising: coacervation, co-extrusion, inclusion, complexation, liposome entrapment, and encapsulation by using supercritical fluid technology and nanoparticles.

In general, it is necessary to take into account that the use of an encapsulation technique may impair the bioavailability of the iron compound. Several factors, such as the coating material, ratio of coating material to iron and the technology and process used for encapsulation, may influence on iron bioavailability. Thus, it is important to assess the bioavailability of the encapsulated compound. Apart from this, it is important to bear in mind that the encapsulation of a compound is an expensive technique and thus increases the price of the added final iron compound.

Table 2.11: Iron compounds that have been encapsulated and the coating material and technology used for this encapsulation

Reference	*Iron compound*	*Coating material*	*Technology*	*Food vehicle*
Oshinowo et al., 2012	Ferrous fumarate	Hydroxypropyl methylcellulose, sodium hexametaphosphate and titanium dioxide	Fluidized bed	Salt
Yadava et al., 2012	Ferrous fumarate	Hydroxypropyl methyl cellulose (HPMC) HPMC + polyethylene glycol + titanium dioxide HPMC + stearic acid + stearic acid + titanium dioxide	Fluidized bed	Salt
Abbasi & Azari, 2011	Ferrous sulphate	Liposomes: lecithin + cholesterol & Polyglycerol monostearate	Emulsification	Low-fat pasteurised milk
Romita et al., 2011	Ferrous fumarate	Not-specified	Spray-drying	Salt
Blanco-Rojo et al., 2011	Ferric pyrophosphate	Lecithin	Not-specified	Juice
Y O Li, Diosady, & Wesley, 2010	Ferrous fumarate	Rice flour & Wheat flour & Durum flour & Durum semolina	Melt extrusion	Salt
Yao O Li, Diosady, & Wesley, 2010	Ferrous fumarate	HPMC + titanium dioxide	Melt extrusion	Salt
Biebinger et al., 2009	Ferrous sulphate	Hydrogenated palm oil	Spray-cooling	Salt
Andersson et al., 2008	Ferric pyrophosphate	Soy stearine, titanium dioxide, hydroxypropyl methylcellulose and sodium hexametaphosphate	Spray-cooling	Salt
Y. Li et al., 2008	Ferrous fumarate	Granulated with dextrin, cellulose, sodium exametaphosphate, coated with approximately 30% soy stearine	Not specified	Ultra Rice
Wegmüller, Zimmermann, Bühr, Windhab, & Hurrell, 2006	Ferric pyrophosphate	Hydrogenated palm fat	Spray-cooling	Salt
Xia & Xu, 2005	Ferrous sulphate	Cholesterol and Tween 80	Thin-film hydration Thin-film sonication, Reverse-phase evaporation Freeze-thawing	Milk
Wegmüller, Zimmermann, & Hurrell, 2003	Electrolytic iron	Partially hydrogenated soybean oil	Not specified	Salt
	Ferric pyrophosphate	Dextrin, glycerol esters of fatty acids, sodium enzymatically hydrolyzed lecithin		
	Ferrous fumarate	Partially hydrogenated palm oil Fully hydrogenated soybean oil Cellulose derivative Granulated with dextrin, cellulose, sodium hexametaphosphate, coated with approximately 30% soy stearine		
	Ferrous sulphate	Edible wax matrix Edible matrix of mono and diglycerides Sodium (heptahydrate) hexametaphosphate Partially hydrogenated soybean oil Mono and diglycerides edible fatty acids Cellulose derivative (monohydrate) Partially hydrogenated palm oil Fully hydrogenated soybean oil Soy stearine (heptahydrate) Stearic acid		
Zubillaga et al., 1996	Ferrous sulphate	Phospholipids	Emulsification	Pasteurized milk

3. JUSTIFICATION AND OBJETIVES

As it has been stated in the review of the literature, the prevalence of iron deficiency aims at the development of new approaches for reducing the incidence of iron deficiency worldwide. To this end, food fortification is regarded as the most long-term cost-effective strategy to prevent and/or overcome iron deficiency. Nevertheless, fortification of food products with iron is a challenge to the food industry as it decreases the food stability by catalysing lipid oxidation and thus impairing the nutritional value and sensory characteristics of the fortified food.

When a food fortification project is planned it is necessary to take some decisions to that aim. The main ones are to decide between mass and targeted fortification and select the proper combination between food vehicle and fortificant.

When the prevalence of iron deficiency in a population is high it is necessary to consider the population as a whole and thus mass fortification is indicated. However, when the prevalence is especially high in a population group, the fortification needs to be addressed to this population group and thus conduct a targeted fortification. Regarding iron deficiency, the specific needs of a concrete population group such as children and women at their reproductive years is different from other populations groups. Therefore, targeted fortification needs to be considered for these groups, as mass iron fortification will not reach their needs. In this regard, the fortification of foods with iron, targeted to children, is of great interest because of the high prevalence in this group.

Concerning the food vehicle, biscuits and pastries are some of the bakery products that are daily consumed in a child's diet. Therefore, this type of food product has been selected in this thesis as a food vehicle in this iron fortification project. Regarding the fortificant, heme iron has the highest bioavailability as its absorption mechanism is different from that of non-heme iron and it is not affected by food ligands. Thus, a heme iron ingredient, which consists on a heme iron polypeptide powder obtained from the blood of healthy pigs, has been selected. Nonetheless, the addition of heme iron to a food product, as other non-heme iron compounds, supposes a technological challenge due to its pro-oxidant activity.

In addition, the selected heme iron ingredient is dark and thus unattractive. However, this ingredient can be masked in chocolate, which, in turn, is very appreciated by children. Therefore, the development of a chocolate bakery product fortified with iron as it can be a sandwich-type cookie can be attractive to the target population, children. It is reasonable to consider that two cookies can be their daily consumption and consequently it is intended that this amount of food will provide the 30% of the recommended iron intake (14 mg according to the European Commission, 2008).

As has been pointed out before, heme iron fortification in bakery products supposes a challenge due to the pro-oxidant character of the iron added. Therefore, the main objective is **to determine which strategies are appropriate to overcome this problem, in order to produce a heme iron fortified bakery product destined to children with an appropriate self-life**.

To overcome the oxidative problem we have selected three different strategies, namely, (i) the antioxidant addition, (ii) the encapsulation by co-spray-drying of the heme iron, and (iii) the combination of both strategies. These strategies and their combinations were first studied in a model consisting in palm oil fortified with heme iron and were finally tested in a sandwich-type cookie filled with a chocolate cream fortified with heme iron. Refined palm oil was chosen as model for iron fortification in bakery products because it is commonly used in the manufacturing of biscuits, cookies, pastries and chocolate fillings. In consequence, we subdivided the overall objective in other more specific:

The first specific objective of this thesis is to **find the better antioxidants** in a palm oil matrix fortified with heme iron (study 1).

The second is to **select the optimal antioxidant combination and concentration** for delaying the onset of oxidation in the heme iron fortified palm oil model (study 2).

The third is to **determine** in lipid models **the protective effect of encapsulating the heme iron** by co-spray-drying with different coatings materials (studies 3 and 4).

The fourth is to **select the better combinations of both strategies**, antioxidant addition and heme iron encapsulation, for delaying the onset of oxidation in the heme iron fortified palm oil model (study 4).

Finally, the last objective was to assay the better strategies and their combinations in order **to produce a heme iron fortified bakery product destined to children with an appropriate self-life** (study 5).

4. EXPERIMETAL DESIGN AND METHODOLOGY

4.1. Experimental design

This thesis project has been sub-divided in five different studies in order to achieve the previously described objectives. Accordingly, all these studies were aimed at delaying the onset of oxidation of bakery products fortified with heme iron. To do so, different strategies were assessed, namely the antioxidant addition and the co-spray-drying of the heme iron with different compounds.

4.1.1. STUDY 1: ANTIOXIDANT SELECTION

The first study aims to evaluate the effectiveness of three antioxidants with different mechanism of action in the prevention of the oxidation of a heme iron fortified palm oil. Refined palm oil was chosen because it is frequently used in the manufacturing of biscuits, cookies, pastries, and fillings. In consequence, the studied system could serve as a model for some bakery products. This palm oil based model was subjected to a thermal treatment at 220 °C during 10 min to mimic the baking conditions of cookies and pastries. The same palm oil bakery model has been used in studies 1, 2 and 4 of this research project thesis.

The three antioxidants selected for this study were ascorbyl palmitate, acting as an oxygen scavenger, citric acid, acting as a metal chelator, and tocopherol extract, acting as a free radical scavenger. Based on a factorial design (2x2x2), each antioxidant was added into the palm oil matrix at two different concentrations. The treatments are resumed in table 4.1.

Table 4.1: Experimental design of the first study

Treatments	*Ascorbyl palmitate [mg/kg]*	*Citric acid [mg/kg]*	*Tocopherol extract [mg/kg]*
Control	0	0	0
AP	500	0	0
CA	0	300	0
TE	0	0	500
AP+CA	500	300	0
AP+TE	500	0	500
CA+TE	0	300	500
AP+CA+TE	500	300	500

AP: Ascorbyl palmitate; CA: Citric acid; TE: Tocopherol extract

In this preliminary study the heme iron ingredient (AproFER 1000™), with a total iron content of 1.07% (96% heme iron), was dispersed in palm oil in 15% (w/w) proportion. Thus, the total amount of iron added to the palm oil model (1.61 mg Fe/g sample) was higher than that of the following studies because we aimed to ensure the selection of the best antioxidant for the iron fortification model.

The preparation of the different treatments was as follows: first, palm oil was melted at 55°C, and subdivided in 8 aliquots corresponding to 8 different treatments. Thereafter, for preparing each treatment, palm oil was heated at 140°C and the different antioxidants and the heme iron ingredient were added under stirring. These conditions were chosen in order to ensure the even dispersion of the different compounds in the matrix. Following, samples were heated at 220°C for 10 min to mimic the baking conditions. Finally, the palm oil mixture was allowed to cool down to 70-80°C and 10 and 20 mL vials were completely filled up with the mixture. To ensure the homogeneous dispersion in each vial, they were shaken till palm oil solidified at room temperature.

In this study, the palm oil used was characterized before preparing the samples by means of the following analyses:

- Tocopherol and tocotrienol content (*n*=4, section 4.2.4.1).
- Fatty acid composition (*n*=2, section 4.2.4.2).

The heme iron ingredient was also characterized by determining the heme iron content as described in section 4.2.5 (*n*=2).

The samples at time 0 of storage (after the heating treatment) were characterized by means of the following determinations:

- Tocopherol and tocotrienol content in the control sample (*n*=4, section 4.2.4.1).
- Heme iron content in all samples (*n*=2, section 4.2.5).
- Susceptibility to oxidation of all samples (FOX induced method) (*n*=2, section 4.2.6.1).

Samples were stored at room temperature in the dark during 200 days. The evolution of oxidation was assessed at 0, 20, 40, 80, 120 and 200 days of storage. After the corresponding storage time, samples were stored till their analyses at -20°C. The oxidation was monitored by means of the following determinations:

- Evolution of primary oxidation compounds (see section 4.2.6.2):
    - Peroxide value (*n*=2, EU standard method).
    - Lipid hydroperoxide content (*n*=2, FOX non-induced method).
- Evolution of secondary oxidation products through the *p*-anisidine value (*n*=2, section 4.2.6.3).

4.1.2. STUDY 2: OPTIMIZATION OF THE CONCENTRATION OF THE SELECTED ANTIOXIDANTS

The second study of this thesis project aims to optimize the concentration of the efficient antioxidants in delaying the onset of oxidation. In the first study, ascorbyl palmitate was found to be very efficient and a synergism between this antioxidant and citric acid was observed. With this study we pursuit to assess this synergistic effect and to optimize the

amounts of antioxidants used for minimizing the costs, while ensuring an appropriate protection against oxidation.

Four different concentrations of ascorbyl palmitate (0, 100, 200 and 400 mg/kg) and three different concentrations of citric acid (0, 250 and 500 mg/kg) were added to the heme iron dispersions in palm oil using a 4x3 factorial design (Table 4.2). This factorial design was replicated twice.

Table 4.2: Experimental design of the second study

Treatments	*Ascorbyl palmitate [mg/kg]*	*Citric acid [mg/kg]*
Control	0	0
CA (250)	0	250
CA (500)	0	500
AP (100)	100	0
AP (100) + CA (250)	100	250
AP (100) + CA (500)	100	500
AP (200)	200	0
AP (200) + CA (250)	200	250
AP (200) + CA (250)	200	500
AP (400)	400	0
AP (400) + CA (250)	400	250
AP (400) + CA (500)	400	500

AP: Ascorbyl palmitate; CA: Citric acid

In this study, we fortified the palm oil with heme iron in order to reach more than a 15% of the of the Recommended Dietary Allowances for iron (i.e. 14 mg Fe/day, European Comunities Commission (2008)) by one chocolate filled biscuit, weighing 25 g and having 7.5 g of chocolate filling with a 25% of palm oil. As the total iron content of the heme iron ingredient (AproFER 1000™) was 1.35%, of which 96% was heme iron, this ingredient was dispersed in palm oil in 8.7% (w/w) proportion. Thus, we intended to add a total amount of iron of 1.18 mg/g sample, much lower than in the previous study (1.61 mg Fe/g sample). However, when the samples were analysed, the total iron found in samples was 0.95 mg Fe/g sample and, therefore, two of the biscuits described above will provide a 25% of iron's RDA instead of the intended 31%. Thus, the iron content was lower than expected (0.95 vs. 1.18 mg/kg) probably because there was a mistake during the dosage of the iron ingredient.

Briefly, the preparation of the different treatments was obtained after melting palm oil at 55°C. Then, palm oil was subdivided in 24 aliquots for the different treatments (12 treatments x 2 replicates). Thereafter, for preparing each treatment, palm oil was heated at 140°C and the different antioxidants and the heme iron ingredient were added under stirring. These conditions were chosen in order to ensure the even dispersion of the different compounds in the matrix. Following, samples were heated at 220°C for 10 min to mimic the baking conditions. Finally, the palm oil mixture was allowed to cool down to 70-80°C and 10 mL vials

were completely filled up with the mixture and allowed to solidify at room temperature. Afterwards, an in order to ensure the homogeneous dispersion in each vial, these were melted again at 55°C and immediately after shaken and introduced on an ice bath for solidifying the palm oil as soon as possible.

In this study, the palm oil used was characterized before preparing the samples by means of the following analyses:

- Tocopherol and tocotrienol content (*n*=6, section 4.2.4.1).
- Fatty acid composition (*n*=4, section 4.2.4.2).

The heme iron ingredient was also characterized by determining the total iron content and heme iron content as described in section 4.2.5 (*n*=6).

The samples at time 0 of storage (after the heating treatment) were characterized by means of the following determinations:

- Tocopherol and tocotrienol content in the control samples (*n*=6, section 4.2.4.1).
- Total iron content and heme iron content in all samples (*n*=6, section 4.2.5).
- Susceptibility to oxidation of all the samples (FOX induced method) (*n*=2, section 4.2.6.1).

Samples were stored at room temperature in the dark for one year, and the evolution of oxidation was followed at days 0, 15, 30, 60, 120, 180, 240, 300 and 360 days. After the corresponding storage time, samples were stored till their analyses at -20°C. The oxidation was monitored by means of the following determinations:

- Evolution of primary oxidation compounds (*n*=2 for each sample of the two replicates, section 4.2.6.2):
    - Peroxide value (EU standard method)
    - Lipid hydroperoxide content (FOX non-induced method)
- Evolution of secondary oxidation products (Section 4.2.6.3):
    - *p*-Anisidine value (*n*=2 for each sample of the two replicates).
    - Hexanal content by static headspace (SHS) (*n*=2 for samples of one replicate, except for samples stored for 180 days).

4.1.3. STUDY 3: SELECTION OF CO-SPRAY-DRYING AGENT

The third study of this work was carried in accelerated storage conditions and pursuits the rapid selection of a compound that co-spray-dried with the heme iron retarded the oxidation in an oil matrix fortified with heme iron. By an effective co-spray-drying it is possible to avoid the contact between the pro-oxidant iron and the fatty matrix thus delaying the onset of oxidation.

For this prupose, we selected two different and common coating materials used as co-spray-drying agents. One of them was a protein agent consisting of calcium caseinate whereas the

other agent was a carbohydrate consisting of a combination (1:1, w/w) between maltodextrin and modified starch (octenyl succinate). Both components were assessed in two proportions with the heme iron concentrate, 1:1 and 2:1 (heme iron concentrate : co-spray-drying agent, w/w) (Table 4.3). The manufacturing procedure of the different heme iron ingredients is presented on section 4.2.1.

Table 4.3: Experimental design of the third study

Treatments	*Heme iron ingredients*	*Iron % of the ingredient*
Control	AproFER 1000™	1.34
CAS 2:1	2:1, w/w, heme iron concentrate : CAS	0.98
CAS 1:1	1:1, w/w, heme iron concentrate : CAS	0.80
ST-MD 2:1	2:1, w/w, heme iron concentrate : ST-MD	0.96
ST-MD 1:1	1:1, w/w, heme iron concentrate : ST-MD	0.74

CAS: Calcium caseinate

ST-MD: Modified starch : maltodextrin (1:1, w/w)

All samples of different treatments were prepared to contain the same iron amount, 1.30 mg Fe/g sample

As this study was planned to select the most effective co-spray-drying agent in a short time, sunflower oil was used instead of palm oil. The sunflower oil was fortified with the different heme iron ingredients and the resulting samples were stored at 60°C to accelerate oxidation. The different treatments were prepared to content the same amount of iron (1.30 mg Fe/g mixture), which means that different percentages of the heme iron ingredients were dispersed in the sunflower oil. However, when the samples were analysed, the total iron found in samples was on average 1.27 mg Fe/g sample.

Mixtures of sunflower oil fortified with heme iron were prepared at 40°C under magnetic stirring. Once the heme iron ingredients were well dispersed, the mixtures were heated at 220°C for 10 min to mimic the typical baking conditions. Thereafter, mixtures were stored at 60°C in an oven. Every few hours, samples were taken by the following sampling procedure: mixtures were removed from oven, homogenized by magnetic stirring for 5 min and vials were filled up with sample, flushed with nitrogen, capped and stored at -20°C till analyses.

The evolution of the oxidation was followed during the next days after samples' preparation and till the control sample reached a peroxide value (EU standard method, section 4.2.6.2) higher than 100 mEq O_2/kg of oil. Besides the peroxide value, the secondary oxidation was monitored by the *p*-anisidine value (section 4.2.6.3). The oxidation determinations were performed in duplicate. Heme and total iron content were determined in the heme iron ingredients (n=5) and in samples at time 0 of storage (n=3, the determinations were done only three times due to the lack of sample) (section 4.2.5).

4.1.4. STUDY 4: COMBINATION BETWEEN THE BEST ANTIOXIDANT & THE BEST CO-SPRAY-DRYING AGENT

In the fourth study of this work, we used the results from the previous studies in order to determine the best strategy on delaying oxidation by combining the best antioxidant, namely ascorbyl palmitate, added at the optimum concentration (400 mg/kg) with the best co-spay-drying agent, namely calcium caseinate, assessed at two proportions.

Three different heme iron ingredients were used: the control heme iron ingredient (AproFER 1000™) and the heme iron co-spray-dried with calcium caseinate at 2:1 and 1:1 proportions (heme iron concentrate : calcium caseinate, w/w) (see section 4.2.1 for heme iron ingredients manufacturing procedure). Besides these ingredients, a treatment with calcium caseinate but this without being co-spray-dried with the heme iron was included in order to assess the antioxidant capability of the protein itself (at 1:1 proportion with the control heme iron ingredient) (Table 4.4).

Table 4.4: Experimental design of the forth study

Treatments	*Heme iron ingredients*	*Ascorbyl palmitate [mg/kg]*
Control	AproFER 1000 ™	0
CAS 2:1	2:1, w/w, heme iron concentrate : CAS	0
CAS 1:1	1:1, w/w, heme iron concentrate : CAS	0
CAS[a]	AproFER 1000 ™	0
AP	AproFER 1000 ™	400
CAS 2:1 + AP	2:1, w/w, heme iron concentrate : CAS	400
CAS 1:1 + AP	1:1, w/w, heme iron concentrate : CAS	400

Treatment abbreviations: CAS, calcium caseinate; AP, ascorbyl palmitate.

[a] Treatment with AproFER 1000™ and CAS (1:1, w/w) without co-spray-drying

The iron content of the heme iron ingredients was different, being 1.07% for the control ingredient AproFER 1000™, 0.75% for the heme iron ingredient at 2:1 ratio and 0.58% for the co-spray-dried heme iron ingredient at 1:1 ratio.

As model for bakery goods, samples were prepared by dispersing the different selected heme iron ingredients in palm oil. The amount of ingredient added to the palm oil was different in each treatment in order to maintain constant the iron quantity of samples (1.20 mg Fe/g samples). In this case, when the samples were analysed, the total iron found in samples was on average the same as the intended (1.20 mg Fe/g sample). Similarly to the second study, by using these dispersions a portion of 2 chocolate filled biscuits, each weighing 25 g and having 7.5 g of chocolate filling with a 25% of palm oil, will provide on average a 32% of the Recommended Daily Allowances for iron (i.e. 14 mg Fe/day, European Comunities Commission (2008)).

As in the previous studies with palm oil, for the preparation of the different treatments the palm oil was melted at 55°C and subdivided in 7 aliquots corresponding to 7 different treatments. Thereafter, for preparing each treatment, palm oil was heated at 140°C and the different antioxidants and the corresponding heme iron ingredient were added under stirring. These conditions were chosen in order to ensure the even dispersion of the different compounds in the matrix. Following, samples were heated at 220°C for 10 min to mimic the baking conditions. Finally, the palm oil mixture was allowed to cool down to 70-80°C, homogenized by a Polytron PT3100 (10 sec, 15000 rpm) and 10 and 20 mL vials were completely filled up with the mixture. To ensure the homogeneous dispersion in each vial, immediately vials were shaken and introduced on an ice bath till palm oil solidified.

In this study, the palm oil used was characterized before preparing the samples by means of the following analyses:

- Tocopherol and tocotrienol content (*n*=5, section 4.2.4.1).
- Fatty acid composition (*n*=5, section 4.2.4.2).

The heme iron ingredients were also characterized by determining the total iron content and heme iron content as described in section 4.2.5 (*n*=5).

The samples at time 0 of storage (after the heating treatment) were characterized by means of the following determinations:

- Tocopherol and tocotrienol content in all samples (*n*=5, section 4.2.4.1).
- Total iron content and heme iron content in all samples (*n*=5, section 4.2.5).
- Susceptibility to oxidation of all the samples (FOX induced method) (*n*=5, section 4.2.6.1).

In this case, samples were stored at room temperature in the dark for one year, and the evolution of oxidation was followed for days 0, 90, 180 and 360 days. After the corresponding storage time, samples were stored till their analyses at -20°C. The oxidation was monitored by means of the following determinations:

- Evolution of primary oxidation compounds (*n*=5, section 4.2.6.2):
    - Peroxide value (EU standard method).
    - Lipid hydroperoxide content (FOX non-induced method).
- Evolution of secondary oxidation products (*n*=5, section 4.2.6.3):
    - *p*-Anisidine value.
    - Hexanal content by static headspace (SHS).

4.1.5. STUDY 5: CHOCOLATE FILLED COOKIES WITH HEME IRON

The last experiment studying the oxidation during storage was done in a real bakery product, which consisted in a sandwich-type chocolate filled cookie. The aim of this study was to assess the effectiveness of the most useful previous strategies, ascorbyl palmitate addition and the co-spray-drying of heme iron with calcium caseinate, on the oxidative stability of a real

bakery product. For this proposes, we studied the effect of the addition of the different heme iron ingredients combined with ascorbyl palmitate into the chocolate filling. As shown in Table 4.5, five different treatments were prepared, one control sandwich-type cookie with no heme iron added, sandwich-type cookies with control heme iron ingredient, AproFER 1000™, alone or in combination with ascorbyl palmitate (400 mg/kg), and sandwich type cookies with the co-spray-dried heme iron (1:1, w/w, heme iron concentrate : calcium caseinate) alone or in combination with ascorbyl palmitate (400 mg/kg).

Table 4.5: Experimental design of the fifth study

Treatments	*Heme iron ingredients*	*Ascorbyl palmitate [mg/kg]*
Control no heme	-	-
Control heme	AproFER 1000™	-
AP	AproFER 1000™	400
CAS 1:1	1:1, w/w, heme iron concentrate : CAS	-
CAS 1:1 + AP	1:1, w/w, heme iron concentrate : CAS	400

CAS: Calcium caseinate; AP: Ascorbyl palmitate

Different cookie producers kindly provide us common formulas for the chocolate cream used to fill this kind of cookies (Table 4.6).

Table 4.6: Composition of the chocolate cream used to fill the cookies.

Ingredient	*%*
Icing sugar	54.3
Palm oil	23.6
Cocoa powder (10% fat)	16.3
Dry milk (1.5% fat)	5.6
Vanilla extract	0.1
Lecithin	0.1

In order to simplify the production of the cookies, we decided to use commercial biscuits (*Marie biscuits,* Carrefour, Spain) filled with chocolate creams elaborated in our laboratory. As in the fourth study, the iron content of the heme iron ingredients was different, being 1.74% iron for the AproFER 1000™ and 0.88% for the heme iron co-spray-dried with caseinate at 1:1 ratio. Thus, different heme iron ingredients amounts were added to the chocolate creams in order to maintain constant its iron content (0.31 mg Fe/g chocolate cream).

Each sandwich-type cookie was filled with 7 g of chocolate cream and weighted a total of 20 g. When the samples were analysed, the total iron found was on average 0.12 mg Fe/g cookie. Therefore, two of these cookies will provide a 34% of the Recommended Daily Allowances for iron (i.e. 14 mg Fe/day, European Comunities Commission (2008)).

To elaborate the cookies, we prepared the fortified heme iron chocolate fillings using a food processor (Thermomix brand, Vorwerk, Germany) (Figure 4.1). Briefly, we melted the palm oil in the food processor at 40ºC, after that, lecithin and, when needed, ascorbyl palmitate were added and thereafter the icing sugar was incorporated till a homogenous white paste was achieved.

While the mixer was blending the palm oil, the lecithin and the icing sugar altogether, the rest of ingredients (cocoa powder, dry milk, vanilla extract and heme iron ingredient) were mixed in a container. When the colour of the powder was homogeneous, it was added carefully and slowly into the mixer. After complete addition of ingredients, the mixture was blended at 40°C during five minutes to ensure a homogeneous mixture. Then, disposable pastry bags where filled with the chocolate cream and 7 g were putted on top of a *Marie biscuit*. Carefully, the cream was covered with another *Marie biscuit* and allowed to cool down. When all the cookies of a treatment were filled and cooled, the cookies where stored in aluminium-plastic zip bags, with very low permeability to water (<0.01g of water /m^2/24 h with 90% of relative humidity at 34°C). The bags were stored in the darkness at room temperature. The whole procedure has been resumed on figure 4.1.

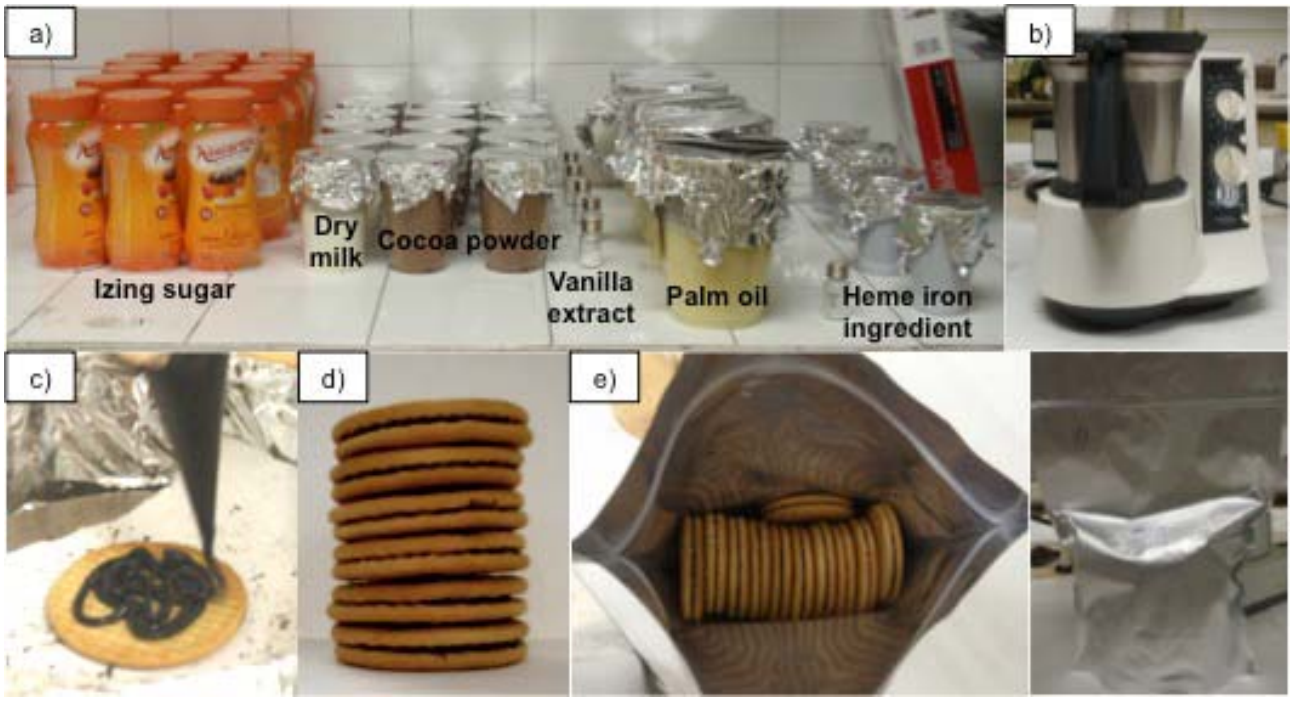

Figure 4.1: Cookies manufacturing process (a) Ingredients; (b) Mixer; (c) Cookie filling; (d) Final cookie and (e) Packaging

In this study, the palm oil used was characterized as follows:

- Tocopherol and tocotrienol content (*n*=3, section 4.2.4.1).
- Fatty acid composition (*n*=5, section 4.2.4.2).

The heme iron ingredients were also characterized by determining their total iron content and heme iron content as described in section 4.2.5 (*n*=5). Also the water absorption and solubility indexes of the heme iron ingredients were measured (*n*=5, sections 4.2.2.1 and 4.2.2.2).

Cookies at time 0 of storage were characterized by means of the following determinations:

- Tocopherol and tocotrienol content of fat extracted from cookies of all treatments (*n*=5, section 4.2.4.1).
- The total iron content and heme iron content was determined in all samples (in grinded cookies) (*n*=5, section 4.2.5).
- Susceptibility to oxidation of the fat extracted from cookies of all treatments (FOX induced method) (*n*=5, section 4.2.6.1).

Both tocopherol and susceptibility to oxidation assays required the previous extraction of the lipid fraction as described in section (4.2.3.2).

In this last oxidation storage study, the oxidation was followed during one year of storage at room temperature in the dark, taking samples at 0, 90, 180 and 360 days. After each storage time, samples were vacuum-packed and stored at -20°C till their analyses. The oxidation was monitored by means of the following determinations:

- Evolution of primary oxidation compounds (*n*=5, section 4.2.6.2):
    - Peroxide value (EU standard method) of the fat extracted from cookies as described in section 4.2.3.2
    - Lipid hydroperoxide content (FOX non-induced method) of the fat extracted from cookies as described in section 4.2.3.2
- Evolution of secondary oxidation products (*n*=5, section 4.2.6.3):
    - *p*-Anisidine value of the fat extracted from cookies as described in section 4.2.3.2
    - Hexanal content by static headspace (SHS) of the grinded cookies.

The colour of the different cookie fillings were measured, at time 0, and compared to chocolate fillings of commercial cookies and chocolate bars with different cocoa percentages (*n*=4, section 4.2.2.3). Also, the evolution of the colour of the different heme iron ingredients over the storage time was measured (*n*=4, section 4.2.2.3).

Additionally, consumer's overall acceptability was assessed by means of a consumers test as described on section 4.2.7 (*n*=32, at each storage time).

4.2. Methodology

4.2.1. PRODUCTION OF CO-SPRAY-DRIED HEME IRON INGREDIENTS

The co-spray-dried heme irons were produced in APC Europe S.A. (Granollers, Spain). The heme iron ingredient used as control along the thesis project is the commercial product AproFER 1000™ from APC Europe.

As described by González-Rosendo et al. (2010), the heme iron ingredient AproFER 1000™ is a dark powder obtained from the blood of healthy pigs. After separating the blood cell fraction by centrifugation, a pressure pump was used to release the haemoglobin contained in the red blood cells. The haemoglobin was then enzymatically hydrolysed using a proteolytic enzyme (Alcalase®, Novo-Nordisk, Denmark) under controlled pH and temperature conditions. Following this, the heme group was concentrated by ultrafiltration and spray-dried.

For obtaining the different co-spray-dried heme iron ingredients, the heme iron concentrate, resulting after ultrafiltration in the production of the AproFER 1000™ ingredient, was used as starting point. Previously, its solid content was determined gravimetrically. Once the solid content was known, the co-spray-drying agent was weighted in the desired proportion. If the co-spray-dried heme iron was prepared at 1:1 ratio, the same amount of co-spray-drying agent as the solid content of the heme iron concentrate was dispersed in the concentrate. If the heme iron prepared was the one at 2:1 ratio, half of the amount of the co-spray-drying agent was added.

In all the productions, the pH of the heme iron concentrate was adjusted to 8 before adding the co-spray-drying agent. The mix was homogenized at 40°C in order to facilitate the dispersion of the co-spray-drying agent into the heme iron concentrate. Finally, all ingredients were spray-dried in an Anhydro (Copenhagen, DK) atomizer.

4.2.2. PHYSICOCHEMICAL CHARACTERISTICS OF HEME IRON INGREDIENTS

4.2.2.1. Water absorption index (WAI)

Firstly, a screw-cap centrifuge tube was weighed (M, g) and approximate 500 mg of the heme iron ingredient (M_1, g) were poured into it. Water (M_2, g) was added to disperse the ingredient with a powder/water ratio of 0.05/1 (w/w) at ambient temperature. The dispersion was incubated in a water bath at 60°C for 30 min and immediately followed by cooling in an ice-water bath for 30 min. Then, the tube was centrifuged at 2800 g for 20 min. The resulting supernatant was removed and the centrifuge tube with sediment (M_3, g) was weighed again. WAI was calculated as following formula: $WAI(g/g) = (M_3 - M)/M_1$.

4.2.2.2. Water solubility index (WSI)

The heme iron ingredient (S_1, g) was dispersed in a centrifuge tube by adding water with a powder/water ratio of 0.02/1 (w/w) at ambient temperature. Then the dispersion was incubated in a water bath at 80°C for 30 min, followed by centrifugation at 4000 g for 10 min. The supernatant was carefully collected in a pre-weighed evaporating dish (S_2, g) and subjected to dry at 103 ± 2°C, and the evaporating dish with residue was weighed again (S_3, g). WSI was calculated as following formula: $WSI(\%) = (S_3 - S_2)/S_1\, x100$.

4.2.2.3. Colour assessment

The colour of the heme ingredients of studies 3, 4 and 5 was measured by a Konica Minolta Chromameter (model CR-410; Konica Minolta Sensing Inc., Osaka, Japan) based on the CIE (Commission International de L'Eclairage) L*a*b* colour space at the initial storage time (being L* lightness; a* redness and b* yellowness values).

Also, the evolution over the storage time (0, 90, 180 and 360 days) of the heme iron ingredients colour was assessed on the fifth study at room temperature.

Finally, the different chocolate fillings colours were also assessed at the initial storage time (0 days). For doing so, sandwich type cookies were heated during 5 seconds on a microwave. Then, cookies were separated and the chocolate filling was removed carefully and transferred to a capsule.

The colour was determined from four different random surfaces of the heme iron ingredients or chocolate creams. The instrument was set for illuminant D-65 and at a 2°-observer angle, and standardized using a standard white plate.

4.2.3. PREPARATION OF SAMPLES FOR CHEMICAL ANALYSES

4.2.3.1. Palm oil and sunflower oil studies (Studies 1 to 4)

In the first study, sample preparation for the various analyses was done in different manners. Tocopherols and tocotrienols of control sample at time 0 and heme iron content of all time 0 samples were assessed in samples without removing the heme iron particles. For the different oxidation determinations the heme iron particles were removed by different procedures. For the peroxide value determination, samples were melted at 55°C, and thereafter centrifuged at 1370 g during 5 minutes. The supernatant was used for peroxide value determination. For the LHP content determination through the FOX method, samples were melted (55°C), weighted in 10 mL volumetric flask (0.2-1.2 g) and the volume was made up with dichloromethane/ethanol (3:2, v/v). Then, the solution was centrifuged at 1370 g for 3 min, and an aliquot of the supernatant (100-600 μL) was used for the determination. Finally,

before *p*-AnV determination, samples were melted at 55°C and filtered through Whatman no. 54 filter paper at room temperature to remove the heme iron particles.

This last procedure for removing heme iron particles through filtration was used in the following studies with palm oil (studies 2 and 4) for all the oxidation and tocopherol and tocotrienol determinations. Also, the same procedure was done on sunflower samples from study 3; however, in this case samples were not melted at 55°C, just removed from the freezer and allowed to thaw at room temperature before filtering them.

Determinations of total and heme iron, and hexanal content were performed on samples without removing the heme iron particles.

4.2.3.2. Sandwich-type cookies study (Study 5)

In the last study, where real cookies were used in the oxidation storage experiment, the oxidation analyses were done on the fat extracted from the whole cookie. As the method of Folch et al. (1957) extracts part of the heme moiety along with the fat, this resulted in a dull colour which interferes with some analyses. Therefore, we selected the method from Rose & Oklander (1965), in which a mixture of isopropanol/chloroform (3:2, v/v) is used for the extraction of lipids.

The final method used for the lipid extraction of cookies was as follows: the cookies at each storage time, each weighting 20 g, were grinded on a Retsch blender Grindomix GM 200 (Düseldorf, Germany). For time 0 and 90 days three cookies were used for analyses, whereas for time 180 and 360 two cookies were used. Cookies were grinded for 10 seconds at 6000 rpm in discontinuous mode (1 second grinding, 1 second stopped) and thereafter 10 seconds in continuous mode.

Five grams of the grinded cookies were destined to the analysis of the volatile content. The remaining amount of ground cookies was transferred to a special glass extraction vessel (GS 50, Kinematica, Lucerne, Switzerland), and 300 mL of the extracting mixture (isospropanol/chloroform, 3:2 v/v) were added. The sample was then homogenized on a Polytron PT 3100 at 19000 rpm for 40 seconds. Samples were allowed to settle and the upper liquid was decanted and filtered through Whatman no.1 filter paper at room temperature under vacuum into a Kitasato flask. Then the solvent was transferred to a round bottom flask. This process was done two times more, the second one with 200 mL and the last one with 100 mL of the solvent mixture, to a total extraction volume of 600 mL. Thereafter, the Kitasato flask was rinsed with 20 mL of the solvent mixture and purred also into the round bottom flask. The extract was then dried on a rotary evaporator at 40°C. At storage times of 180 and 360 days, in which less amount of sample was extracted, the volume of solvent mixture used was proportionally reduced, using a total of 400 mL divided into 200 mL, 120 mL and 80 mL, respectively. The fat extraction yield was equal at all storage times.

Once the extract was dried, it was re-dissolved in 80 mL (60 mL for 180 and 360 days of storage) of a methanol/chloroform (2:1, v/v) mixture. The following steps are similar to those

reported in the Folch method. The extract was sub-divided in two test tubes, 10 mL of 1% salt solution where added to each tube, tubes were manually shaken and samples where centrifuged at 540 *g* during 20 min. After phase separation, the chloroform phase was filtered through sodium sulphate anhydrous to remove any trace of water and the filtrate was collected into a round bottom flask. Thereafter, samples were evaporated on a rotary evaporator at 35°C. Following, the flasks were left overnight under vacuum on a desiccator. Finally, the fat on the flasks was melted at 60°C, transferred to small vials, blew with nitrogen and stored at -20°C till analyses.

Determinations of total and heme iron, and hexanal content were performed directly on the grinded cookie.

4.2.4. PALM OIL CHARACTERIZATION

The palm oils used in the studies 1, 2, 4 and 5 were characterized by its content in tocopherols and tocotrienols and fatty acid composition.

4.2.4.1. Tocopherol and tocotrienol composition

The tocol (tocopherols and tocotrienols) content of the different palm oils used to prepare samples of the different studies was determined. In addition, the content in tocols of control samples (palm oil added with the control heme iron ingredient) after the heating treatment was determined in studies 1 and 2. In studies 4 and 5, the tocol content of all treatments at time 0 of storage was also analysed.

The method used for the determination of tocol composition was adapted from Hewavitharana et al. (2004) and Nuchi et al. (2009). Firstly, an antioxidant solution was prepared in absolute ethanol containing 1% pyrogallol w/v, 0.012% butylated hydroxytoluene w/v, and 0.4% anhydrous citric acid w/v. The whole process must be carried out under attenuated light. Before weighing, samples must be liquid and homogenous and therefore palm oil samples were melted at 55°C and shacked. An amount of sample ranging from 200 to 250 mg of palm oil were weighted in a 50 mL screw capped centrifuge tube and immediately 5 mL of the antioxidant solution where added, mixed gently and kept into an ice bath until 10 mL of 1.6 N methanolic KOH were added to samples. Then samples were immediately mixed using a vortex mixer for 1 min.

After homogenization, saponification was carried out in a water bath at 70°C under gentle agitation during 30 min. Every 8 min the tubes were mixed manually and putted back into the water bath. After the heating treatment, the tubes where allowed to stand into an ice bath for 7 min to reach room temperature. Then, 15 mL of distilled water were added. Following, 10 mL of petroleum ether were added to samples and subsequently mixed with a vortex mixer for 1 min and centrifuged at 450 *g* for 7 min. The upper layer was removed with a Pasteur

pipette and transferred into a round bottom flask. This procedure was repeated two times more, reaching a total volume of 30 mL petroleum ether. The non-saponifiable fraction was then dried on a rotary evaporator at 30°C.

The dry-residue in the round bottom flask was then dissolved with petroleum ether and filtered through a PTFE syringe filter (0.45 μm) under vacuum. The filtrate was collected into a screw-capped test tube and evaporated under nitrogen stream at 30°C. Finally the residue was re-dissolved with 1 mL of n-hexane HPLC grade and 100 μL injected on a liquid chromatograph Series 1100 (Hewlett-Packard, Waldbronn, Germany) for chromatographic separation.

The loop volume was of 20 μL and a Luna column (4.6 mm i.d., 150 mm; Phenomenex, USA) packed with 3 μm–100 *Å* silica was used. Tocols were isocratically eluted with n-hexane:1,4-dioxane (95:5 v/v) and detected using a Hewlett-Packard 1046A spectrofluorometric detector (excitation and emission wavelengths of 290 and 320 nm, respectively). Tocols extraction and injection was done within the same day.

Calibration curves were prepared in n n-hexane (HPLC grade) using standards of the four tocopherols (Calbiochem, San Diego, CA). Tocotrienols were quantified using the calibration curve for the corresponding tocopherol analog. The standards were injected in the same conditions as samples.

4.2.4.2. Fatty acid composition

The fatty acids methyl esters (FAMEs) were prepared from palm oil following the protocol described by Guardiola et al. (1994). Firstly, palm oil was melted (55°C) and homogenized. Then, 100 mg of sample were weighted on a screw cap test tube and 2.5 mL of methanolic solution of sodium methoxide 0.5 N were added. The tube was tightly sealed and placed in a boiling water bath for 20 min or until a homogenous phase was achieved. The tubes were removed from water bath and allowed to cool at room temperature. After triacylglycerol transesterification, basic pH was assessed by the addition of phenolphthalein 1%. Thereafter, 3 mL of methanolic solution of boron trifluoride (14% w/v) were added. The tube was sealed again and returned to the boiling water bath for 15 min. Finally, the tube was allowed to cool down until reaching 30-40°C. FAMEs were extracted by adding 3 mL of n-hexane and 4 mL of a saturated sodium chloride solution to the tube that was agitated for 30 seconds. When the two phases were separated, the hexane phase containing FAME was transferred to another tube with a small amount of anhydrous sodium sulphate. After 1 h, samples were transferred to vials and kept at -20°C till chromatographic determinations were carried out.

FAMEs were analysed on an Agilent (Waldbronn, Germany) 4890D model gas chromatograph, fitted with a flame-ionization detector and split-splitless injector, set at 300 and 270°C, respectively. The sample volume injected was 1 μL. The split ratio was 1:30. Chromatographic separation of FAME was performed on a fused-silica capillary column (60 m - 0.25 mm i.d.) coated with 0.2 μm of a stationary phase of 90% biscyanopropyl plus 10%

cyanopropylphenyl-polysiloxane (SP-2380 from Supelco, USA). Helium, at 30 psi, was used as a carrier gas. The oven was programmed as follows: firstly 2 min at 150°C, then the temperature was increased at 1.5°C/min to 180°C, then at 7.5°C/min to 220°C at which temperature it was held for 6 min, and then finally the temperature was increased at 5°C/min to 250°C and held for 20 min at this temperature.

4.2.5. TOTAL AND HEME IRON QUANTIFICATION

4.2.5.1. Heme iron quantification

The heme iron content was determined in the heme iron ingredients and also in all samples at 0 days of storage by means of the colorimetric method described by Hornsey (1956).

According to this method, the heme pigment (hemin) is extracted by a solution of acidified acetone and thereafter its absorbance is measured at 640 nm. For a complete extraction of the heme moiety, it is important to have a quantity of acetone in the extraction medium close to 80%. Taking into account that the water content of samples studied was negligible, the solution prepared contained 80% of acetone, 2% of hydrochloric acid and water.

150 mg of palm oil fortified with heme iron or 1.5 g of grinded cookies were weighted on a screw capped tube and exactly 30 mL of acidified acetone were added. For the content in the heme ingredients, 40 mg of sample were weighted and exactly 60 mL of acidified acetone were added. During 10 min, samples were extracted in an ultrasound bath and thereafter allowed to stand for one hour in darkness at room temperature under magnetic stirring. Following, extracts were filtered through Whatman paper filter nº42, and their absorbance was assessed at 640 nm.

The heme iron content was calculated as follows:

$$Heme\ iron\ (mg/g\ sample) = 8.54\% * Hemin\ (mg/g\ sample)$$

$$Hemin\ (mg/g\ sample) = \frac{Ab}{4.8}\ x\ \frac{V_{solvent\ added}}{m}\ x\ 652$$

Were:

Ab = Absorbance at 640 nm

$V_{extract}$ = volume of solvent added in mL,

m = weight of oil in g

4.2.5.2. Total iron quantification

The total iron content of the heme ingredients and treatment samples was determined in all samples except for samples of the first study of this thesis project. The total iron content was assessed by a PerkinElmer ICP-AES (Optima 3200 RL model) after the mineralization of the

samples. The quantification of the iron was made by the external standard method, using an iron standard from High-Purity Standards (Charleston, SC) and was traceable to the Standard Reference Materials of NIST.

The calcination of the heme iron ingredients was as follows: firstly, 250 mg of the ingredient was weighted in a glass beaker and 2 mL of H_2SO_4 were added to the sample and allow drying in a sand bath at 250°C. The ingredient was then calcinated in a muffle furnace at 450°C for 10 hours (rate of heating 1°C/min). The following day, 1 mL of H_2SO_4 was added and allowed to dry in a sand bath at 300°C. Finally, a second heating treatment was carried out in the muffle furnace at 450°C for a period of 3-6 hours, re-dissolved in 2 mL of HCl and diluted to a volume of 100 mL with 1% HNO_3.

The calcination process of samples from the study 2 was as follows: 1 g sample was weighed in a glass beaker and 2 mL of H_2SO_4 were added. Then, sample was placed in a sand bath at 250°C. Once it was smoking an additional 2 mL of H_2SO_4 were added. This step was repeated twice, until the amount of added H_2SO_4 was 8 mL. Sample was then allowed to dry in the sand bath before being calcinated in a muffle furnace at 450°C for 10 hours (rate of heating 1°C/min). The following day, 1 mL of H_2SO_4, 2 mL of HNO_3 and 1 mL of H_2O_2 were added (H_2O_2 was added in fractions of 0.2 mL). The beaker was then allowed to boil at 250°C in the sand bath for 30 min. Thereafter, sample was dried and calcinated again overnight in the muffle furnace at 450°C. Finally, the sample was re-dissolved in 2 mL of HCl and diluted to a volume of 100 mL with 1% HNO_3.

The calcination of samples in study 3 and 4 was optimized as follows: firstly, 1 g sample was weighed in a glass beaker and 2 mL of H_2SO_4 were added. Then, sample was placed in a sand bath at 250°C. Once it was smoking, an additional 2 mL of H_2SO_4 were added and allowed to dry in the sand bath. Sample was then calcinated in a muffle furnace at 450°C for 10 hours (rate of heating 1°C/min).The following day, 1 mL of H_2SO_4 was added and allowed to dry on the sand bath at 250 °C and subsequently calcinated at 450°C. As in study 2, the sample was re-dissolved in 2 mL of HCl and diluted to a volume of 100 mL with 1% HNO_3.

Finally, cookies were digested with nitric acid. Because samples contained powder milk, the concentration of calcium was high and it may precipitate as sulphate (from added sulphuric acid). Besides, the iron concentration of this sample was lower than that of the previous ones, which made necessary to change the methodology for improving the sensitivity. Consequently, 250 mg of ground cookies were weighted in quartz tubes and 25 mL of HNO_3 were added. Then samples were left overnight on a thermo block at 60°C. The following morning, the temperature was raised till 120°C, and left for 1 h. Thereafter, samples were evaporated at 160°C till approximately 5 mL. Following, 5 mL of perchloric acid were added and samples were kept at 180°C for one extra hour. Finally, perchloric acid was evaporated at 210°C till 1 mL approximately and samples were transferred to a 50mL capacity flask with 1% nitric acid.

4.2.6. ASSESSMENT OF SUSCEPTIBILITY TO OXIDATION AND OXIDATION STATUS DURING STORAGE TIME

The susceptibility to oxidation was determined in samples at day 0 of storage. The primary and secondary oxidation parameters were determined during the storage at the times defined in each study.

4.2.6.1. Susceptibility to oxidation

Susceptibility to oxidation was assessed on samples at day 0 of storage by the induced version of the FOX-method described by Grau et al. (2000). By means of this method, the formation of lipid hydroperoxides (LHP) over time was measured in samples from studies 1, 2, 4 and 5.

To perform the method, 1.2 g of sample was dissolved in 10 mL of dichloromethane/ethanol (3:2, v/v). Depending on samples' susceptibility to oxidation, different aliquot volumes were used (ranging between 200 and 500 μL). These aliquots were mixed with the FOX reaction media in 1cm path length Teflon-capped glass-cuvettes. The FOX reaction media consisted of: 100 μL of 5 mM aqueous ferrous ammonium sulphate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic xylenol orange and the required quantity of dichloromethane/ethanol (3:2, v/v) to reach the final cuvette volume of 2,000 μL. It is important to note that all glass material was cleaned previously with double deionized water in order to avoid any interference during the measurement of the LHP.

The incubation of the mixture in the cuvettes was carried out at room temperature under attenuated light conditions until absorbance at 560 nm was stable, which means that the difference between two consecutive measurements for all samples was less than 0.02 (incubation times ranged from 54 to 191 hours in the different studies). By using a standard curve prepared with cumene hydroperoxide (CHP), the content of the LHP formed during the incubation was expressed as millimoles of CHP equivalents/kg of each sample.

To better describe samples susceptibility to oxidation, form the LHP formation curve several parameters were calculated as described by Tres et al. (2009). The parameters determined were:

- Initial LHP: initial LHP value measured after 30 min of incubation
- MAXLHP: maximum LHP value
- TMAX: time until the MAXLHP was achieved
- Final LHP: final LHP value, measured when the absorbance at 560 nm was stable
- AUC: area under the curve of LHP formation

4.2.6.2. Primary oxidation

The evolution of the primary oxidation products in samples was determined using the peroxide value (PV) and the non-induced FOX method.

- *Peroxide value (PV)*

The peroxide value of samples was measured as in the method described in the *European Communities Commission* regulation for olive oil (European Communities Commission, 1991). By definition, this method determines all substances (expressed as oxygen active milli equivalents) that oxidize potassium iodide under the conditions of the test. Basically, the substances are assumed to be peroxides.

Depending on the expected peroxide value, 0.5 to 4 g of sample were weighted on a 250 mL Erlenmeyer flask. Following, 10 mL of chloroform and 15 mL of acetic acid were added and mixed. While purging with nitrogen to replace the air in the flask and solvents, 1 mL of potassium iodide saturated solution was added. Then, the flask was shaken for one minute, and incubated at room temperature in the dark for 5 minutes. Thereafter, the reaction was stopped by the addition of 75 mL of distilled water.

Samples and blanks were titrated with 0.01 or 0.002 N sodium thiosulphate. The concentration of the sodium thiosulphate used depended on samples' expected peroxide value. A 1% starch solution was used as indicator. The exact concentration of sodium tiosulphate solution was assessed by its titration against potassium dichromate.

The PV is calculated by the next formula:

$$PV = \frac{V\ x\ N\ x\ 1000}{m}$$

Were:

V= mL of sodium thiosulphate used in the assay
N=exact normality of the sodium thiosulphate solution
m= weight of oil, g

- *LHP content*

The LHP content of samples during the storage period was assessed by non-induced FOX method (Navas et al., 2004). The procedure used is similar to that described for the induced FOX method, with the exception that samples absorbance at 560 nm is measured immediately after 30 min of reaction at room temperature in the dark.

Briefly, from 0.2 -1 g of sample was dissolved in 10 mL of dichloromethane/ethanol (3:2, v/v), depending on the oxidation state of samples. 100 µL of the solution were mixed with the FOX reaction media in 1 cm path length Teflon-capped glass-cuvettes. The FOX reaction media consisted of: 100 µL of 5 mM aqueous ferrous ammonium sulphate, 200 µL of 0.25 M methanolic H_2SO_4, 200 µL of 1 mM methanolic xylenol orange and 1400 µL dichloromethane/ethanol (3:2, v/v) to reach the final cuvette volume of 2,000 µL. It is important to note that all glass material was cleaned previously with double deionized water in order to avoid any interference during the measurement of the LHP.

4.2.6.3. Secondary oxidation

The formation of secondary oxidation compounds in the samples was assessed using the *p*-anisidine value (*p*-AnV; AOCS, 1998) and the hexanal content determined by static headspace.

- *p-Anisidine value*

The official method from the American Oil Chemistry Society Cd 18-90 was assessed on samples. This method, determines the amount of aldehydes (mainly 2-alkenals and 2,4-decadienals) in samples by reaction in an acidic medium of the aldehyde compounds and the *p*-anisidine.

The *p*-anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm path length cuvette of a solution containing 1.00 g of oil in 100 mL of a mixture of solvent and *p*-anisidine according to the method described below.

Between 0.5 and 4 grams of sample were weighted in 25 mL volumetric flasks and dissolved with isooctane. The absorbance of the solution at 350 nm was measured. Then, exactly 5 mL of solution were transferred to test tubes and 1 mL of *p*-anisidine solution in acetic acid (0.25% w/v) was added. After 10 min of reaction, the absorbance of the solution with *p*-anisidine was measured at 350 nm.

The *p*-anisidine value is given by the formula:

$$p - AnV = \frac{25x(1.2As - Ab)}{m}$$

Were:

As=absorbance of the fat solution after reaction with *p*-anisidine reagent
Ab=absorbance of the fat solution
m=weight of oil, g

- *Hexanal content by Static Headspace*

Opposite to the other oxidation parameters, samples used on this assay were neither filtered nor extracted (see section 4.2.3). Therefore, in studies 2 and 4 the sample used was the mixture of palm oil with the heme iron and in study 5 the sample was the grinded cookie. Using a Combi PAL autosampler (CTC Analytics, Switzerland), a number of extraction conditions were assayed to identify the best extraction conditions for volatile compounds. The variables tested for setting up the method for palm oil samples were: samples weight (0.5 - 1.0 - 1.5 g), extraction time (15 - 30 - 45 min) and temperature (40 – 55 – 70°C). For the parameters optimization, we followed a similar approach as described by Romeu-Nadal et al. (2004), which consisted on maintaining two parameters fixed (i.e. 0.5 g of sample and 15 min) and change the third one (temperature of extraction in this case). By means of this methodology, the final selected extraction conditions for volatiles determination consisted in heating 500 mg of palm oil fortified with heme iron sample plus 100 µL of dimethyl sulfoxide (DMSO) at 70°C for 30 min.

Following a similar approach as that for palm oil samples, various conditions were assessed for ground cookies. In this case, we kept the time and the temperature for the extraction (30 min at 70°C) and tried different sample's amount (0.5-1.0-1.5 g) and the addition of water (0 – 0.5 – 1 mL). The selected conditions were, 0.5 g of grinded cookies plus 1 mL of water, plus 100 μL of DMSO, extracted during 30 min at 70°C.

In both cases, 1 mL of the vial headspace was injected into an Agilent 4890D model (Waldbronn, Germany) gas chromatograph equipped with flame ionization detector and a split-splitless injector. The injector was set for 2 min in splitless mode; the split ratio was 1:20 for palm oil samples and 1:10 for cookie samples. The chromatographic separation of volatile compounds was performed on a fused-silica capillary column (30 m × 0.20 mm i.d.) coated with 0.2 μm of a stationary phase of 5% diphenyl- plus 95% dimethyl-polysiloxane (Equity TM-5 from Supelco, USA). Helium, at 20 psi, was used as a carrier gas. The injector and the detector temperatures were 200°C and 325°C, respectively. The oven program was as follows: 1 minute at a temperature of 40°C, which was then increased at 10°C/min to 300°C and then held at this temperature for 5 min.

Hexanal was identified by comparing the retention time with a standard and was quantified using the external standard method. For palm oil samples, to prepare the standard curve, fresh palm oil (without hexanal) was mixed with the heme iron ingredient. A standard curve was prepared for each heme iron ingredient. A total of 500 mg of the mixture was then weighed in vials. 100 μL of solutions with different concentrations of hexanal in DMSO were added to the vials and sealed by screw caps.

The method was validated before assessing the samples from the second study of the thesis. Under these conditions, for palm oil samples, the method displayed a good linearity (R^2=0.993), a good precision (RSD = 1.95%, 4 determination within the same day in a sample containing 285 mg hexanal/kg) and a good recovery (98%). The recovery was assessed in 2 samples that were analysed 4 times. Samples had 285 mg hexanal/kg and 125 mg/kg, respectively, and the hexanal amount added was one third of their initial concentration (95 mg/kg and 42 mg/kg, respectively). Taking into account the analyte content of samples (between 50 - 600 mg/kg), these values fulfil the values recommended by the AOAC international (1998). The limits of detection and quantification of the method were 6 and 19 mg/kg, respectively. Both limits were calculated as 3 and 10 times the standard deviation of the baseline noise, respectively (AOAC international, 1998).

For the ground cookies, the standard curve was prepared on fresh ground cookies (control, control heme and CAS 1:1), with no hexanal, and then adding aliquots of 100 μL of DMSO with different hexanal concentrations. This method had a good linearity (R^2=0.991 - 0.994), a good precision (RSD = 3.04%, 4 determination within the same day in a sample containing 32 μg hexanal/kg) and a good recovery (99%). The recovery was assessed in 2 samples that were injected four times. Concentration of the samples was 32 and 16 μg hexanal /kg. The hexanal standard amount added was one third of their initial concentration (11 μg/kg and 6 μg/kg, respectively). Taking into account the analyte content of samples (between not detected - 180 μg/g) these values meet the values specified by the AOAC for validation of methods. The limit

of detection and quantification of the method were 0.86 and 1.12 μg/kg of sample, respectively.

4.2.7. SENSORY ANALYSIS OF FORTIFIED COOKIES

The sensory analysis of cookies was performed in the last study. The analyses were done at time 0, 180 and 360 days. Overall acceptance was performed by means of a consumer test with 32 participants at each storage time. A 9-point hedonic scale was used, being 9 excellent, 1 very poor, to evaluate the fortified cookies. Answer sheet is presented in annex 9.4.

Samples were served to consumers at room temperature and presented to the participants in plastic white plates coded with three-digit random numbers. Every participant tasted all the different cookies in each session. Spring water was provided for rinsing their palates between samples.

The age of the consumers ranged between 18 and 63 years and both women and men were included. In order to participate, the consumption of chocolate bakery goods has to be at least 12 times per year, among other characteristics (Annex 9.5). The rules given to the participants before attending to the sensory analysis are also given in this annex.

4.2.8. STATISTICAL ANALYSES

In studies 1, 2, 4 and 5, the relationship between PV and LHP (non-induced) was assessed using the Pearson's correlation coefficient. The relationship between *p*-AnV and hexanal was assessed in studies 2, 4 and 5 by the same coefficient. Moreover, the relationship between primary and secondary oxidation values was assessed in the last study.

In study 1, Spearman's correlation coefficients were used to assess the relationship between parameters from the FOX-induced method (MAXLHP, TMAX, FinalLHP and AUC) used to assess the susceptibility of samples to oxidation and the areas calculated under the curves given by PV and LHP content (non-induced FOX method) used to assess primary oxidation during the storage of samples for 200 days.

Multifactorial ANOVA was used to determine whether the studied factors, antioxidant concentration and storage time (in studies 1 and 2) and treatment and storage time (in studies 4 and 5) had any significant effect on PV, LHP content, *p*-AnV and hexanal content of palm oil and cookies samples. ANOVA was applied to each storage time to determine any significant effect of the treatments on PV, LHP content, *p*-AnV and hexanal content. Also, in studies 4 and 5, ANOVA was used to assess the differences between treatments in the tocopherol and tocotrienol content and in the FOX induced parameters of samples after heating (at 0 days of storage). When significant differences were observed the means were

separated using multiple comparison tests (α=0.05). In all cases a $P \leq 0.05$ was considered significant.

5. PUBLICATIONS

5.1. Effectiveness of antioxidants in preventing oxidation of palm oil enriched with heme iron: A model for iron fortification in baked products

JOURNAL: European Journal of Lipid Science and Technology, 2010, 112, 761-769.

IMPACT FACTOR : 2.033.

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TITLE AND ABSTRACT IN SPANISH/ TÍTULO Y RESUMEN EN CASTELLANO:

"Efectividad de antioxidantes en la prevención de la oxidación de manteca de palma enriquecida con hierro hémico: un modelo para la fortificación con hierro de productos de bollería, pastelería y galletería"

Los productos de pastelería, tales como los biscotes, las galletas o los pasteles, representan un buen medio para la foritificación con hierro de alimentos, ya que son consumidos por un alto porcentaje de la población en riesgo de desarrollar deficiencia de hierro, como pueden ser los niños.

Sin embargo, la fortificación con hierro puede promover la oxidación del alimento enriquecido. Para estudiar la incidencia en productos de pastelería, ha sido empleado un modelo consistente en manteca de palma enriquecida con hierro hémico y diferentes antioxidantes. Para reproducir las condiciones de horneado de los productos de pastelería, las muestras de manteca de palma fueron calentadas a 220ºC durante 10 minutos.

En este estudio, los antioxidantes seleccionados fueron un anti-radicalario, como es el extracto de tocoferoles (0 y 500 mg/kg), un secuestrante de oxígeno, como es el palmitato de ascorbilo (0 y 500 mg/kg) y un agente quelante, como es el ácido cítrico (0 y 300 mg/kg). Mediante el empleo de un diseño factorial, estos antioxidantes fueron combinados y comparados con una muestra control a la que no se le añadió ningún antioxidante.

A lo largo de 200 días de almacenamiento a temperatura ambiente y en la oscuridad se analizaron diversos parámetros de oxidación primaria (índice de peróxidos y contenido de hidroperóxidos lipídicos) y secundaria (índice de *p*-anisidina). El tratamiento que combinó el palmitato de ascorbilo y el ácido cítrico fue el más efectivo en retardar el desarrollo de la oxidación. A su vez, el extracto de tocoferoles no fue efectivo en la prevención de la oxidación de las muestras. El índice de *p*-anisidina no aumentó a lo largo del tiempo de almacenamiento, indicando que este parámetro de oxidación no fue el adecuado para el seguimiento de la evolución de la oxidación secundaria en este modelo.

Research Article

Effectiveness of antioxidants in preventing oxidation of palm oil enriched with heme iron: A model for iron fortification in baked products

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Bakery products such as biscuits, cookies, and pastries represent a good medium for iron fortification in food products, since they are consumed by a large proportion of the population at risk of developing iron deficiency anemia, mainly children. The drawback, however, is that iron fortification can promote oxidation. To assess the extent of this, palm oil with added heme iron and different antioxidants was used as a model for evaluating the oxidative stability of some bakery products, such as baked goods containing chocolate. The palm oil samples were heated at 220°C for 10 min to mimic the conditions found during a typical baking processing. The selected antioxidants were a free radical scavenger (tocopherol extract (TE), 0 and 500 mg/kg), an oxygen scavenger (ascorbyl palmitate (AP), 0 and 500 mg/kg), and a chelating agent (citric acid (CA), 0 and 300 mg/kg). These antioxidants were combined using a factorial design and were compared to a control sample, which was not supplemented with antioxidants. Primary (peroxide value and lipid hydroperoxide content) and secondary oxidation parameters (*p*-anisidine value, *p*-AnV) were monitored over a period of 200 days in storage at room temperature. The combination of AP and CA was the most effective treatment in delaying the onset of oxidation. TE was not effective in preventing oxidation. The *p*-AnV did not increase during the storage period, indicating that this oxidation marker was not suitable for monitoring oxidation in this model.

Practical applications: Commonly consumed foods, such as baked food products, are often enriched with iron in efforts to address iron deficiencies. The bioavailability of heme iron is higher than that of inorganic iron. However, as with inorganic iron, heme iron induces oxidation, which affects organoleptic properties and shelf life. Adding antioxidants to fortified products could prevent such undesired changes. In this study, effectiveness of antioxidants in preventing oxidation of iron-fortified palm oil was evaluated. These results are applicable in the baking industry and contribute to efforts to increase the nutritional value of different food products.

Keywords: Antioxidants / Food enrichment / Heme iron / Oxidative stability / Palm oil

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Abbreviations: AP, ascorbyl palmitate; **AUC**, area under the curve; **CA**, citric acid; **CHP**, cumene hydroperoxide; **FA**, fatty acid; **FOX**, ferrous oxidation-xylenol orange; **LHP**, lipid hydroperoxide; **MAXLHP**, maximum lipid hydroperoxide; ***p*-AnV**, *p*-anisidine value; **PV**, peroxide value; **TE**, tocopherol extract; **TMAX**, time to reach the maximum lipid hydroperoxide value

1 Introduction

Iron deficiency is one of the most prevalent health issues in the world [1]. The fortification of foods with iron is a common strategy used to tackle this problem. Among the different iron compounds that can be used, heme iron has a particularly high bioavailability, which is higher than that of inorganic iron mainly because the inhibition of heme iron absorption by food ligands is low [2], and also their absorption process is different [3]. As a result, trials have taken place to fortify different foods such as biscuits [4, 5], cookie fillings

[6], weaning foods [7], flour [8], and black beans [9] with heme iron.

It has been reported that heme iron has the ability to act as a prooxidant [10–12], however, only a small number of authors paid attention to this matter. For instance, Asenjo *et al.* [4] found that fortifying biscuits with 6% bovine heme iron concentrate (a red cell isolate containing the whole molecule of hemoglobin) was acceptable based on a sensory evaluation (although the control ranked better). Asenjo *et al.* [4] monitored the peroxide value (PV) during storage (at 17–20°C) and found that it increased compared with the non-fortified control sample. However, the authors did not provide any data on secondary oxidation products. In another study, Martínez Graciá *et al.* [7] used 0.5% w/w heme concentrate containing the entire molecule of hemoglobin to fortify a commercially available weaning food. After 8 months of storage at 22 and 37°C no rancidity was detected by a sensory evaluation. The low dose of iron used could explain these results, but the authors provided no additional information regarding lipid oxidation. The above examples illustrate the lack of information regarding the evolution of oxidation in products supplemented with heme iron.

A good medium is an important part of a successful iron fortification program [13] as it has to be something that is consumed by a large proportion of the target population. Since iron deficiency anemia is most prevalent in children [1] and because cookies are a daily component of children's diets, cookies and/or pastries are a good choice. Furthermore, a dark ingredient such as heme iron could be disguised well in a chocolate cookie or filling.

The objective of this work was to evaluate the oxidative stability of a refined palm oil sample – to which a heme iron ingredient was added – which was subjected to a heating treatment to mimic the baking conditions of cookies and pastries. In addition, we evaluated the oxidative stability of this matrix after various antioxidants were added with different mechanisms of action (tocopherol extract (TE), ascorbyl palmitate (AP), and citric acid (CA)). A control group to which no antioxidants were added was used for comparison. Primary and secondary oxidation parameters were monitored during storage at room temperature for a period of up to 200 days. Oxidative stability was also assessed by means of an induced method.

2 Materials and methods

2.1 Materials

Palm oil was obtained from Lípidos Santiga S.A. (Santa Perpètua de Mogoda, Spain). AP (99.5% purity) was purchased from Sigma–Aldrich (Madrid, Spain). CA (99.5% purity) and triacetin (99% purity) were purchased from Scharlab S. L. (Barcelona, Spain), and Guardian Toco 70™ which was used as a TE source was donated by Danisco Cultor España S.A. (Barcelona, Spain). The concentration of the Guardian Toco 70™ was 70% TE and 30% sunflower oil. The following concentrations of the different analogs were determined in our laboratory by HPLC: 10.9% alpha-tocopherol, 1.3% beta-tocopherol, 47.7% gamma-tocopherol, and 18.9% delta-tocopherol. The heme iron ingredient (AproFER 1000™) was from APC Europe S.A. (Granollers, Spain). It consists of a dark powder obtained from the blood of healthy pigs. Hemoglobin was liberated, later enzymatically hydrolyzed, and the heme group was concentrated/purified by ultrafiltration and spray-drying as described by Quintero-Gutierrez *et al.* [6]. This heme iron ingredient is a proteinaceous material that has a total iron concentration of 1.07%, of which a 96% is heme iron.

All chemicals used were ACS grade with the exception of the solvents used in the ferrous oxidation-xylenol orange (FOX) method and when determining tocol; the chemicals used for the latter two processes were of HPLC grade.

2.2 Ingredient selection and study design

Samples were made up of a palm oil base in which the heme iron ingredient (15% w/w) was dispersed. Refined palm oil was chosen for this study because it is frequently used in the manufacturing of biscuits, cookies, pastries, and fillings, and so the studied system could serve as a model for some bakery products. In fact, the heme iron is a dark ingredient that can be disguised well in baked goods containing chocolate and in chocolate fillings. Based on the factorial design ($2 \times 2 \times 2$), we compared different combinations of two concentrations of three types of antioxidants: TE (0 and 500 mg/kg), AP (0 and 500 mg/kg), and CA (0 and 300 mg/kg) (Table 1). These antioxidants were chosen because their main mechanisms of action vary – free radical scavenger (tocopherols), oxygen scavenger (AP), and transition metal chelating agent (CA) – and this favors the possibility of synergistic effects among them. Moreover, these compounds are generally recognized as safe (GRAS) and can be used according to the good manufacturing practices [14, 15].

Table 1. Antioxidants used in the different treatments

Treatments	Tocopherol extract (mg/kg)	Ascorbyl palmitate (mg/kg)	Citric acid (mg/kg)
Control	0	0	0
CA	0	0	300
AP	0	500	0
AP + CA	0	500	300
TE	500	0	0
TE + CA	500	0	300
TE + AP	500	500	0
TE + AP + CA	500	500	300

CA, citric acid; AP, ascorbyl palmitate; TE, tocopherol extract.

2.3 Preparation of samples

Palm oil was melted (55°C), mixed, and divided into eight aliquots. In order to favor the solubility and dispersion of the antioxidants (particularly of the AP, which is a fine crystalline powder), each aliquot was heated at 140°C under agitation and then the corresponding antioxidants were added to it. A 10% w/w solution of CA in triacetin was prepared before adding it into the palm oil because of the low solubility of CA in the oil. A 35% w/w of the Guardian Toco 70^{TM} was dissolved in sunflower oil before it was added into the palm oil to favor the dispersion of the tocols in the matrix. After complete dissolution of the antioxidants, the heme iron ingredient was dispersed (15% w/w) in the mixture. The sample was then heated in an oven at 220°C for 10 min to reproduce typical baking conditions. The resulting sample was allowed to cool to 70°C, homogenized, and then distributed into 20 and 10 mL capacity vials. Vials were agitated until the content was solid. This was done to ensure the heme iron ingredient was dispersed evenly inside the vials.

Vials were stored at room temperature in the dark for 0, 10, 20, 40, 80, 120, and 200 days. Afterward, the samples were frozen at −20°C until their analysis.

2.4 Tocol composition

The analysis of the tocol content of the palm oil was adapted from the method described by Hewavitharana *et al.* [16]. Briefly, 5 mL of absolute ethanol containing 1% pyrogallol w/v, 0.012% butylated hydroxytoluene w/v, and 0.4% anhydrous CA w/v were added to 200–250 mg of palm oil. Then, 10 mL of 1.6 N methanolic KOH were added, and saponification was carried out at 70°C for 30 min. Non-saponifiable fractions were then extracted with petroleum ether and filtered through a 0.45 μm teflon membrane. After solvent evaporation under a nitrogen stream at 30°C was achieved, the residue was re-dissolved in 99% *n*-hexane (HPLC grade). For the TE, 133 mg were dissolved in 50 mL of 99% *n*-hexane (HPLC grade) and then 1 mL of this solution was transferred to a 50 mL volumetric flask with 99% *n*-hexane. In both cases, chromatographic separation of tocols was performed using a liquid chromatograph Series 1100 (Hewlett-Packard, Waldbronn, Germany) with a loop volume of 20 μL and a Luna column (4.6 mm i.d. × 150 mm; Phenomenex, USA) packed with 3 μm–100 Å silica. Tocols were isocratically eluted with *n*-hexane: 1,4-dioxane (95:5 v/v) and detected using a Hewlett-Packard 1046A spectrofluorometric detector (excitation and emission wavelengths of 290 and 320 nm, respectively).

2.5 Fatty acid composition

The FAME were prepared from palm oil after a reaction with sodium methoxide followed by boron trifluoride in methanol and were then finally extracted with *n*-hexane [17]. Fatty acid (FA) methyl esters were analyzed on an Agilent (Waldbronn, Germany) 4890D model gas chromatograph, fitted with a flame-ionization detector and split-splitless injector, set at 300 and 270°C, respectively. The split ratio was 1:30. Chromatographic separation of FAME was performed on a fused-silica capillary column (60 m × 0.25 mm i.d.) coated with 0.2 μm of a stationary phase of 90% biscyanopropyl plus 10% cyanopropylphenyl-polysiloxane (SP-2380 from Supelco, USA). Helium, at 30 psi, was used as a carrier gas. The oven was programmed as follows: firstly 2 min at 150°C, then the temperature was increased by 1.5°C/min to 180°C, then by 7.5°C/min to 220°C at which point it was held for 6 min, and then finally the temperature was increased by 5°C/min to 250°C and held for 20 min. The sample volume injected was 1 μL.

2.6 Heme iron

The heme iron content was determined in the heme ingredient and also in samples after being heated at 220°C for 10 min according to the colorimetric method described by Hornsey [18].

2.7 Susceptibility to oxidation

To assess the susceptibility of the samples to oxidation, the induced version of the FOX method [19] was performed with the samples taken after the thermal treatment. This is an induced method that measures the formation of lipid hydroperoxides (LHP) in a sample extract over time [20]. To set up the conditions for this method, preliminary tests were conducted using 500 μL of a dissolution of 1.2 g of the sample in 10 mL of dichloromethane:ethanol (3:2 v/v). This was added to a glass-cuvette containing the FOX reaction media, the composition of which was similar to that proposed by Navas *et al.* [21]: 100 μL of 5 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic xylenol orange, and 1000 μL of dichloromethane:ethanol (3:2 v/v). Cuvettes were capped with Teflon caps, and were maintained in subdued light. The evolution of the LHP formation was monitored until the absorbance at 560 nm was stable (the difference between two consecutive measurements was less than 0.05). Thus the final incubation time of the FOX reaction was 93.75 h. During the incubation of FOX reaction, absorbance was measured at different times according to Tres *et al.* [22] to monitor the susceptibility of the samples to oxidation. The LHP formed were expressed as millimole of cumene hydroperoxide (CHP) equivalents/kg of each sample, with reference to a calibration curve prepared using CHP as standard. To better describe the differences observed, the following parameters were calculated: Initial LHP (the initial LHP value was measured after 30 min of incubation), MAXLHP (the maximum LHP value), TMAX (the time

that the MAXLHP was achieved), Final LHP (the final LHP value measured after 93.75 h of incubation), and AUC (area under the curve) [22].

2.8 Evolution of oxidation status during storage

The oxidation of samples was assessed using the PV [23], the LHP content measured by a non-induced FOX method [21] after 30 min of reaction in the darkness at room temperature, and the *p*-anisidine value (*p*-AnV) [24].

2.9 Statistical analysis

All measurements were replicated twice. The relationship between the PV and the LHP content (non-induced FOX method) was assessed using the Pearson's correlation coefficient. Using the Spearman's correlation coefficients we assessed the relationships between the following parameters: firstly, those calculated in the FOX-induced method applied to thermally treated samples (MAXLHP, TMAX, Final LHP, and AUC), and secondly, the parameters for the areas under the curves representing the evolution of PV and LHP content (non-induced FOX method) in the samples stored for 200 days.

Table 2. Fatty acid composition of the palm oil (expressed as area normalization in percentage, mean ± SD)

Fatty acid	%
10:0	0.05 ± 0.02
12:0	0.31 ± 0.001
14:0	1.06 ± 0.002
15:0	0.05 ± 0.00002
16:0	43.64 ± 0.002
16:1n-9	0.03 ± 0.00006
16:1n-7	0.16 ± 0.0001
17:0	0.11 ± 0.00008
17:1n-7	0.03 ± 0.0003
18:0	4.42 ± 0.0006
18:1t	0.12 ± 0.003
18:1n-9	37.90 ± 0.003
18:1n-7	0.69 ± 0.004
18:2n-6	10.52 ± 0.0007
18:3n-3	0.19 ± 0.0004
20:0	0.39 ± 0.0005
20:1n-9	0.14 ± 0.0006
20:2n-6	0.05 ± 0.004
20:3n-6	0.08 ± 0.00008
24:0	0.08 ± 0.003
Saturated FA	50.10 ± 0.01
Monounsaturated FA	38.94 ± 0.006
Polyunsaturated FA	10.83 ± 0.004
PUFA n-6	10.65 ± 0.004
PUFA n-3	0.19 ± 0.0004
Trans-FA	0.12 ± 0.003

Table 3. Tocol composition of the non-heated palm oil, and of the control sample (palm oil with heme iron added (85% palm oil/15% heme iron ingredient, w/w) after being heated at 220°C for 10 min)

	Tocopherol content (mg/kg)	Tocotrienol content (mg/kg)
Palm oil (non-heated)		
Alpha	124.1 ± 5[a)]	173.3 ± 7
Beta	2.2 ± 0.1	24.4 ± 0.8
Gamma	6.4 ± 0.3	182.1 ± 6
Delta	1.10 ± 0.09	17.3 ± 0.7
Total	133.8 ± 6	397.1 ± 14
Palm oil (non-heated) × 0.85 (dilution factor)		
Alpha	105.5 ± 4	147.3 ± 6
Beta	1.9 ± 0.1	20.7 ± 0.7
Gamma	5.4 ± 0.3	154.8 ± 5
Delta	0.9 ± 0.08	14.7 ± 0.6
Total	113.7 ± 5	337.5 ± 12
Palm oil with heme iron (after heating)		
Alpha	92.8 ± 12	126.6 ± 16
Beta	3.0 ± 0.2	18.4 ± 1.8
Gamma	5.0 ± 0.4	129.6 ± 14
Delta	ND[b)]	16.3 ± 2
Total	100.8 ± 6	290.9 ± 16

[a)] Mean ± SD.
[b)] ND: not detected.

3 Results and discussion

3.1 Palm oil characterization

Table 2 shows the concentration of the different FA, which corresponds with the typical FA composition of palm oil [25]: 50.10% saturated FA, 38.94% MUFA, 10.83% PUFA, and 0.12% *trans* FA.

The tocol content of the palm oil was determined by HPLC (Table 3). The concentration of tocotrienols was high, particularly in alpha- and gamma-tocotrienol, which is characteristic of palm oil [25]. Since palm oil has a total tocol content of 530.9 mg/kg (total tocopherols: 133.8 mg/kg and total tocotrienols: 397.1 mg/kg), all the samples have a certain amount of these antioxidants – including those without added TE.

3.2 Basal content of tocols after the heat treatment

To determine the basal concentration of tocols after the heat treatment, the tocol content of a sample containing 85% palm oil and 15% heme iron ingredient (control) was analyzed. The results are shown in Table 3. The content of the tocol homologs in the heated control sample was lower than in the non-heated palm oil, multiplied by the dilution factor (0.85). It is important to note that, because the control sample contained only 85% palm oil, the tocols were more diluted

than in the palm oil samples. Consequently, some tocol degradation may have occurred in the control sample as a result of the heat treatment. This is in line with the results of a study by Giuffrida *et al.* [26], in which alpha- and delta-tocopherol began decomposing at 165.1 and 179.2°C, respectively. Therefore, it is possible that some of the naturally occurring palm oil tocols may have been decomposed in the samples exposed to temperatures of 220°C.

3.3 Susceptibility to oxidation

The induced FOX method was applied to samples at day 0, just after the heat treatment. By measuring the evolution of the LHP values during the incubation period of these samples, we were able to assess their susceptibility to oxidation thus providing valuable information on their future oxidative stability [20, 22]. The evolution of the LHP values during the incubation is shown in Fig. 1. Because different profiles were recorded upon incubation, the following parameters: Initial LHP, MAXLHP, TMAX, Final LHP, and AUC (Table 4) were calculated as explained in Section 2. These parameters described numerically the samples' susceptibility to oxidation.

The susceptibility to oxidation parameters obtained from Fig. 1 indicated that in those treatments in which AP was added the levels of the AUC and Initial LHP were lower than in other treatments (Table 4). Among these parameters, as reported previously [22], the AUC showed to be a good marker of the susceptibility to oxidation. However, in certain matrices such as the one studied in this paper, alternative parameters as the Initial LHP are able to discriminate very efficiently the differences in samples' susceptibility to oxidation. As Initial LHP is considered to measure the levels of LHP already present in the samples, we can conclude that during the heat treatment the presence of AP provided a certain level of protection against the onset of oxidation.

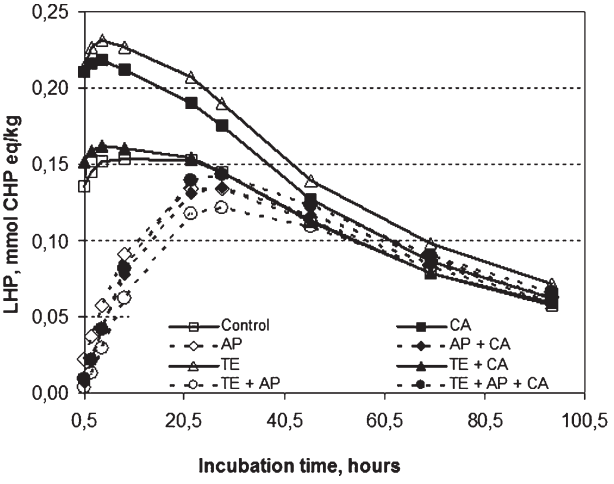

Figure 1. Susceptibility to oxidation (FOX-induced method): Evolution of the lipid hydroperoxide values during the incubation of FOX reactions containing palm oil in which heme iron and different antioxidants ((Control) without antioxidants, (CA) 300 mg/kg citric acid, (AP) 500 mg/kg ascorbyl palmitate, (AP + CA) 500 mg/kg ascorbyl palmitate + 300 mg/kg citric acid, (TE) 500 mg/kg tocopherol extract, (TE + CA) 500 mg/kg tocopherol extract + 300 mg/kg citric acid, (TE + AP) 500 mg/kg tocopherol extract + 500 mg/kg ascorbyl palmitate, (TE + AP + CA) 500 mg/kg tocopherol extract + 500 mg/kg ascorbyl palmitate + 300 mg/kg citric acid) were added.

The AUC and MAXLHP values were higher in samples to which TE or CA were added alone, whereas in samples with added AP (AP, AP + CA, TE + AP, and TE + AP + CA) a lower MAXLHP value was found. It is interesting to note that the time required to reach this peak concentration (TMAX) was different for each sample; treatments containing TE and/or CA (CA, TE, and TE + CA) required only 4 h to reach the TMAX, whereas those containing AP (AP, AP + CA, TE + AP, and TE + AP + CA) required more than 20 h. However, the Final LHP values (at 93.75 h) were relatively similar in all samples. In conclusion, the samples that contained AP had lower Initial LHP values, reached lower MAXLHP values, at a longer incubation time (TMAX), thus presenting a lower AUC. Therefore, the addition of AP reduced the susceptibility to oxidation of the matrix studied.

3.4 Assessment of primary oxidation during storage

To monitor the evolution of primary oxidation in samples during storage, PV and LHP content (non-induced FOX method) were measured.

Figure 2A shows the evolution of PV over storage time. We observed that in the treatments without the addition of AP (Control, CA, TE, and TE + CA) PV was generally higher than the rest. In addition, the PV of these samples started to increase from the beginning, whereas the PV of the treatments with added AP (AP, AP + CA, TE + AP, and TE + AP + CA) were still below 0.4 mEq O_2/kg during the first 40 days. We therefore conclude that the addition of AP delayed the onset of oxidation, whereas in the rest of the treatments oxidation started to occur relatively quickly. The effectiveness of AP in delaying the onset of oxidation has been previously described by Masson *et al.* [27]. They observed that the level of PV in potato chips fried in palm oil containing 500 mg of AP/kg was lower than in chips fried in the control group (without addition of AP).

After 80 days of storage, the treatments containing AP and CA (AP + CA and TE + AP + CA) showed lower PV (0.3 and 0.5 mEq O_2/kg, respectively) than those treatments containing AP without CA (AP and TE + AP that recorded 3.0 and 8.9 mEq O_2/kg, respectively) thus indicating a synergism between both antioxidants.

After 200 days of storage, the addition of AP (an oxygen scavenger) alone and its combination with CA (a chelating agent) inhibited the formation of primary oxidation compounds (as measured by PV) by 38 and 87%, respectively,

Table 4. Susceptibility to oxidation (FOX-induced method): Initial lipid hydroperoxide value (Initial LHP), maximum lipid hydroperoxide value (MAXLHP), time to reach the maximum lipid hydroperoxide value (TMAX), final lipid hydroperoxide value (Final LHP), and the area under the curve (AUC) found in a palm oil matrix to which heme iron and different antioxidants were added

Treatments	Initial LHP (mmol CHP eq/kg)	MAXLHP (mmol CHP eq/kg)	TMAX (h)	Final LHP (mmol CHP eq/kg)	AUC ((mmol CHP eq/kg) × h)
Control	0.14	0.15	8.67	0.06	10.37
CA	0.21	0.22	4	0.06	12.55
AP	0.02	0.13	21.75	0.06	9.13
AP + CA	0.01	0.14	28	0.06	9.07
TE	0.21	0.23	4	0.07	13.76
TE + CA	0.15	0.16	4	0.06	10.52
TE + AP	0.00	0.12	28	0.06	8.26
TE + AP + CA	0.01	0.14	28	0.07	9.51

CA, citric acid; AP, ascorbyl palmitate; TE, tocopherol extract.

compared with the control. The addition of TE did not reduce the PV, except when combined with AP + CA.

Despite some differences found after long storage times, the evolution of the LHP content during storage measured using the non-induced FOX method (Fig. 2B) corroborates the ability of AP to delay the oxidation in certain samples observed by means of the PV (Fig. 2). In samples containing only AP or AP combined with other compounds (AP, AP + CA, TE + AP, and TE + AP + CA) the LHP content was from the beginning lower than in the rest of the samples (Control, CA, TE, and TE + CA). As with the PV, there was a synergism between AP and CA between days 80 and 120. For example, at around day 120, the addition of AP alone was able to inhibit LHP formation by 38%, whereas the combination of AP and CA resulted in a 70% inhibition.

Moreover, looking at the two analytical parameters (PV and LHP content) in relation to the concentration of LHP in the samples, we found that they were significantly correlated (Pearson's $r = 0.833$, $p < 0.01$, $N = 56$). Therefore any of the two methods could be used to monitor changes in LHP in the samples. Despite this, after a period of 200 days the antioxidant effect of AP was more evident when the hydroperoxides were measured using the PV. In addition, it is observed that those samples containing the TE without AP recorded similar LHP than the control in different storage times. The high tocol content present in the palm oil (Table 3) may explain the lack of effect of exogenous addition of tocopherols. Because samples are endogenously protected with radical scavengers, the presence of antioxidants acting by different mechanisms explained the protective effects of AP and CA in comparison to the control. Overall, CA seemed to be less efficient than AP thus indicating that the major prooxidant factor in this system is the presence of oxygen rather than the presence of transition metals.

Samples contained iron due to the addition of the heme ingredient. The iron from this ingredient is already chelated in the porphyrin ring and likely coated by hydrolyzed proteinaceous compounds. The integrity of the heme moiety in

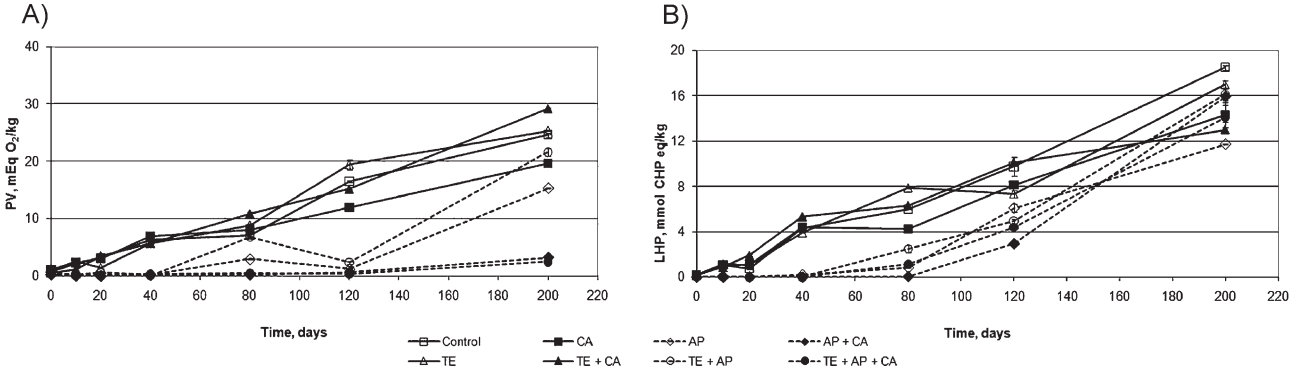

Figure 2. Oxidation during storage time: evolution of the (A) peroxide value and (B) lipid hydroperoxide value (non-induced FOX method) in a palm oil in which heme iron and different antioxidants ((Control) without antioxidants, (CA) 300 mg/kg citric acid, (AP) 500 mg/kg ascorbyl palmitate, (AP + CA) 500 mg/kg ascorbyl palmitate + 300 mg/kg citric acid, (TE) 500 mg/kg tocopherol extract, (TE + CA) 500 mg/kg tocopherol extract + 300 mg/kg citric acid, (TE + AP) 500 mg/kg tocopherol extract + 500 mg/kg ascorbyl palmitate, (TE + AP + CA) 500 mg/kg tocopherol extract + 500 mg/kg ascorbyl palmitate + 300 mg/kg citric acid) were added.

samples after being heated at 220°C for 10 min seemed to be high because the recovery of the heme iron content averaged 103%. This indicates that relatively low amounts of free iron are released to the system after heating and this explains why the chelator CA is not as efficient as the presence of an oxygen scavenger such as AP.

3.5 Correlation between FOX-induced parameters, and PV and LHP content during storage

During storage of samples (0, 20, 40, 80, 120, and 200 days) primary oxidation was measured by the PV [23] and the non-induced FOX method [21]. Apart from this, the FOX-induced method [22] was performed with samples after the heat treatment (day 0), to asses their susceptibility to oxidation (Fig. 1) and compare these results with the primary oxidation developed during real storage (Fig. 2). To achieve this, at each storage time (10, 20, 40, 80, 120, and 200 days), it was calculated the AUC given by both primary oxidation parameters (Fig. 2). Spearman's correlation coefficients were used to assess the relationship between these areas and the MAXLHP, TMAX, Final LHP value, and the AUC obtained by the induced FOX method in the samples after the heating treatment (Table 5). Highly significant correlations were observed between the TMAX and the areas under the curves of the PV and the LHP content (non-induced FOX method) for all storage times. These areas were also significantly correlated with the MAXLHP value and the AUC up until days 40 and 120, respectively. The TMAX, MAXLHP, and AUC calculated in this induced method are consistent with the formation of primary oxidation compounds during the actual storage of samples. In rabbit meat samples from animals fed fresh or heated sunflower oils and with or without tocopheryl acetate supplements, the same parameters TMAX, MAXLHP, and AUC have also been useful to assess the oxidability [22]. Therefore, the FOX-induced method is a useful tool that can be used to evaluate the susceptibility to oxidation of not only these particular palm oil samples (+ heme iron and antioxidants) but also other kinds of samples. Overall, the AUC is highly correlated at all storage times with the AUC calculated from the PV and non-induced LHP values because they measure the cumulative formation of LHP during incubation or storage. However, from our point of view, it is advisable to determine all the parameters reported when studying the susceptibility to oxidation because depending on each type of matrix some can also provide valuable information. However, in this case the Final LHP value calculated in the FOX-induced method is not a useful parameter since it does not correlate with the PV or LHP content (non-induced FOX method) for any of the storage times (Table 5).

3.6 Assessment of secondary oxidation during storage

Secondary oxidation products were monitored during storage by measuring the *p*-AnV. The results of this analysis revealed low values during all the storage times monitored (Fig. 3). The samples containing AP (AP, AP + CA, TE + AP, and TE + AP + CA) have a lower *p*-AnV than the other samples thus indicating the effectiveness of AP as an antioxidant, as it was encountered by primary oxidation measurements. Peroxides decompose over time and lead to the formation of secondary oxidation products. However, the synergistic effect of AP and CA recorded in this study

Table 5. Spearman correlation coefficients between parameters from the FOX-induced method used to assess the susceptibility of samples to oxidation and the area calculated under the curves given by peroxide and LHP values (non-induced FOX method) used to assess primary oxidation during the storage of samples

		Peroxide value						LHP value (non-induced FOX method)					
Days of storage		10	20	40	80	120	200	10	20	40	80	120	200
Parameters from induced FOX method													
MAXLHP[a)]	*r*	0.833	0.762	0.714	0.619	0.667	0.690	0.905	0.857	0.786	0.667	0.667	0.619
	(*p*)	(0.010)	(0.028)	(0.047)	(0.102)	(0.071)	(0.058)	(0.002)	(0.007)	(0.021)	(0.071)	(0.071)	(0.102)
	n	8	8	8	8	8	8	8	8	8	8	8	8
TMAX	*r*	−0.901	−0.851	−0.851	−0.851	−0.851	−0.851	−0.751	−0.901	−0.951	−0.851	−0.851	−0.751
	(*p*)	(0.002)	(0.007)	(0.007)	(0.007)	(0.007)	(0.007)	(0.032)	(0.002)	(0.000)	(0.007)	(0.007)	(0.032)
	n	8	8	8	8	8	8	8	8	8	8	8	8
Final LHP	*r*	−0.048	−0.214	−0.357	−0.548	−0.357	−0.262	−0.024	−0.095	−0.214	−0.357	−0.357	−0.405
	(*p*)	(0.911)	(0.610)	(0.385)	(0.160)	(0.385)	(0.531)	(0.955)	(0.823)	(0.610)	(0.385)	(0.385)	(0.320)
	n	8	8	8	8	8	8	8	8	8	8	8	8
AUC	*r*	0.881	0.905	0.857	0.714	0.714	0.667	0.905	0.833	0.786	0.667	0.714	0.714
	(*p*)	(0.004)	(0.002)	(0.007)	(0.047)	(0.047)	(0.071)	(0.002)	(0.010)	(0.021)	(0.071)	(0.047)	(0.047)
	n	8	8	8	8	8	8	8	8	8	8	8	8

[a)] See Table 4 for abbreviations.

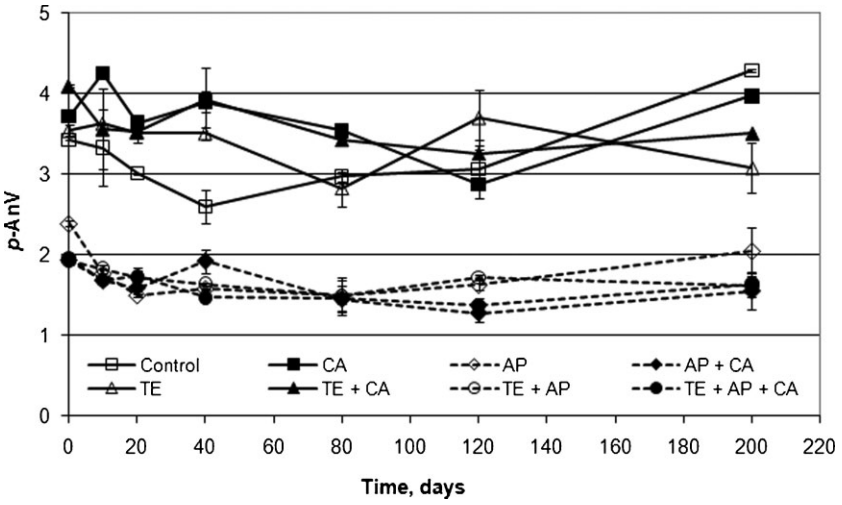

Figure 3. Oxidation during storage time (*p*-anisidine value): Evolution of the p-AnV over storage time in a palm oil in which heme iron and different antioxidants ((Control) without antioxidants, (CA) 300 mg/kg citric acid, (AP) 500 mg/kg ascorbyl palmitate, (AP + CA) 500 mg/kg ascorbyl palmitate + 300 mg/kg citric acid, (TE) 500 mg/kg tocopherol extract, (TE + CA) 500 mg/kg tocopherol extract + 300 mg/kg citric acid, (TE + AP) 500 mg/kg tocopherol extract + 500 mg/kg ascorbyl palmitate, (TE + AP + CA) 500 mg/kg tocopherol extract + 500 mg/kg ascorbyl palmitate + 300 mg/kg citric acid) were added.

when primary oxidation products were monitored was not observed when *p*-AnV was studied, even though samples were monitored for a long period of time. In addition, no increase in the *p*-AnV was observed during 200 days of storage.

Similarly, in an experiment performed with olive oil (which has a high content of oleic FA and a low concentration of PUFA), Lee *et al.* [28] observed very little variation in the *p*-AnV when oxidizing the oil at different temperatures. For example, after 180 days at 25°C the *p*-AnV varied from 5.8 to 3.7; whereas after 18 days at 60°C the change in the *p*-AnV was from 5.8 to 6.2.

The formation of secondary oxidation products such as aldehydes depends on the FA composition of the fat. In addition, different aldehydes react differently with *p*-anisidine. For example, the reaction of 2,4-dienals with *p*-anisidine had the highest molar absorbance, followed by 2-alkenals. Alkenals have the lowest molar absorbance [29]. Therefore, the *p*-AnV may not be useful for monitoring oxidation in the present type of sample matrix. It may be necessary to combine it with another secondary oxidation parameter.

Tompkins and Perkins [30] determined the *p*-AnV and headspace volatiles of partially hydrogenated soybean oil used for frying. This oil had a similar oleic content (40.49%) to the palm oil used in the present study (Table 2) but a much higher linoleic acid content (37.9%). The authors found a highly significant correlation between the *p*-AnV and the following volatile aldehydes: 2-alkenals, *tt*-2,4-decadienal, hexanal, and heptanal. Most of these aldehydes would be primarily formed by the breakdown of linoleic acid [31]. However, nonanal, which was the only selected aldehyde that originates primarily from the breakdown of oleic acid [31], did not significantly correlate with the *p*-AnV [30]. As palm oil has a very low concentration of linoleic acid and a high proportion of oleic acid, it is possible that the analysis of nonanal was more effective as a secondary oxidation marker than other volatile aldehydes and *p*-AnV.

4 Conclusions

The use of AP – alone or in combination with other antioxidants – delayed the onset of oxidation measured by the PV and LHP content (non-induced FOX method) in a palm oil matrix, which is a simple model for some bakery products, such as baked goods containing chocolate. In addition, there was a synergistic effect between AP and CA after 80 days of storage at room temperature. The TE was not useful to delay the oxidation in the iron fortified palm oil used in this study. This could be related to the high tocopherol and tocotrienol content of the palm oil used. Therefore, the combined use of AP (oxygen scavenger) and CA (chelating agent) is highly recommended to extend the shelf life of heme iron-enriched food products containing palm oil.

The application of the induced FOX method allowed us to predict the ability of AP to delay lipid oxidation. The advantage of using this method is that the results were obtained in 4 days, instead of the longer period needed to monitor oxidation during storage. However, the synergistic effect between AP and CA mentioned above could not be observed with this induced method.

The *p*-AnV was lower for the samples containing AP. This value did not change much during 200 days of storage at room temperature, showing that this oxidation parameter was not suitable for monitoring the oxidation of palm oil during storage. Alternatively, the measurement of headspace volatiles such as nonanal, might be better for monitoring secondary oxidation of fats rich in oleic acid.

The authors have declared no conflict of interest.

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5.2. The effect of citric acid and ascorbyl palmitate in palm oil enriched with heme iron: A model for iron fortification in bakery products

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TITLE AND ABSTRACT IN SPANISH/ TÍTULO Y RESUMEN EN CASTELLANO:

"Efecto del ácido cítrico y el palmitato de ascorbilo en manteca de palma enriquecida con hierro hémico: un modelo para la fortificación con hierro de productos de bollería, pastelería y galletería"

La fortificación de alimentos con hierro es una estrategia común para la prevención de la deficiencia de hierro. Esto es importante debido a que una gran parte de la población, principalmente niños y mujeres (en edad reproductiva), están en riesgo de desarrollar anemia por deficiencia de hierro.

Varios productos de pastelería, tales como galletas, pastas y bollos, son consumidos regularmente por los niños, haciendo su fortificación una estrategia efectiva para prevenir esta deficiencia. A pesar de que el hierro hémico es altamente bio-disponible, promueve la oxidación al igual que otras formas de hierro.

En el presente estudio, con el objetivo de prevenir la oxidación de productos horneados, se emplea un modelo de manteca de palma enriquecida con hierro hémico para la optimización de las dosis de palmitato de ascorbilo (0, 100, 200 ó 400 mg/kg) y ácido cítrico (0, 250 ó 500 mg/kg). Ambos antioxidantes fueron combinados empleando un diseño factorial.

Las muestras enriquecidas con hierro fueron calentadas a 220ºC durante 10 minutos para simular las típicas condiciones de horneado. A lo largo de un año de almacenamiento a temperatura ambiente y en la oscuridad fueron medidas tanto la oxidación primaria (índice de peróxidos y contenido en hidroperóxidos lipídicos) como la secundaria (índice de *p*-anisidina y contenido en hexanal).

Los resultados de la oxidación, tanto primaria como secundaria, indicaron que a lo largo del almacenamiento, el palmitato de ascorbilo añadido retrasó la oxidación. Opuestamente, el ácido cítrico añadido actúo como pro-oxidante, por lo que no se observó un sinergia entre ambos antioxidantes.

Research Article

The effect of citric acid and ascorbyl palmitate in palm oil enriched with heme iron: A model for iron fortification in bakery products

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Iron fortification in food products is a common strategy used to prevent iron deficiency. This is important because a large proportion of the population, mainly young children and women (of childbearing age) are at risk of developing iron deficiency anemia. Certain baked goods (biscuits, cookies) are regularly consumed by children making their iron fortification an effective strategy to prevent this deficiency. Although heme iron has high bioavailability it promotes oxidation, as other iron forms. In the present study, a model consisting of a refined palm oil, enriched with heme iron, was used to optimize the dose of ascorbyl palmitate (0, 100, 200, or 400 mg/kg) and citric acid (0, 250, or 500 mg/kg) to prevent oxidation in baked products. Both antioxidants were combined using a factorial design. Iron enriched samples were heated at 220°C for 10 min to mimic typical baking conditions. Primary (peroxide value and lipid hydroperoxide content) and secondary (*p*-anisidine value and hexanal content) oxidation were measured over 1 year of storage at room temperature in the darkness. Results of primary and secondary oxidation indicated that ascorbyl palmitate delays oxidation during storage. Conversely, citric acid acted as prooxidant and no synergistic effect between the two antioxidants was observed.

Practical applications: The fortification of food with iron represents a potentially effective strategy to overcome iron deficiency. A heme iron ingredient was used in this study because it has higher bioavailability than that of inorganic iron. As heme iron is a dark ingredient it could be disguised in baked products that contain chocolate. The heme iron ingredient was mixed with palm oil, which is a fat widely used in bakery. Nevertheless, as with inorganic iron, heme iron induces oxidation, and affects nutritional value, organoleptic properties and the shelf-life of fortified foods. This study demonstrates that ascorbyl palmitate could be useful to prevent such undesired changes in iron fortified baked products.

Keywords: Antioxidants / Food fortification / Heme iron / Oxidative stability / Palm oil

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1 Introduction

Iron deficiency is one of the most common health issues in the world, mainly affecting infants, children and women [1]. Three major intervention strategies are currently used to tackle this problem, namely iron supplements, diet diversification, and food fortification. Every strategy has its pros and cons; though food fortification is regarded as the most cost effective long-term strategy to reduce the prevalence of iron deficiency [2, 3]. The success of a fortification program depends on various factors, which include the selection of the food delivery system, the requirements and eating habits of the consumer, the price, any technological barriers, and changes in sensory properties [4].

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Abbreviations: AP, ascorbyl palmitate; **AUC,** area under the curve; **CA,** citric acid; **CHP eq.,** cumene hydroperoxide equivalents; **FOX,** ferrous oxidation-xylenol orange; **LHP,** lipid hydroperoxide; **MAXLHP,** maximum lipid hydroperoxide; ***p*-AnV,** *p*-Anisidine value; **PV,** peroxide value; **SFA,** saturated fatty acids; **TMAX,** time to reach the maximum lipid hydroperoxide value

The incidence of iron deficiencies is particularly high in children, making them an important target population for iron fortification [1]. For the program to be successful, an effective food vehicle – one which is consumed by a large proportion of the target audience – must be selected [2, 5]. Indeed, national programs in Chile and Brazil have already demonstrated that the use of wheat cookies fortified with heme iron have improved the iron status of school-age children [6, 7].

Both heme and inorganic iron forms have been used in fortification programs [8, 9]. Although heme iron ingredients have not been yet authorized for iron fortification in the European Union, in other countries such as Japan and Mexico its addition to food products has been already authorized [10, 11]. Heme iron has a higher bioavailability than inorganic iron, mainly because their absorption is different [12] and that the inhibition of heme iron absorption by food iron ligands is low [13]. Heme iron has been used in various trials to fortify different foods despite its inherent dark color, which limits its use and product acceptability [7, 8]. Since chocolate is very popular among children, this allows the delivery of the intense colored heme iron being well disguised in any chocolate topping or filling in baked goods. The main drawback of free iron and heme iron is that they are both potent prooxidants [14–16]. In the case of free iron, this has been addressed by studying the efficiency of different strategies, such as the addition of antioxidants and chelating agents, and the use of different encapsulation techniques [17–19]. In contrast, the issue is not addressed to the same extent in the case of heme iron, even though several authors have reported that heme iron had detrimental effects on the sensory properties of fortified foods [8, 20].

In order to minimize this problem, Aleman et al. [21] used a model for iron fortification in baked products, which consisted of adding 15% w/w of a heme iron ingredient (with 1.07% of total Fe, of which the 96% was heme iron) and various antioxidants to a refined palm oil matrix. This was heated at 220°C for 10 min to mimic typical conditions during the baking process. In brief, the authors [21] used a factorial design to study how the addition of ascorbyl palmitate (AP) (0 or 500 mg/kg), citric acid (CA) (0 or 300 mg/kg) and tocopherol extract (0 or 500 mg/kg) effected the oxidation of this heme iron enriched palm oil matrix. The impacts were measured over a storage period of 200 days at room temperature in the dark. By using this model, the authors reported that the tocopherol extract and CA alone were ineffective, whereas 500 mg/kg of AP was very effective in preventing oxidation of the samples during the storage period. Additionally, a synergistic effect was observed when AP and CA were used in combination.

Given the results of the previous study, the above-mentioned model for iron fortification in baked goods was used for the present project to attempt to optimize the dose of AP and CA with the aim of preventing lipid oxidation.

2 Materials and methods

2.1 Materials

Refined palm oil was donated by Lípidos Santiga S.A. (Santa Perpètua de Mogoda, Spain). AP (99.5%), FAME standards (>98%), cumene hydroperoxide (80%) and hexanal (98%) were purchased from Sigma–Aldrich (Madrid, Spain). CA (99.5%), dimethyl sulfoxide (DMSO) and triacetin (99%) were purchased from Scharlab S.L. (Barcelona, Spain). The tocopherol standard was purchased from Calbiochem (San Diego, CA). The heme iron ingredient, AproFer 1000^{TM} was donated by APC Europe S.A. (Granollers, Spain). As described by González-Rosendo et al. [22], the heme iron ingredient is a dark powder obtained from the blood of healthy pigs. After separating the blood cell fraction by centrifugation, a pressure pump was used to release the hemoglobin contained in the red blood cells. The hemoglobin was then enzymatically hydrolyzed using a proteolytic enzyme (Alcalase®, Novo-Nordisk, Denmark) in controlled pH and temperature conditions. Following this, the heme group was concentrated by ultrafiltration and spry dried. This heme iron ingredient is a proteinaceous material. The batch used had a total iron content of 1.35%, of which 96% was heme iron.

All chemicals used were of ACS grade with the exception of the solvents used in the ferrous oxidation-xylenol orange (FOX) method, in the tocopherol and tocotrienol determination and in the heme iron determination, which were of HPLC grade. The reagents in the determination of the total iron content were Baker Instra-analyzed. The iron standard solution was purchased from High-Purity Standards (Charleston, SC) and was traceable to the Standard Reference Materials of NIST.

2.2 Ingredient selection and study design

As model for bakery goods, the samples were prepared by dispersing the heme iron ingredient (8.7% w/w) into palm oil. Tentatively and by using this heme iron dispersion in palm oil, a portion of two chocolate filled biscuits, each weighing 25 g and having 7.5 g of chocolate filling with a 25% of palm oil will provide 31% of the RDA of iron (i.e., 14 mg Fe/day) [23].

Refined palm oil was chosen because it is commonly used in the manufacturing of biscuits, cookies, pastries and chocolate fillings amongst others. Four different concentrations of AP (0, 100, 200, and 400 mg/kg) and three different concentrations of CA (0, 250, and 500 mg/kg) were added to the heme iron dispersion in palm oil using a factorial design (Table 1).

2.3 Preparation of samples

The preparation of the samples was carried out as described elsewhere [21] with some modifications. Palm oil was melted

Table 1. Factorial design: antioxidant concentrations added to the heme iron dispersion in palm oil

Treatments	Ascorbyl palmitate (mg/kg)	Citric acid (mg/kg)
Control	0	0
CA (250)	0	250
CA (500)	0	500
AP (100)	100	0
AP (100) + CA (250)	100	250
AP (100) + CA (500)	100	500
AP (200)	200	0
AP (200) + CA (250)	200	250
AP (200) + CA (250)	200	500
AP (400)	400	0
AP (400) + CA (250)	400	250
AP (400) + CA (500)	400	500

CA, citric acid; AP, ascorbyl palmitate.

(55°C), mixed and divided into 12 aliquots. Due to the low solubility of CA in oils, a 10% w/w solution of CA in triacetin was prepared before adding it to the palm oil. Thus, the same amount of triacetin was added to the samples that did not contain CA. To increase the dispersion and solubility of the antioxidants (especially AP, which is a fine crystalline powder), each aliquot was heated to 140°C before adding the antioxidants under agitation. After complete dissolution of the antioxidants in the heated palm oil, the heme iron ingredient was dispersed (8.7% w/w) in the mixture. The sample was then heated again in an oven at 220°C for 10 min to reproduce typical baking conditions. Subsequently, the resulting samples were allowed to cool to 70–80°C and were then homogenized for 10 s at 15 000 rpm using a Polytron PT 3100 and immediately distributed into 10 mL capacity vials.

To ensure the even dispersion of the heme iron in the vial, they were allowed to cool before being melted again at 55.5°C. Before the palm oil could solidify again, the vials were shaken until they were completely homogenized and, immediately after, were then immersed in a bath of ice in order to solidify the palm oil dispersion as quickly as possible.

Vials were stored at room temperature (19–23°C) in the dark for 0, 15, 30, 60, 120, 180, 240, 300, and 360 days. Afterwards, the samples of each storage period were stored at −20°C until their analysis. As the factorial design was replicated twice the number of different samples was 216. To do some determinations (susceptibility to oxidation, primary oxidation parameters, *p*-anisidine value, and tocopherol analogues content), sample vials were heated at 55°C for 15 min and then filtered through Whatman no. 54 filter paper at room temperature to remove the heme iron particles. The rest of the determinations (heme and total iron, and hexanal) were carried without removing the iron particles from the samples.

2.4 Palm oil characterization

2.4.1 Fatty acid composition

The FAME were prepared from 20 mg of palm oil as described elsewhere [24] and were analyzed on an Agilent 4890D model (Waldbronn, Germany) gas chromatograph equipped with a FID and a split-splitless injector. Chromatographic separation of FAME was performed on a fused-silica capillary column (60 m × 0.25 mm i.d.) coated with 0.2 μm of a stationary phase of 90% biscyanopropyl- plus 10% cyanopropylphenyl-polysiloxane (SP-2380 from Supelco, USA). Helium, at 30 psi, was used as a carrier gas. The injector and the detector temperatures were 270°C and 300°C, respectively, with a split ratio of 1:30.

The oven program was as follows: the initial temperature of the oven was 150°C and was held for 2 min, the temperature was then increased by 1.5°C/min to 180°C and then by 7.5°C/min to 220°C. It was held at this temperature for 6 min, then heated at 6.5°C/min to 250°C at which point it was kept for 20 min. The sample volume injected was 1 μL. The palm oil sample was analyzed four times within the same day.

2.4.2 Tocopherol and tocotrienol composition

The tocopherol and tocotrienol content was determined in 200 mg of palm oil samples (the palm oil used to prepare the different treatments and the filtered control sample) by normal-phase HPLC as described elsewhere [21]. Each sample was analyzed six times on different days. Briefly, 5 mL of absolute ethanol containing 1% pyrogallol w/v, 0.012% butylated hydroxytoluene w/v and 0.4% anhydrous CA w/v were added to the palm oil samples. Ten milliliters of 1.6 N methanolic KOH were then added and saponification was carried out at 70°C for 30 min. The unsaponifiable fraction was then extracted with petroleum ether and the solution was filtered through a 0.45 μm Teflon membrane. After solvent evaporation under a nitrogen stream at 30°C was achieved, the residue was re-dissolved in *n*-hexane and 100 μL injected to the chromatograph. Separation of tocopherols and tocotrienols was performed using a liquid chromatograph Series 1100 (Hewlett-Packard, Waldbronn, Germany) with a loop volume of 20 μL and a Luna column (4.6 mm i.d. × 150 mm; Phenomenex, USA) packed with 3 μm–100 Å silica. Tocopherols and tocotrienols were isocratically eluted with *n*-hexane: 1,4-dioxane (95:5, v/v) and detected using a Hewlett-Packard 1046A spectrofluorometric detector (excitation and emission wavelengths were 290 and 320 nm, respectively).

2.5 Heme and total iron content

The heme iron content was determined according to the colorimetric method described by Hornsey [25] in the heme

iron ingredient and also in the samples from each treatment after being heated at 220°C for 10 min. The total iron content of the heme ingredient and samples from each treatment was determined by a PerkinElmer ICP-AES (Optima 3200 RL model) after the calcination of the samples. All samples were randomly analyzed six times on different days.

The calcination process of samples from each treatment was carried out as follows: 1 g sample was weighed in a glass beaker and 2 mL of H_2SO_4 were added. The sample was then placed in a sand bath at 250°C. Once the sample was steaming, an additional 2 mL of H_2SO_4 were added. This procedure was repeated until the amount of H_2SO_4 added reached 8 mL. The sample was then allowed to dry in the sand bath before being calcinated in a muffle furnace at 450°C for 10 h (rate of heating 1°C/min). After the heating treatment, 1 mL of H_2SO_4, 2 mL of HNO_3, and 1 mL of H_2O_2 were added (H_2O_2 was added in fractions of 0.2 mL). The beaker was then allowed to boil at 250°C in the sand bath for 30 min. Following this, the sample was dried and calcinated again overnight in the muffle furnace at 450°C. Finally, the sample was re-dissolved in 2 mL of HCl and diluted to a volume of 100 mL with 1% HNO_3.

The calcination of the heme iron ingredient was as follows: 250 mg of the ingredient was weighed in a glass beaker. Following this, 2 mL of H_2SO_4 were added to the sample. The sample was then placed in a sand bath at 250°C until dry. As for the samples from each treatment, the ingredient was then calcinated in a muffle furnace at 450°C for 10 h (rate of heating 1°C/min). After the heating treatment, 1 mL of H_2SO_4 was added and allowed to dry in a sand bath at 300°C. Finally, a second heating treatment was carried out in the muffle furnace at 450°C for a period of 3–6 h. The ingredient sample was re-dissolved in 2 mL of HCl and diluted to a volume of 100 mL with 1% HNO_3.

2.6 Susceptibility to oxidation

The induced version of the FOX method was performed to assess the susceptibility of the samples to oxidation after the thermal treatment [26, 27]. In this assay, the formation of lipid hydroperoxides (LHP) in each sample was measured over an incubation period. To develop this assay, different aliquot sizes were used – either 200 or 500 μL, depending on the samples' susceptibility to oxidation – of 1.2 g of the sample dissolved in 10 mL of dichloromethane:ethanol (3:2, v/v). Briefly, the aliquots were mixed in 1 cm Teflon-capped glass-cuvettes with the FOX reaction media (consisting of 100 μL of 5 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic xylenol orange) and the required quantity of dichloromethane:ethanol (3:2, v/v) to reach the final cuvette volume of 2000 μL [28]. The incubation of the mixture in the cuvettes was carried out at room temperature in attenuated light until absorbance at 560 nm was stable (the difference between two consecutive measurements for all samples was <0.02). The incubation time was set at 54 h and absorbance was measured at different times according to Tres et al. [29]. By using a standard curve prepared with cumene hydroperoxide (CHP), the content of the LHP formed during the incubation was expressed as millimoles of CHP equivalents/kg of each sample. The following parameters were determined as described elsewhere [29] to better describe the differences observed in the susceptibility towards oxidation of the samples: Initial LHP (the initial LHP value was measured after 30 min of incubation), MAXLHP (the maximum LHP value), TMAX (the time until the MAXLHP was achieved), Final LHP (the final LHP value measured after 54 h of incubation) and AUC (area under the curve of LHP formation). All the samples were analyzed at the same time in duplicate.

2.7 Determination of the oxidation during the storage

The formation of primary oxidation compounds in samples stored for different periods was assessed using the peroxide value (PV) [30] and by measuring the LHP content through the non-induced FOX method after 30 min of reaction in the darkness at room temperature [28]. To determine the LHP content, samples of between 0.1 and 1.2 g, depending on the oxidation degree of the sample, were weighed in a 10 mL volumetric flask, which was then filled up with dichloromethane/ethanol (3:2, v/v). Between 100 and 500 μL of the solution was used to determine the LHP content as previously described [28]. The formation of secondary oxidation compounds in the samples was assessed using the *p*-anisidine value (*p*-AnV) [31]. The hexanal content was determined by static headspace. As previously mentioned, PV, LHP content and *p*-AnV were determined in filtered samples.

To identify the best extraction conditions for volatile compounds, different parameters were tested, following a similar approach as described by Romeu-Nadal et al. [32]. The extraction conditions assessed were: sample weight (0.5, 1.0, or 1.5 g), extraction time (15, 30, and 45 min) and temperature (40, 55, and 70°C). For this propose, a Combi PAL autosampler (CTC Analytics, Switzerland) was employed. The final extraction conditions selected were as follows: 500 mg of sample (without filtering) was weighed in a vial and sealed by a screw cap. The extraction of the volatile compounds was carried out over a period of 30 min at 70°C. Following this, 1 mL of the headspace of the vial was injected into an Agilent 4890D model (Waldbronn, Germany) gas chromatograph equipped with FID and a split-splitless injector. The injector was set for 2 min in splitless mode; the split ratio was 1:20.

Chromatographic separation of volatile compounds was performed on a fused-silica capillary column (30 m × 0.20 mm i.d.) coated with 0.2 μm of a stationary phase of 5% diphenyl- plus 95% dimethyl-polysiloxane (Equity TM-5 from Supelco, USA). Helium, at 20 psi, was used as a carrier

gas. The injector and the detector temperatures were 200 and 325°C, respectively. The oven program was as follows: 1 min at a temperature of 40°C, which was then increased by 10°C/min to 300°C and then kept at this temperature for 5 min.

Hexanal was identified by comparing the retention time with a standard and was quantified using the external standard method. To prepare the standard curve, fresh palm oil (without hexanal) was mixed with the heme iron ingredient (8.7%). 500 mg of the mixture was then weighed in vials. 100 μL of solutions with different concentrations of hexanal in DMSO were added to the vials (concentration range, 22–380 mg hexanal/kg sample) and sealed by screw caps. To ensure that the vials of the samples and the vials of the standard curve had the same matrix, 100 μL of DMSO were added to all treatment samples.

Under these conditions, the method displayed a good linearity ($R^2 = 0.993$), a good precision (RSD = 1.95%, four determination within the same day in a sample containing 285 mg hexanal/kg) and a good recovery (98%). The recovery was assessed in two samples, one with 285 mg hexanal/kg and other with 125 mg/kg, four times with and addition of hexanal standard of one-third of the initial concentration (95 mg/kg and 42 mg/kg, respectively). Taking into account the analyte content of samples (between 50 and 600 mg/kg), these values fulfill the values recommended by the AOAC (1998). Besides, the limit of detection and quantification of the method were 6 and 19 mg/kg, respectively. Both limits were calculated as three and ten times the SD of the base line noise, respectively [33].

As mentioned above, the experimental design was replicated twice (216 samples). In both replicates, PV, LHP content and *p*-AnV were analyzed in duplicate within the same day. The hexanal content was also analyzed in duplicate within the same day but only in one of these replicates (except at 180 days of storage, due to the lack of sample; 96 samples).

2.8 Statistical analysis

Pearson's correlation coefficients were used to study the relationships between PV and LHP content ($n = 216$) and between *p*-AnV and hexanal ($n = 96$).

Multifactorial ANOVA was used to determine whether the studied factors had any significant effect on PV, LHP content, *p*-AnV and hexanal content. The main factors studied were AP concentration (0, 100, 200, and 400 mg/kg), CA concentration (0, 250, and 500 mg/kg) and the storage time (0, 15, 30, 60, 120, 180, 240, 300, and 360 days, without time 180 days for hexanal content). Interactions between more than two factors were not considered. In all cases, $p \leq 0.05$ was considered significant. When significant differences were produced by the main factors, the least-squares means were separated using Duncan's test ($\alpha = 0.05$).

3 Results and discussion

3.1 Palm oil characterization

The concentration of the different fatty acids (FA) is shown in Table 2. The composition found is in agreement with the typical FA composition of palm oil [34]: 50.52 ± 0.26% SFA, 39.87 ± 0.20% MUFA, 9.62 ± 0.05% PUFA, and 0.09 ± 0.01% *trans* FA.

The high concentration of tocotrienols present in palm oil (Table 3), particularly in alpha- and gamma-tocotrienol, is characteristic of this oil [34, 35]. The total content of tocopherols and tocotrienols was 466 mg/kg (total tocopherols: 146 mg/kg and total tocotrienols: 320 mg/kg), meaning that all samples had a certain amount of these antioxidants present.

The content of the tocopherol and tocotrienol analogues in the heated control sample was 20.2% lower than in the same non-heated palm oil (Table 3). Consequently, degradation of tocopherol (19.6%), and tocotrienol (21.1%) occurred as a result of submitting the samples to the heat treatment (220°C for 10 min) in the presence of iron. This is

Table 2. Fatty acid composition of the palm oil used to prepare the different treatments (expressed as area normalization in percentage)

Fatty acid[a)]	Mean ± SD (%)
10:0	0.03 ± 0.01
12:0	0.28 ± <0.01
14:0	1.12 ± 0.01
15:0	0.05 ± <0.01
16:0	44.11 ± 0.30
16:1n−9	0.03 ± <0.01
16:1n−7	0.17 ± <0.01
17:0	0.10 ± <0.01
17:1	0.03 ± <0.01
18:0	4.36 ± 0.03
18:1t	0.09 ± 0.01
18:1n−9	38.74 ± 0.20
18:1n−7	0.66 ± 0.02
18:2n−6	9.37 ± 0.05
18:3n−3	0.14 ± <0.01
20:0	0.38 ± <0.01
20:1n−9	0.15 ± <0.01
20:2n−6	0.03 ± <0.01
20:3n−6	0.07 ± <0.01
24:0	0.08 ± <0.01
SFA	50.52 ± 0.30
MUFA	39.87 ± 0.20
PUFA	9.62 ± 0.05
PUFA n−6	9.47 ± 0.05
PUFA n−3	0.14 ± <0.01
Trans FA	0.09 ± 0.01

[a)]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; *Trans* FA, *trans* fatty acids.

Table 3. Tocopherol and tocotrienol composition of the palm oil used to prepare the different treatments and that of the palm oil obtained after filtering the control treatment to remove the heme iron (heated at 220°C for 10 min)

	Tocopherol content (mg/kg)	Tocotrienol content (mg/kg)
Palm oil (non-heated)		
Alpha	141.0 ± 5.6[a)]	155.2 ± 5.5
Beta	2.4 ± 0.07	9.8 ± 0.8
Gamma	3.0 ± 0.3	148.5 ± 7.3
Delta	ND[b)]	9.2 ± 0.3
Total	146.4 ± 5.6	322.7 ± 11.5
Filtered palm oil (after heating)		
Alpha	113.4 ± 11.5	122.4 ± 14.9
Beta	2.4 ± 0.7	7.9 ± 0.8
Gamma	2.5 ± 0.3	113.3 ± 11.6
Delta	ND	9.3 ± 0.3
Total	118.3 ± 12.6	252.9 ± 14.7

[a)]Mean ± SD.
[b)]ND, not detected.

in agreement with the results obtained in a previous study where the degradation of tocopherols and tocotrienols during heating at 220°C for 10 min was a 13.2% [21].

3.2 Heme iron and total iron content

The heme iron and total iron content was measured in all samples at time zero. The average final content of the samples was 0.94 mg heme iron/g of sample and 0.95 mg total iron/g of sample. These results indicate that heme iron was the main form present in the samples (98%) and that the heme moiety was well preserved after heating samples for 10 min at 220°C. This is in accordance with our previously reported results [21].

3.3 Susceptibility to oxidation

Using the induced FOX method, sample's susceptibility to oxidation was measured at time zero. This method measured the LHP development over an incubation period [28]. As the curves of LHP formation in the samples during incubation followed different patterns, various parameters were determined as described elsewhere [29] to more accurately describe the samples' susceptibility to oxidation (Table 4).

The initial LHP value indicates the amount of LHP already present in the samples [29]. Based on the initial LHP values (Table 4), it appears that during heat treatment, the presence of AP at higher concentrations (200 or 400 mg/kg) alone or in combination with CA, provided a certain level of protection against LHP formation. However, AP at 100 mg/kg, alone or in combination with CA, seemed to have no effect in preventing LHP formation during the heating of samples, since the value found for the control sample was either lower or similar. From these results it seems that AP is the only antioxidant effective at 200 and 400 mg/kg. Finally, the treatments containing only CA recorded higher initial LHP values than those found in the control indicating that CA acts as a prooxidant during heat treatment.

Table 4. Susceptibility to oxidation measured using the FOX-induced method

Treatments	Initial LHP (mmol CHP eq/kg)	MAXLHP (mmol CHP eq/kg)	TMAX (h)	Final LHP (mmol CHP eq/kg)	AUC ((mmol CHP eq/kg) × h)
Control	0.17 ± 0.01[a)]	0.94 ± 0.02	2.00	0.40 ± 0.01	31.73 ± <0.01
CA (250)	0.39 ± 0.01	1.52 ± 0.02	2.00	0.63 ± 0.02	49.21 ± 0.01
CA (500)	0.39 ± <0.01	1.62 ± <0.01	2.00	0.63 ± <0.01	50.57 ± <0.01
AP (100)	0.25 ± <0.01	0.47 ± <0.01	2.00	0.30 ± <0.01	19.59 ± 0.01
AP (100) + CA (250)	0.17 ± 0.01	0.23 ± 0.01	4.15	0.14 ± <0.01	10.18 ± 0.01
AP (100) + CA (500)	0.21 ± <0.01	0.85 ± <0.01	2.00	0.40 ± <0.01	30.06 ± <0.01
AP (200)	0.01 ± 0.01	0.08 ± 0.02	26.15	0.08 ± 0.01	3.72 ± 0.01
AP (200) + CA (250)	0.02 ± 0.01	0.09 ± <0.01	18.45	0.09 ± 0.01	4.20 ± <0.01
AP (200) + CA (500)	0.01 ± <0.01	0.08 ± <0.01	26.15	0.08 ± <0.01	3.51 ± <0.01
AP (400)	0.01 ± 0.01	0.11 ± 0.05	18.45	0.10 ± 0.01	4.98 ± <0.01
AP (400) + CA (250)	ND[b)]	0.08 ± <0.01	26.15	0.08 ± 0.01	3.84 ± <0.01
AP (400) + CA (500)	0.01 ± 0.01	0.09 ± 0.01	26.15	0.09 ± <0.01	4.23 ± <0.01

See Table 1 for treatment abbreviations.
The parameters determined from the curve of formation of lipid hydroperoxides were: initial lipid hydroperoxide value (initial LHP), maximum lipid hydroperoxide value (MAXLHP), time to reach the maximum lipid hydroperoxide value (TMAX), final lipid hydroperoxide value (final LHP) and the area under the curve (AUC) found for palm oil samples to which heme iron and different antioxidants were added. Susceptibility towards oxidation was measured in filtered samples. Therefore, results are expressed per weight of filtered sample.
[a)]Mean ± SD.
[b)]ND, not detected.

Similar conclusions can be drawn when MAXLHP, TMAX, and Final LHP values are studied (Table 4). Those treatments in which AP was added at higher concentrations (200 or 400 mg/kg) with or without CA had lower MAXLHP and Final LHP values than those found in samples in which AP was present at the lowest concentration (100 mg/kg), alone or in combination with CA. Additionally, treatments in which CA was added alone (CA 250 and CA 500) had the highest MAXLHP and Final LHP values. It is important to note that the time taken to reach the maximum LHP concentration (TMAX) varied between the samples. The control and the treatments containing CA alone (250 and 500 mg/kg) and AP at 100 mg/kg, alone or in combination with CA, required from 2 to 4.15 h to reach the MAXLHP. In contrast, much longer periods were required to reach TMAX (18.45–26.15 h) for treatments that contained AP at higher concentrations (200 and 400 mg/kg) with or without CA.

The AUC is a good indicator for measuring the susceptibility towards oxidation in different food matrices [21, 29]. In this case, this parameter provided similar information to those parameters previously described. Those samples in which AP was added at higher concentrations (200 and 400 mg/kg), alone or combination with CA, seemed to decrease the extent of oxidation and thus result in much lower AUC values than the control. In contrast, treatments with CA alone had higher AUC values than the control indicating a prooxidant effect. Overall, these results are in agreement with those previously reported by Aleman et al. [21] using a similar model.

3.4 Assessment of oxidation during storage

The formation of LHP in samples stored for different periods was assessed by means of PV and LHP content measured through the non-induced FOX method. The formation of secondary oxidation compounds during storage was assessed by measuring *p*-AnV and the hexanal content.

3.4.1 Peroxide value

The addition of AP reduced the PV of the samples (Table 5). However, this reduction was not affected by the AP concentration (100, 200, or 400 mg/kg). In contrast, the PV increased when CA was added at 500 mg/kg.

A significant interaction between the addition of AP and CA was found for PV ($p \leq 0.001$). In some samples, the AP was unable to counteract the prooxidant effect of the CA and showed higher PV than the control (data not shown). However, when no CA was added to the samples, the antioxidant effect of the AP was clear: the PV decreased as AP concentration increased (Fig. 1).

These results are consistent with those previously reported by Alemán et al. [21], who observed that the addition of AP at 500 mg/kg reduced PV during the storage of palm oil enriched with heme iron. However, in the present study CA acts as prooxidant, while in the previous study a synergistic effect was found between AP and CA. These controversial results can be related to various relevant differences between the two studies. These differences include the batch and the amount of heme iron used (1.61 vs. 0.94 mg/g per sample), and the age of citric acid used. The citric acid used in the present study was 300 days older than that of the previous study. However, citric acid was stored hermetically sealed at room temperature in the darkness, as recommended by the supplier, and used 3 years before its expiration date.

Table 5. Effect of AP and CA concentrations and storage time on the development of primary (PV and LHP content) and secondary (*p*-AnV and hexanal content) oxidation parameters of samples stored at room temperature in the darkness

	PV[a] (meq O_2/kg)	LHP content[a] (mmol CHP/kg)	*p*-AnV[a]	Hexanal content[b] (mg/kg)
AP concentration				
0 mg/kg	22.2*a*	14.9	5.0*a*	126.5*a*
100 mg/kg	17.8*b*	12.1	3.8*b*	95.4*b*
200 mg/kg	16.5*b*	12.1	3.9*b*	136.9*a*
400 mg/kg	17.1*b*	13.6	3.6*b*	96.1*b*
SEM[c]	*1.9*	*1.4*	*0.39*	*14*
CA concentration				
0 mg/kg	15.5*a*	10.8*a*	3.8	105.7
250 mg/kg	17.5*a*	13.2*b*	4.2	118.1
500 mg/kg	22.3*b*	15.5*b*	4.3	117.5
SEM	*1.7*	*1.2*	*0.34*	*13*
Storage time				
0 days	1.5*a*	0.9*a*	2.1*a*	6.6*a*
15 days	3.4*ab*	2.3*ab*	2.5*a*	29.1*ab*
30 days	4.8*ab*	3.7*ab*	2.7*ab*	62.5*bc*
60 days	8.8*b*	*6.6b*	3.3*abc*	99.2*cd*
120 days	17.9*c*	13.4*c*	3.8*bc*	122.3*d*
180 days	22.1*c*	14.5*c*	4.2*c*	
240 days	30.7*d*	20.2*d*	5.6*d*	165.2*e*
300 days	36.5*e*	26.5*e*	6.1*d*	187.6*e*
360 days	39.9*e*	*30.6e*	6.5*d*	237.5*f*
SEM	*2.9*	*2.2*	*0.59*	*20.3*

[a] Values correspond to least-squares means obtained from multifactor ANOVA ($n = 216$).
[b] Values correspond to least-squares means obtained from multifactor ANOVA ($n = 96$).
[c] Standard error of the least-squares means.
Values corresponding to a certain factor with different letters differ significantly ($p \leq 0.05$).
PV, peroxide value; LHP content, lipid hydroperoxide content measured through the non-induced FOX method; *p*-AnV, *p*-anisidine value; AP, ascorbyl palmitate; CA, citric acid.
PV, LHP content and *p*-AnV were determined in filtered samples. Hexanal content was determined in the whole samples (non-filtered samples, containing heme iron particles). Results are expressed per weight of filtered or whole sample, accordingly.

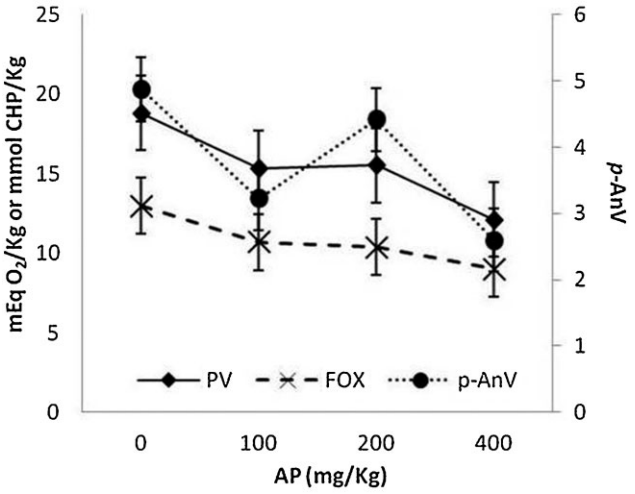

Figure 1. Effect of AP concentration, without CA addition, on the oxidation parameters (PV, LHP content, and *p*-AnV). See Table 5 for abbreviations.

Finally, PV clearly increased during the storage of the samples at room temperature in the dark.

3.4.2 Lipid hydroperoxide content

The LHP content and PV in the samples were highly correlated ($r = 0.935$; $p < 0.0001$, $n = 216$). Therefore, the effects of the studied factors on both parameters were similar (Table 5). The addition of CA at 250 and 500 mg/kg increased the LHP content of the samples, confirming that CA promotes the formation of LHP.

Additionally, a significant interaction between the addition of AP and CA was observed for LHP content ($p \leq 0.001$), confirming that in some samples the AP was unable to counteract the prooxidant effect of the CA (data not shown) and that AP was very effective in preventing LHP formation when no CA was added (Fig. 1). The effect of AP on LHP content is similar to that reported in our previous study [21].

As was the case with PV, the LHP content clearly increased during storage. Therefore, both PV and LHP content measured through the non-induced FOX method reveals similar information about the formation of lipid hydroperoxide in these samples.

3.4.3 *p*-Anisidine value

Irrespective of the concentration, the addition of AP clearly reduced *p*-AnV in the samples (Table 5). The effect of AP on the reduction of *p*-AnV is similar to that reported in our previous study [21]. Conversely to what happens for both primary oxidation parameters, the addition of CA did not significantly increase *p*-AnV in the samples.

As observed for PV and LHP content, a significant interaction between the addition of AP and CA was found for *p*-AnV ($p = 0.001$), confirming that in some samples AP was unable to counteract the prooxidant effect of the CA (data not shown) and that AP was effective in preventing the formation of secondary oxidation products when no CA was added (Fig. 1).

Conversely to what was observed in our previous study [21], *p*-AnV increased during storage of the samples (Table 5). Therefore, in the present study this parameter is useful to follow up oxidation during the storage of palm oil enriched with heme iron.

3.4.4 Hexanal content

The hexanal content and *p*-AnV of the samples were correlated ($r = 0.531$; $p < 0.0001$, $n = 96$). Therefore, the effects of the studied factors on both parameters were similar (Table 5). Finally, as is the case for the rest of the oxidation parameters, hexanal content increased in the samples during storage at room temperature in the darkness (Table 5). Although the variability of hexanal content between sample duplicates was slightly higher than that of the other oxidation parameters, results revealed that hexanal determination may be more sensitive than the rest of the parameters to follow up lipid oxidation during the storage of samples.

Overall, the determination of primary and secondary oxidation parameters during the storage of samples at room temperature in the darkness shows that AP is effective in preventing lipid oxidation, whereas CA acts as prooxidant.

In a previous study carried out on palm oil enriched with heme iron, CA at 300 mg/kg was slightly effective in preventing lipid oxidation, while AP at 500 mg/kg was very effective [21]. As the system had a very low amount of free iron, the chelator (CA) was much less effective than an oxygen scavenger such as AP. In the present study, the amount of free iron in the samples (after heating) was only a 2%. In these conditions, CA was prooxidant, while AP (an oxygen scavenger) was effective in preventing lipid oxidation. The antioxidant effect of AP is consistent with the fact that oxygen is highly soluble in oil [36, 37]. However, it is difficult to explain the prooxidant effect of the CA as the model is very similar to the previous model used [21]. Relevant differences between the two studies that could contribute to explain the different behavior of CA were described above.

Additionally, the chemistry of the iron–citrate complexes is particularly intricate [38]. Some authors have demonstrated that certain conditions (Fe:citric molar ratio, pH and temperature) influence on the formation of ferric citrate species in aqueous solutions [38]. The same authors [39] also reported that depending on conditions, the presence of iron and citric acid in cold drinks can favor the formation of hydroxyl radical. In other research, similar results have been reported for aqueous solutions [40]. Moreover, the prooxidant effect of the citric acid has been reported in a number of systems containing heme iron [41, 42]. For instance, the addition of CA (1% w/w) in a salmon pâté enriched with cod

liver oil to increase the n−3 PUFA content favored the formation of volatiles. The authors suggested this was due to a prooxidant effect of the CA [42]. CA (1 mM) also promoted lipid oxidation (conjugated dienes and TBARS) mediated by fish hemoglobin (3 μM) in liposomes [41]. However, it is important to bear in mind that all the systems previously described are different from our model.

Another difference between the two studies is that in the previous study [21] *p*-AnV did not increase with time, while in the present study a clear increase was observed (Table 5). It is possible that this difference is a response to the variations in the refined palm oils, iron doses and heme iron batches used in both studies. However, it is important to note that both palm oils were obtained from the same refining plant and used immediately upon receipt. In addition, the fatty acid composition of both palm oils was very similar.

The amounts of linoleic and linolenic acid in the palm oil were slightly higher in the previous study than in the present (10.52 vs. 9.37% and 0.19 vs. 0.14%, respectively), which is unhelpful in explaining the variation in *p*-AnV between the two studies. Moreover, the tocopherol and tocotrienol content of both palm oils were similar before heating. In the previous study, total tocopherols and total tocotrienols were 134 and 397 mg/kg, respectively; in the present study they were 146 and 320 mg/kg, respectively. However, the loss of tocopherol and of tocotrienol analogues during the heating of control samples was much lower in the previous study than in the present study (13.2 vs. 20.2%). This may be caused by variations in susceptibility to oxidation of both control samples (palm oil enriched with heme iron). In addition, the oxidation parameters measured in the control samples of both studies, immediately after heating and after 200 or 180 days of storage at room temperature in the dark, were slightly higher in the present study (Table 6). These results indicate that samples from the previous study are slightly less susceptible to oxidation than those of the present study, which could explain the varying *p*-AnV between the two studies. In addition, the susceptibility towards oxidation of the control samples measured using the FOX-induced method was lower in the previous study [21] than in the present (Table 4). As the dose of iron was much higher in the previous study, the variations in the refined palm oils used may explain the varying *p*-AnV between the two studies.

Table 6. Oxidation parameters measured in the control samples from both studies after heating (time 0) and after 200 or 180 days of storage at room temperature in the dark

	Storage time (days)	PV (meq O_2/kg)	LHP content (mmol CHP/kg)	*p*-AnV
Previous study [21]	0	0.7 ± 0.01[a)]	0.1 ± <0.01	3.4 ± 0.01
	200	24.6 ± 0.37	18.5 ± 0.21	4.3 ± 0.01
Present study	0	3.3 ± 0.21	1.8 ± <0.01	2.9 ± 0.06
	180	25.3 ± 0.69	20.1 ± 0.51	6.3 ± 0.04

[a)] Mean ± SD.

PV, peroxide value; LHP content, lipid hydroperoxide content measured through the non-induced FOX method; *p*-AnV, *p*-anisidine value.

In the present study, these oxidation parameters were assessed in filtered samples. In the previous study, the heme iron particles were removed from the samples prior to the determinations as follows: for *p*-AnV particles were removed by filtering the samples, as in the present study, and for PV, particles were removed by centrifuging the thawed samples (at 55°C) at 1370*g* for 5 min. To determine the LHP content, the iron particles were removed during the analysis. Samples were thawed in a water bath at 55°C. Between 0.2 and 1.2 g were weighed into a 10 mL volumetric flask and the volume was made up with dichloromethane/ethanol (3:2, v/v). The solution was then transferred into a screw cap tube and centrifuged at 1370*g* for 3 min. Between 100 and 600 μL of the supernatant were used for the determination of the LHP content as previously described [26]. Therefore, only LHP contents from the previous study are expressed per weight of the whole sample. The rest of the results in this table are expressed per weight of the sample, excluding heme iron particles.

4 Conclusions

AP was effective in preventing lipid oxidation in palm oil enriched with heme iron, which was used as a simple model to fortify bakery products containing chocolate with heme iron. In contrast, CA acted as a prooxidant in this model and no synergistic effect with AP was observed.

When no CA was added to the samples, the values of the oxidation parameters decreased as AP concentrations increased. Thus, the AP alone at 400 mg/kg was the most effective treatment in preventing lipid oxidation.

All primary and secondary oxidation parameters were useful to follow the oxidation in samples during storage (Table 5). The results indicate that the most and less sensitive parameters to find significant differences along the storage time were the hexanal content and *p*-AnV, respectively. For this proposal, the sensitivity of the PV and LHP content was very similar and intermediate between the abovementioned secondary oxidation parameters.

Conversely, in a previous and similar study [21], it was observed that *p*-AnV was not useful to follow the oxidation in samples during storage. The different behavior of this parameter during storage of very similar samples indicates that, as there are many factors that may affect lipid oxidation, it is always advisable to use various determinations to more effectively monitor the oxidation of samples during storage.

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5.3. Co-spray-drying of a heme iron ingredient as strategy to decrease its pro-oxidant effect in lipid-containing foods

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TITLE AND ABSTRACT IN SPANISH/ TÍTULO Y RESUMEN EN CASTELLANO:

"Co-atomización de un ingrediente hémico para disminuir su efecto pro-oxidante en alimentos que contienen una fracción lipídica apreciable"

La fortificación de productos con hierro, bien con hierro hémico o no-hémico, es una estrategia común para combatir la deficiencia de hierro debida a la dieta. El hierro hémico posee una gran biodisponibilidad, pero como otras formas de hierro, promueve la oxidación.

La manteca de palma es ampliamente empleada en la formulación de productos de pastelería y rellenos de chocolate. El presente estudio tiene como objetivo el retraso de la oxidación de una matriz de manteca de palma fortalecida con hierro hémico, usada como modelo de productos de pastelería, mediante el uso del palmitato de ascorbilo (en dos concentraciones, 0 y 400 mg/kg) y la co-atomización del ingrediente hémico con caseinato de calcio en dos proporciones (concentrado hemico : caseinato de calcio, 2:2 y 1:1, p/p).

A lo largo de un año de almacenamiento a temperatura ambiente y en la oscuridad han sido determinadas tanto la oxidación primaria (índice de peróxidos y contenido en hidroperóxidos lipídicos) como la secundaria (índice de *p*-anisidina y contenido en hexanal).

Los resultados de la oxidación primaria y secundaria demuestran que la combinación del palmitato de ascorbilo a 400 mg/kg y el ingrediente hémico co-atomización con caseinato de calcio en proporción 1:1 fue el tratamiento que mejor protegió a las muestras de la oxidación a lo largo del tiempo de almacenamiento.

Co-spray-drying of a heme iron ingredient to decrease its pro-oxidant effect in lipid-containing foods

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Running title: Decreased pro-oxidant effect of heme iron co-spray-dried

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1. Abstract

Fortification of food products with non-heme or heme iron is a common strategy to overcome nutritional iron deficiency. Heme iron is highly bioavailable but it promotes oxidation, as do other iron forms. Palm oil is widely used in the formulation of bakery products and chocolate fillings. The work reported here aims to delay the onset of oxidation of a palm oil matrix fortified with heme iron, as a model for bakery products, through the use of ascorbyl palmitate (0 and 400 mg/kg) and the co-spray-drying of the heme iron with calcium caseinate in two ratios (heme iron concentrate:caseinate, 2:1 and 1:1, w/w). Primary (peroxide value and lipid hydroperoxide content) and secondary (*p*-anisidine value and hexanal content) oxidation were measured over one year of storage at room temperature in the dark. The combination of ascorbyl palmitate at 400 mg/kg and the co-spray-dried heme iron in a 1:1 ratio was the treatment that best protected samples from oxidation during the storage time.

Keywords: Heme iron / Food fortification /Co-spray-drying/ Antioxidant / Oxidative stability

List of abbreviations used: AP, ascorbyl palmitate; AUC, area under the curve; CA, citric acid; CAS, calcium caseinate; CHP eq., cumene hydroperoxide equivalents; FAME, fatty acid methyl esters; FID, flame ionization detector; FOX, ferrous oxidation-xylenol orange; ICP-AES, inductively-coupled plasma atomic emission spectroscopy; LHP, lipid hydroperoxide; MAXLHP, maximum lipid hydroperoxide; MUFA, monounsaturated fatty acids; *p*-AnV, *p*-anisidine value; PUFA, polyunsaturated fatty acids; PV, peroxide value; RT, room temperature; SFA, saturated fatty acids; ST-MA, mixture of modified starch (sodium octenyl succinate):maltodextrin (1:1, w/w); trans FA, trans fatty acids; TMAX, time to reach the maximum lipid hydroperoxide value.

Practical applications: Food fortification with iron has been related as a good strategy for overcoming iron deficiency. In this study, a heme iron ingredient has been selected due to it is higher bioavailability than inorganic iron forms. Nevertheless, as other iron forms, heme iron is a potent pro-oxidant, which may affect the nutritional value, the organoleptics properties and shelf-life of the fortified product. Thus, in this study the co-spray-drying of the heme iron with several coatings was assessed in order to reduce its pro-oxidant activity. The different heme irons were mixed with palm oil, which is a fat widely used in bakery. The combination of co-spray-dried heme iron and ascorbyl palmitate delayed the onset of oxidation, which demonstrates its effectiveness in further iron fortified bakery products.

2. Introduction

Iron deficiency is one of the most prevalent health issues in the world. It mainly affects infants, children and women [1]. However, its prevalence in pre-school age children is higher than that of any other population group [2]. This nutritional deficiency occurs during childhood as a result of a sustained negative iron balance, which may be caused by inadequate dietary intake or absorption of iron and increased requirements during the growth period. In later stages of iron depletion, the haemoglobin concentration decreases possibly leading to anaemia, which is the most severe consequence as it has been linked to childhood morbidity, impaired cognitive development and inadequate school performance.

Three major intervention strategies are used to overcome iron deficiency: diet diversification, iron supplementation and food fortification. Food fortification is known to be the most cost-effective long-term strategy to reduce the prevalence of iron deficiency [3]. A successful fortification programme requires proper selecting of the target population, the iron form and the food vehicle [4]. It is essential that the food vehicle either forms part of the regular diet of the target population or is easy to introduce into it [4]. Biscuits fortified with heme iron have been shown to be effective at improving the iron status of school-age children [5, 6].

Both heme and inorganic forms of iron have been used in food fortification programmes [7, 8]. The bioavailability of heme iron is higher than that of inorganic iron due to its different absorption process [9] and it is not affected by food ligands commonly present in typical diets [10]. Despite its dark colour, which limits its use and product acceptability, heme iron has been used in different food matrixes [11]. Products containing chocolate, besides being well accepted by children, mask the dark colour of the heme iron ingredient. However, all iron forms are potent pro-oxidants which can affect the shelf life, sensory properties and nutritional value of the fortified product [12]. To ameliorate this problem several strategies, such as antioxidant addition, iron encapsulation or addition of chelating agents, have been employed in foods fortified with either heme or inorganic iron [13–15].

In order to reduce these drawbacks, we studied in two previous works a model for iron fortification in baked products consisting of refined palm oil fortified with a heme iron ingredient (AproFER 1000™) and antioxidants [16, 17]. In both studies, the model was heated at 220 °C for 10 minutes to mimic typical conditions during baking processing. In the first of two studies, the palm oil matrix was used to assess the effectiveness at delaying the onset of oxidation during 200 days' storage at room temperature (RT) in the dark of three antioxidants: ascorbyl palmitate (AP), citric acid (CA) and tocopherol extract. The tocopherol extract and CA alone were ineffective; whereas AP proved to be highly effective in preventing oxidation of samples during the storage period. In addition, a slight synergistic effect was observed when AP and CA were combined [16]. Taking these results into account, in a second study, the same model was used to attempt to optimize the dose of AP and CA to prevent oxidation. Four different concentrations of AP were combined using a factorial design with three different concentrations of CA. Primary and secondary oxidation parameters were monitored in samples over a 360-day storage period at RT in the dark. AP (400 mg/kg) proved to be highly effective in delaying oxidation during storage; however, in

that study CA acted as a pro-oxidant and no synergistic effect between the two antioxidants was observed [17].

In the present study, the same model was used to assess how the co-spray-drying of the heme iron with calcium caseinate (CAS) affects its pro-oxidant effect. The heme iron was co-spray-dried with CAS at two ratios (2:1 and 1:1, w/w, heme iron concentrate:CAS). In addition, the antioxidant effect of AP (at 400 mg/kg) alone and combined with the co-spray-dried heme iron was also studied. CAS was selected because in a preliminary study the heme iron co-spray-dried with CAS was less pro-oxidant than when a mixture of modified starch and maltodextrin was used as the co-spray-drying agent.

3. Materials and methods

3.1. Materials

Refined palm oil was a gift from Lípidos Santiga S.A. (Santa Perpètua de Mogoda, Spain). Sunflower oil was purchased in a local market. AP (99.5%) and hexanal (98%) were acquired from Sigma-Aldrich (Madrid, Spain). CAS, and a mixture of modified starch (sodium octenyl succinate) and maltodextrin (1:1, w/w) (ST-MA) were purchased from Cargill S.L.U. (Rubí, Spain). Dimethyl sulphoxide (headspace grade) was acquired from Scharlab S.L. (Barcelona, Spain).

All the chemicals used were of ACS grade with the exception of the solvents used in the ferrous oxidation-xylenol orange (FOX) method, in the tocopherol and tocotrienol determination and in the heme iron determination, which were of HPLC grade. The iron standard solution was purchased from High-Purity Standards (Charleston, SC) and was traceable to the Standard Reference Materials of NIST.

3.2. Producing heme iron ingredients

Heme iron ingredients were produced at APC Europe S.A. (Granollers, Spain). The heme iron ingredient used as a control (AproFER 1000™) is a dark powder obtained from the blood of healthy pigs as described by González-Rosendo et al. (2010).

For the preliminary study, five different ingredients were manufactured: the heme iron control and four co-spray-dried ingredients: two with CAS and two with ST-MA at two ratios (2:1 and 1:1, w/w, heme iron concentrate:co-spray-drying agent). For the final study, three different ingredients were prepared: the heme iron control and two co-spray-dried ingredients with CAS at the ratios 2:1 and 1:1, w/w, heme iron concentrate:CAS. More detailed information on heme iron ingredient manufacture is given as supplementary information.

3.3. Study design and sample preparation

3.3.1. Preliminary study (accelerated storage conditions).

In this study, our aim was to select one of the co-spray-drying agents for the following study with palm oil stored at RT. To this end, we used a model containing sunflower oil, which is more susceptible to oxidation than palm oil, stored at 60°C.

Mixtures of sunflower oil fortified with heme iron were prepared at 40°C under magnetic stirring. The 5 heme iron ingredients were dispersed in the sunflower oil matrix in different percentages in order to maintain the iron content of all the samples constant (1.30 mg Fe/g mixture). The iron content was higher in this study than in the study with palm oil because we aimed to ensure the selection of the best co-spray-drying agent.

Once the heme iron ingredients were homogenously dispersed in the sunflower oil, the mixtures were heated at 220°C for 10 minutes to mimic the typical baking conditions. Thereafter, the mixtures were stored at 60°C in an oven. Sampling was conducted as follows: at specific times, the sunflower oil mixtures were removed from the oven and homogenized by magnetic stirring for 5 minutes and, immediately after, vials were filled with sample aliquots, flushed with nitrogen, capped and stored at -20°C till analysis.

The evolution of the oxidation of the samples was followed by means of the peroxide value (PV) (European Communities Commission, 1991) and *p*-anisidine index (*p*-AnV) [20]. Before performing the assays, the samples were thawed at RT and filtered through Whatman paper no. 54 under vacuum. The oxidation was followed till the control sample reached a PV higher than 100 mEq O_2/kg. Both the heme and total iron content of the samples were assessed without removing heme iron particles.

3.3.2. Final study (storage at room temperature).

Refined palm oil was chosen because it is commonly used to manufacture biscuits, pastries and chocolate fillings, amongst others. Three different heme iron ingredients were evaluated: the control AproFER 1000™ and two ingredients obtained by co-spray-drying of heme iron with CAS in two ratios (2:1 and 1:1). Both ingredients with CAS were selected because they had showed a high capacity to retard the onset of oxidation in the preliminary trial.

As the different heme iron ingredients contained different amounts of iron, different quantities of the heme iron ingredients were dispersed into the palm oil in order to maintain constant the iron content of all samples (1.20 mg Fe/g sample). Theoretically, by using these heme iron dispersions in palm oil to prepare chocolate filling for chocolate-filled biscuits, each weighing 25 g and having 7.5 g of chocolate filling containing 25% palm oil, 2 such biscuits will provide approximately 32% of the iron RDA (14 mg Fe/day) (European Communities Commission, 2008).

The three heme iron ingredients were combined with two concentrations of AP (0 and 400 mg/kg). The antioxidant AP was assayed at 400 mg/kg as previous studies [16, 17] showed its capacity to delay the onset of oxidation in similar palm oil models fortified with heme iron at this concentration. One further treatment was included that combined the heme iron

control ingredient and CAS in a ratio of 1:1 (without co-spray-drying) in order to test the antioxidant properties of CAS by itself (Table 1).

Samples were prepared as described elsewhere [17] with few modifications. The palm oil was melted (55°C), mixed and divided into seven aliquots. AP or CAS was dispersed; first under stirring at 140°C and thereafter the heme iron ingredients were dispersed. The samples were then heated at 220°C for 10 min to reproduce typical baking conditions. The samples were allowed to cool to 70°C-80°C, homogenized using a Polytron PT3100 (10 sec, 15000 rpm) and distributed into 10 and 20 mL vials. Thereafter, the vials were closed, shaken and introduced into an ice bath until the content was solid to ensure an even dispersion of the heme iron ingredients. The vials were stored at RT in the dark for 0, 90, 180 and 360 days. After each storage period, samples were frozen at -20°C until analysis. In various determinations (tocopherol content, PV, lipid hydroperoxide (LHP) content and *p*-AnV), the vials were heated to 55°C for 15 min and then the samples were filtered through Whatman no. 54 filter paper at RT to remove the heme iron particles. Heme iron, total iron and hexanal content were determined without removing the iron particles from the sample.

3.4. Palm oil characterization

The fatty acid (FA) composition, and the tocopherol and tocotrienol content of the refined palm oil used to prepare the heme iron dispersions were determined. Fatty acid methyl esters (FAME) were prepared as described elsewhere [22] under the chromatographic conditions described by Aleman et al. (2010). The tocopherol and tocotrienol content was determined in 200 mg samples (of the palm oil used to prepare all the treatments and the palm oils obtained by filtration of the treatment samples stored for 0 days) by normal-phase HPLC as described by Aleman et al. (2010). Also, PV (European Communities Commission, 1991), LHP content (using the non-induced FOX method) [23], *p*-AnV [20] and hexanal content [17] were assessed in the fresh palm oil.

3.5. Heme and total iron content

The heme iron content of the different heme iron ingredients and also of the samples at time 0 was determined following Hornsey (1956). The total iron content of the heme iron ingredients and samples was determined after their mineralization using a PerkinElmer ICP-AES (Optima 3200 RL model). The mineralization processes were performed as described elsewhere [17], with few modifications for samples (see supplementary information).

3.6. Susceptibility to oxidation

In the palm oil study, the induced version of the FOX method was used to assess the susceptibility to oxidation of the filtered palm oil mixtures after the thermal treatment [25, 26]. In this assay, the amount of LHP in each filtered sample was measured over an incubation period, using the reaction conditions reported by Navas et al. (2004). The incubation time was set at 168 hours and absorbance was measured at different times, following Tres et al. (2009). To better describe the differences observed in the susceptibility to oxidation of the samples, the following parameters were calculated [27]: Initial LHP (the

initial LHP value was measured after 30 minutes of incubation), MAXLHP (the maximum LHP value), TMAX (the time to achieve the MAXLHP), Final LHP (the final LHP value measured after 168 hours of incubation) and AUC (the area under the curve of LHP formation).

3.7. Determination of oxidation during storage

In the preliminary study, primary oxidation was monitored by means of the PV (European Communities Commission, 1991) and secondary oxidation by means of *p*-AnV [20].

Formation of primary oxidation compounds in palm oil study was assessed by means of PV (European Communities Commission, 1991) and measuring the LHP content through the non-induced FOX method after 30 minutes of reaction in the dark at RT [23]. The formation of secondary oxidation compounds was assessed by means of *p*-AnV [20] and hexanal content determined by static headspace [17].

3.8. Statistical analysis

Pearson's correlation coefficients were used to study the relationships between PV and LHP content and between *p*-AnV and hexanal content in the palm oil study. One-way ANOVA was used to determine the effect of the different treatments on (I) tocopherol and tocotrienol content after heating at 220°C for 10 minutes; and (II) the parameters of the induced FOX method. In addition, MANOVA was used to determine significant effects produced by the different treatments and storage times on PV, LHP content, *p*-AnV and hexanal content. The effect of the different treatments on PV, LHP content, *p*-AnV and hexanal content at each storage time was assessed by one-way ANOVA.

When significant differences were found, the means were separated using Scheffe's test (α=0.05). In all cases, $P \leq 0.05$ was considered significant.

4. Results and discussion

4.1. Preliminary study with sunflower oil fortified with heme iron stored at 60°C

The samples containing the heme iron ingredients co-spray-dried with CAS or ST-MA presented lower PV than the control sample (not co-spray-dried) over the storage time (Figure 1.A). Furthermore, all the heme iron ingredients co-spray-dried at a 1:1 ratio were more effective at delaying the onset of PV than their respective heme iron ingredients co-spray-dried at a 2:1 ratio. It is important to note that the greatest delay in the onset of primary oxidation was achieved in samples with the heme iron ingredient co-spray-dried with CAS.

Secondary oxidation, as measured by *p*-AnV (Figure 1.B), increased from the beginning of the storage time in all the treatments, with the control sample reaching the highest *p*-AnV at the end of the storage time (340 hours). Moreover, samples with the heme iron co-spray-dried in a 1:1 ratio with CAS presented the lowest *p*-AnV throughout the storage period.

In conclusion, the heme iron co-spray-dried with CAS at a 1:1 ratio showed the greatest delay in the onset of primary and secondary oxidation in the sunflower oil matrix stored at 60°C. Therefore, CAS was selected as the co-spray-drying agent for the subsequent study with palm oil fortified with heme iron.

4.2. Palm oil fortified with heme iron as a model for iron fortification in bakery products

4.2.1. Palm oil characterization and tocopherols and tocotrienols content after the heating treatment

The palm oil FA composition (Table 2) was typical [28]: 49.83% SFA; 39.93% MUFA; 10.24% PUFA and 0.11% trans FA.

A high concentration of tocotrienols (Table 3), particularly alpha- and gamma-tocotrienol, is characteristic of this oil [28]. The total content of tocopherol and tocotrienol in the fresh palm oil used to prepare the samples was 632.4 mg/kg (total tocopherols: 193.2 mg/kg and total tocotrienols: 439.2 mg/kg).

The tocopherol and tocotrienol content of the different treatments of palm oil after the heating treatment (220°C for 10 minutes) is shown in Table 3. Those samples with added AP had the same amount of total tocopherols as the non-heated palm oil. However, samples without AP (control, 1:1 and CAS) presented lower total tocopherol content than the non-heated palm oil. Thus, AP prevents the loss of tocopherols during heating, especially in the case of α-tocopherol, which is the most abundant. Similar results were found for the total tocotrienol content and total tocopherols plus tocotrienols, which indicate the protective effect of AP during heating.

Regarding the oxidation status, fresh palm oil had a PV and LHP content of 0.04±0.0001 mEq O_2/kg oil and 0.14±0.006 mmol CHP Eq/kg oil, respectively ($n=5$); while hexanal was not detected and the *p*-AnV was 1.24±0.05 (n=5).

4.2.2. Heme iron and total iron content

Table 4 shows the heme and total iron content of the ingredients and samples prior to storage in both the sunflower and palm oil studies. The average total iron content was close to the target in both studies (1.27 and 1.20 mg Fe/g sample).

The main differences between the heme iron ingredients used in each study was the percentage of heme iron. In the preliminary study, heme iron was 92% of total iron in the ingredients, while in the palm oil study it was 83%. This heme iron content is lower than in the previous studies where heme iron accounted for 96% of the total iron content [16, 17].

Heme iron was the main form present in the samples in both studies. In the palm oil study, the heating of the samples (10 min at 220°C) liberated iron from the heme moiety and the

heme iron percentage was reduced to 73% (Table 4). This resulted in large amounts of free iron (27%) in the samples, which differs from the preliminary study, where the maximum amount of free iron in the samples was 6%-10% (from the heme iron ingredients), since iron liberation during the heating of samples was negligible (Table 4). The previous studies also showed that iron liberation during heating was negligible with free iron in the treatment samples accounting for 2%-4% of the total iron content [16, 17]. The differences in iron liberation and free iron content could be due to small changes in the production conditions of the ingredients (e.g., ultrafiltration pressures and temperatures), since the production of some of these batches was used by the heme iron producer to assay new processing conditions.

4.2.3. Susceptibility to oxidation

The susceptibility to oxidation of the samples was assessed prior to storage. Table 5 presents the parameters calculated from the different curves of LHP formation. The initial LHP value (measured after 30 minutes of incubation) indicates the amount of LHP already present in the samples [27]. These values show that the addition of CAS in a ratio of 1:1 with the heme iron, co-spray-dried or not, favoured the formation of LHP during the heating. These two treatments also presented higher MAXLHP and AUC values together with a lower TMAX. The AUC has been described as a good marker of susceptibility to oxidation in different matrices [16, 27]; therefore these results support the idea that samples with CAS in a 1:1 ratio without AP are more prone to oxidation than the other samples.

It is important to note that the time taken to reach the maximum LHP concentration (TMAX) varied between the samples. Those treatments that combined AP with the co-spray-dried ingredients presented much higher TMAX (48-43.5 h), especially compared with CAS and CAS 1:1 treatments (2.5 h). This result coincides with those found in previous studies [16, 17], where the TMAX was higher for palm oil matrixes fortified with heme iron with AP added.

4.2.4. Assessment of oxidation during storage

The development of LHP over the storage period was monitored by means of the PV and the LHP content (non-induced FOX method). Likewise, the formation of secondary oxidation products was assessed by the *p*-AnV and the hexanal content of the samples (Table 6).

4.2.4.1. Primary oxidation: PV and LHP content

The LHP content and PV of the samples were highly correlated ($r=0.921$; $p<0.0001$, $n=140$). Therefore, the effects of the factors studied were similar on both parameters.

As opposed to what was found in the preliminary study, the co-spray-drying of the heme iron with CAS (irrespective of the ratio) did not protect the samples against primary oxidation (Table 6). Moreover, in previous studies of palm oil fortified with heme iron, the addition of AP prevented samples from primary oxidation [16, 17]; however, in this study this protective effect of AP was not seen as there were no differences between the control

and the AP treatment. This is probably due to the increased free iron in this study (27% compared to 2%-4% in the previous ones [16, 17].

Both PV and LHP content clearly increased during the storage of the samples at RT in the dark (Table 6). At time 0, both control and CAS samples presented higher PV, whereas LHP content was higher in samples with CAS in a 1:1 ratio, co-spray-dried or not; which agrees with the results for susceptibility to oxidation. At the end of the storage period, there was no difference between the control and the AP treatment regarding primary oxidation.

4.2.4.2.Secondary oxidation: p-AnV and Hexanal content

The *p*-AnV and hexanal content of the samples were correlated (r=0.442; $p<0.0001$, n=140). Therefore, the effects of the factors studied were similar on both parameters.

Those treatments that combined AP and the co-spray-dried ingredients (at any ratio) presented lower *p*-AnV than the rest of the treatments, even showing synergism between AP and the co-spray-dried ingredients (Table 6). Similarly, the hexanal content was reduced when AP was combined with the co-spray-dried ingredient in a 1:1 proportion. Furthermore, the *p*-AnV increased with time till the end of the storage period, whereas the hexanal content of the samples increased till day 180. The decrease in concentration of hexanal observed at the end of the storage period might be due to the formation of adducts between hexanal and the peptides from the heme iron ingredient [29].

At the beginning of the storage period (Table 6), the *p*-AnV of samples with AP was lower than for the other treatments; this supports the idea that AP offers oxidative protection during heating treatment of samples at 220°C during 10 minutes. Similar conclusions were drawn regarding the tocopherol and tocotrienol content (Table 3), where samples with AP presented similar values to those of the non-heated palm oil, confirming the antioxidant effect of AP during heating. These results agree with those found in previous studies were AP protected palm oil fortified with heme iron from oxidation during the heating treatment [16, 17].

After 360 days of storage, no differences in secondary oxidation were observed between the CAS 1:1 treatments and the control, which differs from the findings of the preliminary study. Nevertheless, the samples that combined CAS 1:1 and AP reached the lowest *p*-AnV and hexanal content after 360 days of storage.

Overall, AP showed a protective effect during heating in this study. However, it was not observed to be effective over the storage time; which is the opposite of what was found in previous studies [16, 17]. This must be related to differences between the matrixes used in the different studies carried out in our laboratory with heme iron (Table 7).

Both the FA and tocopherol compositions in the different studies were typical for palm oil (Table 7). Differences between the percentages of MUFA, PUFA, linoleic and linolenic acid were small and do not help explain the different AP effect between the studies. Conversely, we found important differences in total tocopherol and tocotrienol content of the palm oils,

with the palm oil used in this study having the highest content. This may cause differences in the susceptibility to oxidation of samples from different studies, but both the loss of tocopherols and tocotrienols, and the AUC values in this study are similar to those in study 1 (Table 7). Moreover, the oxidation parameters in these two studies were similar, which contrasts with the difference in tocopherol and tocotrienol content. However, there is an important difference between the present study and study 1, which is the amount of free iron. In this study, free iron content was much higher (27%) than in the first study (4%). Therefore, the relative importance of the oxidation pathways would have been different, rendering AP, an oxygen scavenger, almost ineffective when added alone in the present study. Also, the higher free iron content in this study may have masked the effect of the higher tocopherol and tocotrienol content on the oxidation of the samples.

Indeed, if we compare studies 1 and 2, where the free iron content is similar (4% vs. 2%), differences in both susceptibility to oxidation and oxidation status are observed (with samples from study 2 being more prone to oxidation); which may be explained by the lower tocopherol and tocotrienol content in study 2 (Table 7).

5. Conclusions

The co-spray-drying of the heme iron, with either CAS or ST-MD, delayed the onset of both primary and secondary oxidation in sunflower oil fortified with heme iron stored at 60°C. The more effective co-spray-drying agent was CAS.

However, in the palm oil study, the co-spray-drying of the heme iron with CAS did not delay the onset of oxidation. Moreover, the use of AP alone did not prevent either primary or secondary oxidation during storage of palm oil fortified with heme iron ingredients. Both these findings contradict what was found in previous studies and may be due to the fact that the matrix in this palm oil study contained a much higher amount of free iron than in the previous studies with palm oil (27% vs. 2%-4%) or sunflower oil (27% vs. 6%-10%).

Nonetheless, the combination of AP and co-spray-drying of the heme iron with calcium caseinate protected the palm oil samples from oxidation; which indicates synergism between AP and co-spray-dried heme ingredients. Moreover, the samples that combined CAS 1:1 and AP reached the lowest *p*-AnV and hexanal content after 360 days of storage.

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7. Conflict of interest

The authors have declared no conflict of interest.

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Supplementary information

Production of heme iron ingredients: The control heme iron (AproFER 1000™) is a dark powder obtained from the blood of healthy pigs. After separating the blood cell fraction by centrifugation, a pressure pump was used to release the haemoglobin contained in the red blood cells. Then, the haemoglobin was enzymatically hydrolysed using a proteolytic enzyme (Alcalase®, Novo-Nordisk, Denmark) under controlled pH and temperature conditions. The heme group was then concentrated by ultrafiltration and spray-dried.

The co-spray-dried heme iron ingredients were prepared from the heme iron concentrate obtained by ultrafiltration. After analysing its solid content, the concentrate was mixed with the appropriate amount of co-spray-drying agent. In the case of the co-spray-dried ingredients in a ratio of 1:1, the same amount of co-spray-drying agent as of the solid content of the concentrate was added. In the case of the co-spray-dried ingredients in a ratio of 2:1, half the amount of co-spray-drying agent was added to the concentrate. In both cases, the co-spray-drying agents were dispersed at 40°C with continuous stirring; thereafter the pH was set at 8.00, as is normal during the production of the AproFER 1000™. All the dispersion mixtures, with or without co-spray-drying agents, were dried on a pilot plant spray-dryer (Anhydro Compact Spray Dryer, Anhydro A/S, Copenhagen, Denmark).

Mineralization of samples and heme iron ingredients: For heme iron dispersions in sunflower oil and palm oil, 1 g samples were weighed in a glass beaker and 2 mL of H_2SO_4 was added. Then the samples were placed in a sand bath at 250 °C. When the samples were smoking, an additional 2 mL of H_2SO_4 was added. The samples were then again placed in the sand bath at 250°C and allowed to dry. Afterwards, the samples were calcined in a muffle furnace at 450°C for 10 hours (rate of heating 1 °C/min). The following day, 1 mL of H_2SO_4 was added, allowed to dry and subsequently calcined again for 2 h in the muffle furnace at 450°C. Finally, the samples were re-dissolved in 2 mL of HCl and diluted to a final volume of 100 mL with 1% HNO_3.

The different heme iron ingredients were mineralized as follows: 250 mg of the ingredient was weighed in a glass beaker. Following this, 2 mL of H_2SO_4 was added to the sample. The sample was then placed in a sand bath at 250°C until dry. The ingredient was then calcined in a muffle furnace at 450°C for 10 h (rate of heating 1 °C/min). After the heating treatment, 1 mL of H_2SO_4 was added and allowed to dry in the sand bath at 300°C. Finally, a second heat treatment was applied in the muffle furnace at 450°C for a period of 3–6 h. The samples were then re-dissolved in 2 mL of HCl and diluted to a final volume of 100 mL with 1% HNO_3.

Table 1. Heme iron ingredients and antioxidant concentrations added to the palm oil.

Treatments	Heme iron ingredients[a]	AP [mg/kg]
Control	AproFER 1000™	0
CAS 2:1	2:1, w/w, heme iron concentrate:CAS	0
CAS 1:1	1:1, w/w, heme iron concentrate:CAS	0
CAS[b]	AproFER 1000™ + CAS	0
AP	AproFER 1000™	400
CAS 2:1 + AP	2:1, w/w, heme iron concentrate:CAS	400
CAS 1:1 + AP	1:1, w/w, heme iron concentrate:CAS	400

Abbreviations: CAS, calcium caseinate; AP, ascorbyl palmitate.

[a] Manufacturing of heme iron ingredients is described in supplementary information

[b] Treatment with AproFER 1000™ and CAS (1:1, w/w) without co-spray-drying

All the samples from the different treatments had the same iron concentration

Figure 1: Evolution of peroxide value (A) and *p*-anisidine value (B) during storage at 60°C of sunflower oil fortified with different heme iron ingredients (n=2).

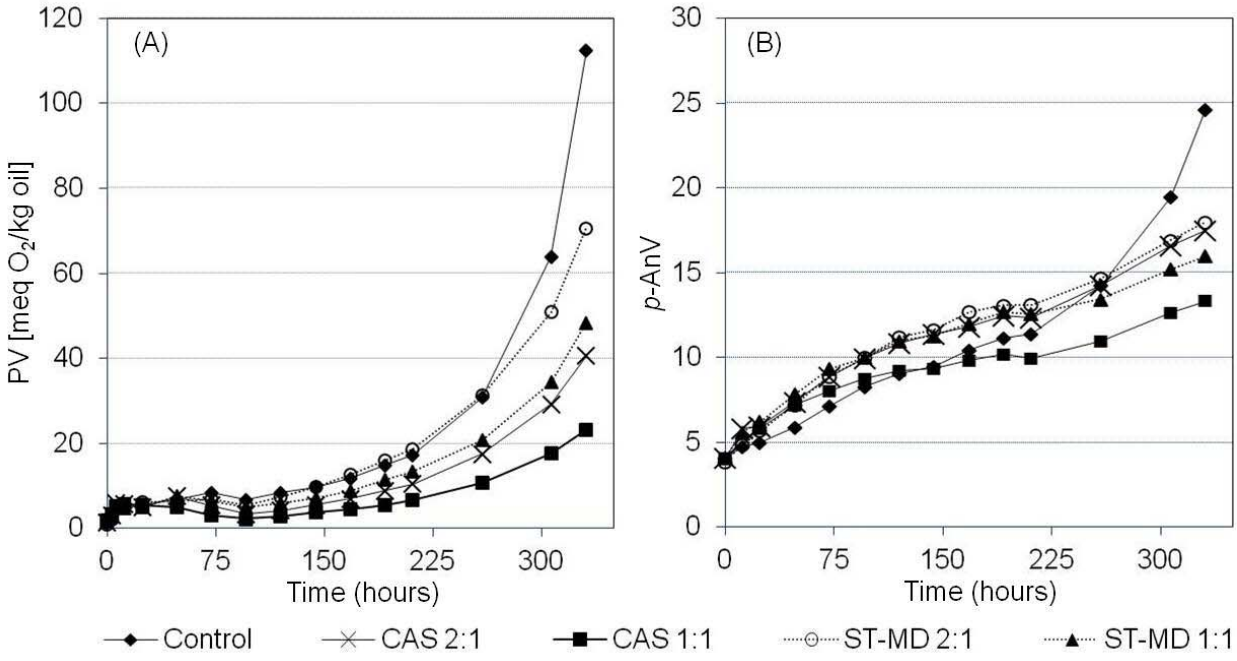

Table 2. Fatty acid composition of the palm oil used to prepare the different treatments (expressed as area normalization in percentage).

Fatty Acid[a]	Mean [%] ± SD
10:0	0.03 ± <0.01
12:0	0.42 ± <0.01
14:0	1.14 ± 0.01
15:0	0.05 ± <0.01
16:0	42.84 ± 0.1
16:1n-9	0.03 ± <0.01
16:1n-7	0.16 ± <0.01
17:0	0.10 ± <0.01
17:1	0.02 ± <0.01
18:0	4.76 ± 0.02
18:1t	0.11 ± 0.01
18:1n-9	38.71 ± 0.09
18:1n-7	0.75 ± 0.06
18:2n-6	9.99 ± 0.02
18:3n-3	0.15 ± <0.01
20:0	0.41 ± <0.01
20:1n-9	0.15 ± <0.01
20:2n-6	0.03 ± <0.01
20:3n-6	0.08 ± <0.01
24:0	0.08 ± <0.01
SFA	49.83 ± 0.08
MUFA	39.93 ± 0.06
PUFA	10.24 ± 0.01
PUFA n-6	10.09 ± 0.01
PUFA n-3	0.15 ± <0.01
Trans FA	0.11 ± 0.01

[a] SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, *Trans* FA = *trans* fatty acids

Table 3. Tocopherol and tocotrienol composition of the palm oil used to prepare the different treatments and that of the palm oil obtained after filtering the different treatment samples prior to storage (after heating at 220°C for 10 minutes).

	Palm oil (not heated)	Control	CAS 2:1	CAS 1:1	CAS	AP	CAS 2:1 + AP	CAS 1:1 + AP
Tocopherol [mg/kg]								
α	188.8±3[a]*w*	165.1±5*yz*	176.4±5*wxy*	159.0±6*z*	167.6±6*xyz*	184.4±6*wx*	178.7±6*wxy*	175.7±12*wxyz*
β	1.4±0.2*wx*	1.7±0.5*wx*	1.3±0.1*wx*	0.9±0.5*x*	1.2±0.4*wx*	1.9±0.5*w*	1.1±0.2*wx*	1.4±0.5*wx*
γ	3.0±0.1*wx*	2.5±0.3*xy*	2.8±0.2*xy*	2.2±0.3*y*	2.8±0.4*xy*	3.6±0.4*w*	2.7±0.2*xy*	2.9±0.3*wx*
δ	ND[b]	ND	ND	ND	ND	ND	ND	ND
Total	193.2±3*w*	169.2±5*xy*	180.0±5*wx*	162.1±7*y*	171.6±4*xy*	190.0±7*w*	182.5±7*wx*	180.0±13*wx*
Tocotrienol [mg/kg]								
α	213.2±8*w*	169.2±9*xy*	188.0±9*wx*	157.9±10*y*	175.5±8*xy*	214.4±14*w*	195.8±11*wx*	193.8±19*wx*
β	21.4±0.2*wx*	19.4±1*wx*	20.5±0.8*wx*	17.4±0.8*x*	19.2±0.9*wx*	22.6±1*w*	22.7±4*w*	21.2±2*wx*
γ	192.4±5*wx*	167.4±10*xyz*	180.4±8*wxy*	154.9±8*z*	172.2±7*xyz*	202.8±12*w*	186.9±9*wxy*	185.6±15*wxy*
δ	12.1±0.2	12.1±0.6	12.4±0.5	11.3±0.6	11.5±0.5	12.9±1	12.5±0.9	12.3±2
Total	439.2±13*w*	368.0±19*xy*	401.3±18*wx*	341.6±16*y*	378.4±15*xy*	452.8±27*w*	417.8±22*wx*	412.8±38*wx*
Total tocopherols + tocotrienols [mg/kg]	632.4±15*w*	537.3±24*xy*	582.0±24*wx*	503.6±22*y*	550.1±21*xy*	642.7±34*w*	600.3±29*wx*	592.9±50*wx*

[a]Mean ± SD; n=5.

[b] ND: not detected

In treatment samples, results are expressed per weight of filtered sample.

See Table 1 for treatment abbreviations. Values in the same row with different letters (w,x,y,z) differ significantly ($P \leq 0.05$).

Table 4. Total iron and heme iron content of ingredients and samples (prior to storage) used in the preliminary and final studies.

	Ingredient		Samples	
	% IRON	**% HEME IRON**	**TOTAL IRON [mg Fe/g sample]**	**% HEME IRON**
Sunflower oil study				
Control	1.34±0.05[a]	93±0.2[a]	1.20±0.02[b]	107±0.1[b]
CAS 2:1	0.98±0.09[a]	92±0.1[a]	1.35±0.01[b]	99±<0.1[b]
CAS 1:1	0.80±0.04[a]	92±0.1[a]	1.31±0.02[b]	107±0.1[b]
ST-MD 2:1	0.96±0.06[a]	90±0.1[a]	1.25±0.04[b]	106±<0.1[b]
ST-MD 1:1	0.74±0.05[a]	94±0.1[a]	1.24±0.04[b]	99±<0.1[b]
Average	-	92±1	1.27±0.06	104±4
Palm oil study				
Control	1.07±0.01[c]	84[c]	1.30±0.25[c]	73±0.2[c]
CAS 2:1	0.75±0.00[d]	80[d]	1.12±0.00[d]	71±<0.1[d]
CAS 1:1	0.58±0.01[e]	86[e]	1.13±0.04[e]	74±0.1[e]
Average	-	83±3	1.20±0.16	73±2

[a] Mean ± SD; n=5.

[b] Mean ± SD; n=3.

[c] Means calculated with all treatments with AproFER 1000™ (n=15): control, CAS and AP.

[d] Means calculated with all treatments with CAS 2:1 heme iron ingredient (n=10):CAS 2:1 and CAS 2:1 + AP.

[e] Means calculated with all treatments with CAS 1:1 heme iron ingredient (n=10):CAS 1:1 and CAS 1:1 + AP.

Samples results are expressed per weight of whole sample (non-filtered samples, containing heme iron particles).

See Table 1 and Figure 1 for treatment abbreviations.

Table 5. Susceptibility to oxidation of samples prior to storage measured by means of the FOX-induced method (n=5). The parameters determined from the curve of formation of lipid hydroperoxides were: initial lipid hydroperoxide value (Initial LHP), maximum lipid hydroperoxide value (MAXLHP), time to reach the maximum lipid hydroperoxide value (TMAX), final lipid hydroperoxide value (Final LHP) and the area under the curve (AUC).

Treatment	Initial LHP [mmol CHP eq/kg]	MAXLHP [mmol CHP eq/kg]	TMAX [h]	Final LHP [mmol CHP eq/kg]	AUC [(mmol CHP eq /kg) x h]
Control	0.08±0.02[a]x	0.13±0.02x	24	0.04±<0.01xy	14.26±1.18x
CAS 2:1	0.05±<0.01x	0.10±<0.01x	24	0.03±0.01x	11.65±1.15x
CAS 1:1	0.21±<0.01y	0.23±<0.01y	2.5	0.06±0.01yz	19.00±1.20y
CAS	0.26±0.04z	0.27±0.04y	2.5	0.07±0.01z	21.10±1.85y
AP	0.07±0.02x	0.13±0.01x	24	0.05±0.01xyz	14.42±1.09x
CAS 2:1 + AP	0.03±<0.01x	0.11±0.01x	43.5	0.05±0.01xyz	12.54±0.77x
CAS 1:1 + AP	0.04±<0.01x	0.12±0.01x	48	0.05±0.01xyz	13.62±0.89x

[a] Mean ± SD; n=5.

Values in the same column with different letters (x,y,z) differ significantly ($P \leq 0.05$).

Results are expressed per weight of filtered sample.

See Table 1 for treatment abbreviations.

Table 6. Effect of treatment and storage time on primary (PV and LHP content) and secondary (*p*-AnV and hexanal content) oxidation parameters of samples stored at room temperature in the dark for 360 days.

	PV [mEq O_2/kg]	LHP content [mmol CHP/kg]	*p*-AnV	Hexanal content [mg/kg]
Treatment[a]				
Control	11.9*u*	11.5*v*	4.0*x*	137.5*wx*
CAS 2:1	16.1*v*	14.9*w*	3.5*w*	107.9*v*
CAS 1:1	20.9*w*	16.9*w*	4.9*z*	72.8*u*
CAS	10.1*u*	8.5*u*	4.6*y*	125.9*vwx*
AP	8.7*u*	9.5*uv*	3.7*wx*	145.3*x*
CAS 2:1+AP	20.1*w*	17.1*w*	2.5*v*	114.8*vw*
CAS 1:1+AP	10.2*u*	11.5*v*	2.2*u*	60.8*u*
SEM[b]	*0.98*	*0.72*	*0.08*	*7.2*
Storage time[a]				
0 days	0.1*u*	0.1*u*	3.0*u*	ND[c]*u*
90 days	10.2*v*	9.5*v*	3.3*v*	21.2*v*
180 days	17.1*w*	16.1*w*	3.7*w*	220.5*w*
360 days	28.6*x*	25.6*x*	4.4*x*	195.5*x*
SEM[b]	*0.74*	*0.54*	*0.06*	*5.4*
Time 0[d]				
Control	0.38*w*	0.08*v*	3.4*v*	ND
CAS 2:1	0.05*u*	0.05u*v*	3.4*v*	ND
CAS 1:1	0.09*u*	0.21*w*	5.3*w*	ND
CAS	0.24*v*	0.26*w*	3.2*v*	ND
AP	0.09*u*	0.07u*v*	2.0*u*	ND
CAS 2:1 + AP	0.04*u*	0.03*u*	1.9*u*	ND
CAS 1:1 + AP	0.05*u*	0.04u*v*	1.9*u*	ND
SEM[e]	*0.02*	*0.01*	*0.09*	*0*
Time 360 [d]				
Control	23.1*uv*	22.6*uvw*	5.2*xy*	233.9*v*
CAS 2:1	32.9*vw*	28.3*vwx*	4.2*w*	189.5*uv*
CAS 1:1	41.6*w*	30.8*wx*	4.9*x*	152.2*uv*
CAS	19.9*u*	12.8*u*	5.5*y*	207.1*uv*
AP	15.1*u*	19.1*uv*	4.9*x*	233.1*v*
CAS 2:1 + AP	41.2*w*	33.6*x*	3.7*v*	216.9*uv*
CAS 1:1 + AP	26.6*uv*	32.3*wx*	2.7*u*	135.5*u*
SEM[e]	*3.1*	*2.6*	*0.12*	*22*

[a] Values correspond to least-squares means obtained from multifactor ANOVA (n=140).
[b] Standard error of the least-squares means.
[c] ND: not detected
[d] Values correspond to means obtained from ANOVA (n=35)
[e] Standard error of the means
See Table 1 for treatment abbreviations. PV: peroxide value; LHP content: lipid hydroperoxide content measured through the non-induced FOX method; *p*-AnV: *p*-anisidine value.
PV, LHP content and *p*-AnV were determined in filtered samples. Hexanal content was determined in the whole samples (non-filtered samples, containing heme iron particles). Results are expressed per weight of filtered or whole sample, accordingly.
Values corresponding to a certain factor with different letters (u,v,w,x,y,z) differ significantly ($P \leq 0.05$).

Table 7. Palm oil characteristics and oxidation status of control samples from different studies after heating at 220°C for 10 minutes (prior to storage) and after 180 or 200 days of storage at room temperature in the dark.

		Study 1 (Aleman et al., 2010)	*Study 2 (Alemán et al., 2014)*	*Present study*
Characteristics of palm oil used to prepare samples fortified with heme iron				
MUFA %		38.94±<0.01[a]	39.87±0.20	39.93±0.06
PUFA %		10.83±<0.01	9.62±0.05	10.24±0.01
% Linoleic acid		10.52±<0.01	9.37±0.05	9.99±0.02
% Linolenic acid		0.19±<0.01	0.14±<0.01	0.15±<0.01
Total tocopherols [mg/kg]		133.8±6	146.4±6	193.2±3
Total tocotrienols [mg/kg]		397.1±14	322.7±12	439.2±13
Total tocopherols + tocotrienols [mg/kg]		530.9±14	469.1±17	632.4±14
Susceptibility to oxidation of control samples (time 0)				
Loss of tocopherols + tocotrienols during heating [%]		13.2±2	20.2±5	12.7±2
AUC [(mmol CHP/kg)xh]		10.37±<0.01	31.73±<0.01	14.27±0.01
Oxidation parameters of the control samples fortified with heme iron over storage time				
PV [mEq O_2/kg]	**0 days**	0.7±0.01	3.3±0.21	0.4±<0.01
	180 or 200 days	24.6±0.37	25.3±0.69	13.8±0.24
LHP [mmol CHP/kg]	**0 days**	0.1±<0.01	1.8±<0.01	0.08±0.02
	180 or 200 days	18.5±0.25	20.1±0.51	14.7±0.94
***p*-AnV**	**0 days**	3.4±0.01	2.9±0.06	3.4±0.05
	180 or 200 days	4.3±0.01	6.3±0.04	4.0±0.61
Average iron content of samples prior to storage				
Total iron content [mg Fe/g sample]		1.61	0.95±0.51	1.20±0.16
% free iron		4	2	27

[a] Mean ± SD

[b] ND: not detected

AUC, area under the curve (induced FOX method); PV, peroxide value; LHP content, lipid hydroperoxide content measured through the non-induced FOX method; p-AnV, p-anisidine value.

In the present study and in study 2, these oxidation parameters were assessed in filtered samples. In study 1, the PV and *p*-AnV were determined after removal of iron particles, while AUC and LHP content were determined in the whole samples, without removing the iron particles Therefore, only AUC and LHP contents from study 1 are expressed per weight of the whole sample; the rest of oxidation results in this table are expressed per weight of the sample, excluding iron particles.

The total iron content results are expressed per weight of whole sample (non-filtered samples, containing heme iron particles).Samples in study 1 were stored for 200 days and compared with samples stored for 180 days in the other studies.

5.4. Oxidative stability of heme-iron fortified cookies: effectiveness of ascorbyl palmitate and/or co-spray-drying of a heme iron ingredient

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TITLE AND ABSTRACT IN SPANISH/ TÍTULO Y RESUMEN EN CASTELLANO:

"Estabilidad oxidativa de galletas fortificadas con hierro hémico: efectividad del palmitato de ascorbilo y/o co-atomización del hierro hémico"

La deficiencia de hierro es el problema nutricional más extendido en el mundo, siendo los grupos de población que presentan la mayor incidencia los niños y las mujeres en edad de reproducción. La fortificación de alimentos con hierro es una estrategia empleada comúnmente para prevenir y/o superar la deficiencia de hierro. En este aspecto, la selección de la apropiada combinación entre el compuesto de hierro y el alimento fortalecido es crucial para que un programa de fortificación sea exitoso.

Así mismo, es importante tener en cuenta que cualquier forma de hierro es un potente pro-oxidante y por ello su adición provocará flavores desagradables y la reducción de la vida útil del producto alimenticio fortalecido. En este estudio un ingrediente hémico altamente bio-disponible ha sido seleccionado como fortificante para fortalecer una crema de chocolate empleada en el relleno de galletas tipo sándwich.

Para evitar el efecto catalítico del hierro hémico sobre la oxidación lipídica dos estrategias distintas han sido estudiadas: (I) la adición de palmitato de ascorbilo y (II) la co-atomización del hierro hémico con caseinato de calcio. A lo largo de un año de almacenamiento a temperatura ambiente y en la oscuridad ha sido monitorizado el desarrollo de la oxidació.

La adición de palmitato de ascorbilo protegió de la oxidación a las galletas fortalecidas y previno la pérdida de tocoferoles y tocotrienoles. En general, ambas estrategias, adición de palmitato de ascrobilo y la co-atomización del hierro hemico, mostraron su efectividad en retrasar el desarrollo de la oxidación. Así mismo, la combinación de ambas estrategias condujo a que las galletas fortalecidas fuesen aceptadas por los consumidores a lo largo de un año de almacenamiento.

Oxidative stability of a heme iron-fortified bakery product: effectiveness of ascorbyl palmitate and co-spray-drying of heme iron with calcium caseinate

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Running title: Oxidative stability of a heme iron-fortified bakery product

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Summary

Iron deficiency is the most prevalent nutritional disorder worldwide, particularly affecting women of childbearing age and children. Iron fortification of food is a common strategy employed to prevent and/or overcome iron deficiency; however, the selection of a suitable combination of iron compound and food vehicle is crucial for a successful fortification programme. Any iron form is a potent pro-oxidant and thus its addition will cause off-flavours and reduce the product's shelf-life. In this study, a highly bioavailable heme iron ingredient was selected to fortify a chocolate cream used to fill sandwich-type cookies. Two different strategies were assessed for avoiding the catalytic effect of heme iron on lipid oxidation: addition of ascorbyl palmitate and co-spray-drying of the heme iron with calcium caseinate. Oxidation development was monitored in the cookies over a one year period of storage at room temperature in the dark. The overall acceptability of the iron-fortified cookies was also assessed. The addition of ascorbyl palmitate protected from oxidation and from the loss of tocopherols and tocotrienols during cookies preparation In general, ascorbyl palmitate, either alone or in combination with the co-spray-dried heme iron, was effective in preventing oxidation during storage. Moreover, the combination of these two strategies also resulted in cookies that were acceptable to consumers after 1 year of storage.

Keywords: Heme iron / Food fortification /Co-spray-drying/ Antioxidants / Oxidative stability

List of abbreviations used: AP, ascorbyl palmitate; AUC, area under the curve; CAS, calcium caseinate; CAS 1:1, co-spray-dried heme iron ingredient with calcium caseinate in 1:1 ratio; CHP eq., cumene hydroperoxide equivalents; CIE, Commission International de L'Eclairage; FA, fatty acid; FAME, fatty acid methyl esters; FID, flame ionisation detector; FOX, ferrous oxidation-xylenol orange; ICP-AES, inductively coupled plasma atomic emission spectroscopy; LHP, lipid hydroperoxide; MAXLHP, maximum lipid hydroperoxide; MUFA, monounsaturated fatty acids; *p*-AnV, *p*-Anisidine value; PUFA, polyunsaturated fatty acids; PV, peroxide value; RDA, recommended daily allowances; SFA, saturated fatty acids; TMAX, time to reach the maximum lipid hydroperoxide value; WAI, water absorption index; WSI, water solubility index.

1. Introduction

Iron deficiency is the most prevalent health disorder in the world (WHO, 2001), and it is estimated that at least 20% of the world's population is affected by this micronutrient deficiency. Moreover, iron deficiency is prevalent in both developed and developing countries (Benoist, McLean, Cogswell, Egli & Wojdyla, 2008).

Food fortification is generally recognised as a good strategy to counteract this nutritional deficiency; there is a large body of evidence indicating its efficacy, and it is regarded as the most cost-effective long-term strategy (Baltussen, Knai & Sharan, 2004; Zimmermann & Hurrell, 2007). For an iron fortification programme to be effective, it is essential that the iron form selected is highly bioavailable. At the same time, organoleptic modifications of the food vehicle should be minimal. This supposes a challenge for the food industry, as many iron forms are potent pro-oxidants (Lotfi, Mannar, Merx, Naber-van & Heuvel, 1996). In general, the more bioavailable non-heme iron compounds are also the most reactive ones (Allen, De Benoist, Dary & Hurrel, 2006).

There are two main forms of dietary iron, namely heme and non-heme iron. Heme iron is known to be the most bioavailable iron form, since its absorption process is different from that of non-heme iron and it is not affected by food ligands (WHO/FAO, 2004).

Selection of the food vehicle should take the target population's diet into account (Allen et al., 2006). If the entire population presents iron deficiency, staple foods such as wheat flour or rice would be suitable food vehicles for iron fortification. However, such mass fortification may be insufficient for some women of childbearing age and children (Lotfi et al., 1996). Those population groups have increased iron needs, mainly due to growth or menstruation, but their total food intake is lower than other population groups and hence the intake of iron-fortified staple foods may not meet their iron requirements. Thus, targeted fortification seems an optimum approach for these population groups.

In previous studies (Aleman et al., 2010; Alemán et al., 2014a; Alemán et al., 2014b), different strategies were tested to avoid the oxidation of a palm oil fortified with heme iron as a model for bakery product fortification. The strategies studied were: the addition of antioxidants and the encapsulation of heme iron by co-spray-drying it with different coatings. The effectiveness of several antioxidants at different concentrations was assessed in that model and ascorbyl palmitate (AP) at 400 mg/kg palm oil was found to be the most effective (Aleman et al., 2010; Alemán et al., 2014a). Subsequently, the same model was used to assess the additional advantages of co-spray-drying heme iron with calcium caseinate either at a 2:1 or a 1:1 ratio (heme iron concentrate:caseinate, w/w). The authors reported that the combination of co-spray-dried heme iron with calcium caseinate and AP was the most effective strategy to prevent oxidation during storage (Alemán et al., 2014b).

The aim of the present study is to assess the oxidative stability and overall acceptability of sandwich-type cookies filled with a chocolate cream fortified with heme iron. Not only would

this food product be easy to introduce into children's diets but the chocolate could help disguise the dark colour of the heme iron ingredients. In order to avoid oxidation, the previous strategies that proved effective in a model for iron fortification of bakery products were combined: the addition of AP at 400 mg/kg of palm oil in the cookie filling, and the co-spray-drying of heme iron with calcium caseinate at a 1:1 ratio (CAS 1:1). The evolution of oxidation and the overall acceptability of these cookies were monitored over a one year period of storage at room temperature in the dark.

2. Material and methods

2.1. Materials

Refined palm oil was donated by Lípidos Santiga S.A. (Santa Perpètua de Mogoda, Spain), cocoa powder (10% fat) by Nutrexpa (Barcelona, Spain), lecithin and calcium caseinate by Cargill (Martorell, Spain) and food grade AP by Induxtra (Banyoles, Spain). Dry-milk (1.5% fat) and vanilla extract were a gift from BDN S.L. (Barcelona, Spain). Commercial Marie biscuits and icing sugar were purchased in a local supermarket. The aluminium-coated plastic ziplock bags used for packing the sandwich-type cookies were of very low permeability to water (<0.01g of water/m^2/24 h with 90% of relative humidity at 33.7ºC) and were purchased from Flexico (Barcelona, Spain).

Fatty acid methyl ester (FAME) standards (purity >98%), cumene hydroperoxide (80%) and hexanal (98%) were purchased from Sigma-Aldrich (Madrid, Spain), dimethyl sulphoxide (DMSO) was purchased from Scharlab S.L. (Barcelona, Spain) and tocopherol standards (>95%) were purchased from Calbiochem (San Diego, CA).

All chemicals used were of ACS grade, with the exception of the solvents used in the ferrous oxidation-xylenol orange (FOX) method, in the tocopherol and tocotrienol determination and in the heme iron determination, which were of HPLC grade. The iron standard solution was purchased from High-Purity standards (Charleston, SC) and was traceable to the Standard Reference Materials of NIST.

2.2. Manufacture and characterisation of heme iron ingredients

The heme iron ingredient used as control was the commercial product AproFER 1000™ obtained from APC Europe S.A. (Granollers, Spain). The heme iron co-spray-dried ingredient, namely CAS 1:1 (heme iron concentrate:calcium caseinate, 1:1, w/w) was produced by APC Europe (as described in the Supplementary data). Colour evolution of heme iron ingredients during storage was measured using a Konica Minolta Chroma-meter (model CR-410; Konica Minolta Sensing Inc., Osaka, Japan) based on the CIE L*a*b* colour space. Water absorption (WAI) and water solubility (WSI) indexes were determined in the heme iron ingredients used in this study as described elsewhere (Zhang et al., 2012). The methods used for characterisation of the heme ingredients are provided in the Supplementary data.

2.3. Manufacture of sandwich-type cookies

Different chocolate creams with and without heme iron fortification were used to fill sandwich-type cookies according to the treatments described in Table 1. A manufacturer of this kind of cookies kindly provided a common formulation for chocolate creams, consisting of 54.3% icing sugar, 23.6% palm oil, 16.3% cocoa powder (10% fat), 5.6% dry milk (1.5% fat), 0.1% vanilla extract and 0.1% lecithin.

The total iron content of the control heme iron ingredient (AproFER 1000™) was 1.74% whereas the content of the co-spray-dried heme iron ingredient (CAS 1:1) was 0.88%. In order to maintain the quantity of iron in samples constant (0.31 mg Fe/g cream or 0.11 mg Fe/g cookie), different amounts of both heme iron ingredients were added to the chocolate cream fillings. These creams were prepared in our laboratory and then used to sandwich two commercial Marie biscuits together. The complete procedure for preparing e the heme iron-fortified sandwich-type cookies is given in the Supplementary data.

The resulting sandwich-type cookies were stored at room temperature in the dark for 0, 90, 180 and 360 days. At the end of each storage period, the corresponding cookies were vacuum packed and stored at -20°C until performing analyses.

Our aim was for each chocolate-filled biscuit weighing 20 g with 7 g of chocolate filling to provide more than 15% of the Recommended Daily Allowance (RDA) of iron (i.e. 14 mg Fe/day) (European Community Commission, 2008). When the samples were analysed, the total iron found was on average 0.12 mg Fe/g cookie. Therefore, one of these cookies would provide 17% of the RDA of iron.

2.4. Lipid extraction from cookies

The lipid fraction of cookies was extracted in order to characterise its tocopherol and tocotrienol content and to assess oxidation status at the different storage periods. The extraction procedure employed was adapted from Rose & Oklander (1965). Further details of the lipid extraction procedure are given in the Supplementary data.

2.5. Palm oil characterisation and tocopherol and tocotrienol content of cookies prior to storage

FAME were prepared from 100 mg of palm oil as described elsewhere (Guardiola, Codony, Rafecas, Boatella & López, 1994) and the chromatographic conditions were those described by Aleman et al. (2010). In addition, the peroxide value (PV) (European Communities Commission, 1991), lipid hydroperoxide (LHP) content (by non-induced FOX method) (Navas et al., 2004), *p*-AnV (AOCS, 1998) and hexanal content were assessed in the palm oil used to prepare the chocolate filling. Five replicates were performed for all determinations.

Tocopherol and tocotrienol content was determined in 200 mg of fresh palm oil and in the same amount of lipids extracted from cookies prior to storage by normal-phase HPLC as described by Aleman et al. (2010). These determinations were conducted in triplicate.

2.6. Heme and total iron content

The heme iron content of the heme iron ingredients and cookies prior to storage was determined according to the colourimetric method described by Hornsey (1956). The total iron content of these samples was determined using a PerkinElmer ICP-AES (Optima 3200 RL model) following mineralisation. This was achieved as follows: first, 250 mg of either heme-iron ingredient or ground cookie was weighed into quartz digestion tubes. Second, 25 mL of HNO_3 was added and tubes were placed in a digestion block and left at 60°C overnight. Thereafter, the temperature was raised to 120°C and left stand for 1 hour. Then, nitric acid was evaporated at 160°C until approximately 5 mL was left. Next, 5 mL of $HClO_4$ was poured into the quartz tube and the temperature was raised to 180°C. After one hour, $HClO_4$ was evaporated at 210°C until 1 mL was left. Finally, the solution was transferred to a 50 mL volumetric flask and filled up with HNO_3 1%. Five replicates were performed for heme and total iron determinations.

2.7. Susceptibility to oxidation

Prior to storage of cookies, the induced version of the FOX method was performed to assess sample susceptibility to oxidation (Bou, Codony, Tres, Decker & Guardiola, 2008; Grau, Codony, Rafecas, Barroeta & Guardiola, 2000). The reaction conditions were those described in Navas et al. (2004). This assay measures the formation of LHP in the fat extracted from cookies during an incubation period that was set at 191 hours. By using a standard curve prepared with cumene hydroperoxide (CHP), the content of the LHP formed during incubation was expressed as millimoles of CHP eq/kg in each sample. The following parameters were determined as described elsewhere (Tres, Nuchi, Bou, Codony & Guardiola, 2009) to better describe the differences observed in the susceptibility to oxidation of the samples: Initial LHP (the initial LHP value was measured after 30 minutes of incubation), MAXLHP (the maximum LHP value), TMAX (the time until MAXLHP was achieved), Final LHP (the final LHP value measured after 191 hours of incubation) and AUC (area under the curve of LHP formation). Susceptibility to oxidation was assessed in 5 different lipid extracts.

2.8. Evolution of oxidation during storage

The formation of primary oxidation compounds in cookies stored for different periods was assessed in the fat extracted using the PV (European Communities Commission, 1991) and by measuring the LHP content by means of the non-induced FOX method version (Navas et al., 2004). The formation of secondary oxidation compounds in samples was assessed by the *p*-anisidine value (*p*-AnV) (AOCS, 1998) and the hexanal content determined by static headspace analysis. This method was set up following a similar approach to that described in Alemán et al. (2014a).

The extraction times and temperatures (30 minutes at 70°C) were the same as in previous studies (Alemán et al. 2014a). However, to identify the best extraction conditions for the determination of hexanal content, we assessed different sample amounts (0.5-1.0-1.5 g) and

the addition of water (0 – 0.5 – 1 mL) using a Combi PAL autosampler (CTC Analytics, Switzerland). The final extraction conditions selected were as follows: 500 mg of ground cookie was weighed into vials to which 1 mL of water was then added. The vials were sealed with screw caps. Extraction of the volatile compounds was carried out over 30 minutes at 70°C. Following this, one millilitre from the vial headspace was injected into an Agilent 4890D model (Waldbronn, Germany) gas chromatograph equipped with a flame ionisation detector and a split-splitless injector. The injector was set for 2 minutes in splitless mode; the split ratio was 1:10.

Chromatographic separation of volatile compounds was performed in a fused-silica capillary column (30 m × 0.20 mm i.d.) coated with 0.2 μm of a stationary phase of 5% diphenyl-plus95% dimethyl-polysiloxane (Equity TM-5 from Supelco, USA). Helium, at 20 psi, was used as a carrier gas. The injector and detector temperatures were 200°C and 325°C, respectively. The oven programme was as follows: 1 minute at a temperature of 40°C, which was then increased by 10°C/min to 300°C and then kept at this temperature for 5 minutes.

Hexanal was identified by comparing the retention time with a standard and was quantified using the external standard method. To prepare the standard curves, fresh ground cookies (control, control heme and CAS 1:1) were used as matrixes. 100 μL of solutions with different concentrations of hexanal in DMSO was added to the vials containing the ground cookies (concentration range, 1.5-200 μg hexanal/kg sample) and sealed with screw caps. To ensure that the samples and the vials of the standard curves had the same matrix, 100 μL of DMSO was added to all treatment samples.

This method displayed a good linearity (R^2=0.991-0.994), a good precision (RSD = 3.04%, 4 determinations within the same day in a sample containing 32 μg hexanal/kg) and a good recovery (99%). The recovery was assessed in 2 samples, which were each injected four times. Sample hexanal concentrations were 32 μg/kg and 16 μg/kg. The hexanal standard added was approximately one third of the initial concentration (final hexanal concentration 43 μg/kg and 22 μg/kg, respectively). Given the analyte content of samples (between not detected - 180 μg/kg), these values comply with AOAC recommendations for validation of methods (AOAC international, 1998). Furthermore, the limit of detection and quantification of the method were 0.86 and 1.12 μg/kg of sample, respectively. Both limits were calculated as 3 and 10 times the standard deviation of the base line noise, respectively.

PV, LHP content and *p*-AnV were determined in the lipid extracts from cookies, whereas the hexanal content was assessed in ground cookies. All the oxidation parameters were determined in quintuplicate.

2.9. Chocolate creams filling colours

The colour of the chocolate cream fillings was measured prior to storage using a Konica Minolta Chroma-meter (model CR-410; Konica Minolta Sensing Inc., Osaka, Japan) based on the CIE L*a*b* colour space. To this end, sandwich -type cookies were heated for 5 seconds in

a microwave. Then, biscuits were separated and the chocolate filling was carefully removed and transferred to a capsule.

The colour was determined from four different random surfaces of the chocolate creams. The instrument was set for illuminant D-65 and at a 2°- observer angle, and standardised using a standard white plate. In addition, the colour of the chocolate cream fillings was compared with commercial chocolate cream fillings and chocolate bars with different percentages of cocoa (33%, 72% and 85%).

2.10. Sensory analysis

Sensory analysis of the sandwich-type cookies was performed after 0, 180 and 360 days of storage. A panel of 32 volunteers assessed the overall acceptability of fortified cookies at each storage time using a 9-point hedonic scale, where 9 = excellent and 1 = very bad. Samples were served at room temperature and presented to consumers on coded white plastic plates. Each consumer tasted all the different cookies in each session. Spring water was provided for participants to rinse their palates between samples. Only volunteers who consumed chocolate bakery products at least 12 times per year were considered for participation. The selected panel included men and women aged between 18 and 63 years.

2.11. Statistical analyses

Pearson's correlation coefficients were used to study the relationships between PV, LHP content, *p*-AnV and hexanal content. One-way ANOVA was used to determine the effect of the different treatments on tocopherol and tocotrienol content and FOX -induced parameters. Multifactorial ANOVA was used to determine whether the studied factors had a significant effect on PV, LHP content, *p*-AnV and hexanal content of cookies. The main factors studied were the treatments applied (Table 1) and the storage time (0, 90, 180 and 360 days). In addition, a series of one-way ANOVAs was applied at each storage time to determine any significant effect of the treatments on PV, LHP content, *p*-AnV and hexanal content. In all cases, $P \leq 0.05$ was considered significant. When significant differences were produced by the main factors, the least-squares means or means were separated using Scheffé's test ($\alpha=0.05$).

3. Results and discussion

3.1. Characterisation of palm oil and heme iron ingredients

The fatty acid (FA) composition of the palm oil used to prepare the chocolate cream filling was typical of this kind of oil (Tres, van der Veer, Alewijn, Kok & van Ruth, 2011): 49.95% SFA, 39.74% MUFA, 10.51% PUFA and 0.08% *trans* FA (the complete FA composition is given in the Supplementary data). Palm oil is characterised by its high content of both tocopherol and tocotrienol, especially alpha- and gamma-tocotrienol (Sambanthamurthi, Sundram & Tan, 2000; Tres et al., 2011). In this case, the total tocopherol and tocotrienol content was 673.9 mg/kg (total tocopherols: 218.6 mg/kg, total tocotrienols: 455.3 mg/kg) (Table 2).

Regarding oxidation status, the fresh palm oil had a PV and LHP content of 0.06±0.0001 meq O_2/kg oil and 0.17±0.005 mmol CHP eq/kg oil, respectively (n=5). Hexanal was not detected whereas the *p*-AnV was 2.30±0.36 (n=5).

The heme iron ingredients could be described as dark. Nevertheless, we found differences between the colour of the co-spray-dried heme iron and the control heme iron (see Supplementary data). In addition, both water absorption and solubility indexes of the co-spray-dried heme iron were higher than those of the control heme iron (see Supplementary data).

3.2. Tocopherol and tocotrienol content of fat extracted from cookies prior to storage

Table 2 shows the tocopherol and tocotrienol content of the lipids extracted from the different cookies prior to storage. The tocopherol and tocotrienol content of control cookies with heme iron was much lower than that of the palm oil used to prepare the chocolate fillings and than that of the control cookies without heme iron. Thus, heme iron addition induces a higher tocopherol and tocotrienol degradation during preparation of the chocolate fillings.

When comparing cookies, those fortified with heme iron without added AP (control heme iron and CAS 1:1) presented a lower tocopherol and tocotrienol content than the control cookies without heme iron (48% and 53%, respectively) and also than that of the samples with AP (AP and CAS 1:1 + AP cookies). Indeed, the tocopherol and tocotrienol content of cookies with AP did not differ from control cookies (without added heme iron). Therefore, the addition of AP protected against the degradation of tocopherols and tocotrienols induced by the addition of heme iron during preparation of the chocolate fillings, which involves heating to 40°C for approximately 40 minutes. This result is in accordance with previous results in palm oil samples fortified with heme iron, where the addition of AP reduced tocopherol and tocotrienol loss during heating treatments (Alemán et al., 2014b). However, it should be noted that samples from this previous study were used as a model for bakery products and were thus subjected to higher temperatures (220°C for 10 minutes) to mimic typical baking conditions.

3.3. Heme and total iron content

The control heme iron ingredient (AproFER 1000™) contained 1.74% of iron, of which 97.3% was heme iron. The heme iron ingredient encapsulated by co-spray-drying (CAS 1:1) contained 0.88% of iron, of which 91.8% was heme iron.

The heme and total iron content of samples was also assessed in cookies prior to storage. On average, the fortified heme iron cookies contained 0.10 ± <0.01 mg heme iron/g of cookie and 0.12 ±<0.01 mg total iron/g cookie. Therefore, two cookies, which could be considered a normal serving size, would provide on average 4.63±0.16 mg of total iron/serving (33% of the RDA of iron) (European Community Commission, 2008), with heme iron as the main source (84-88%). Given that the total iron content of the non-fortified cookie was 0.03 ±<0.01 mg

iron/g cookie, negligible amounts of iron were liberated from the heme moiety of the heme iron ingredient during the preparation of the chocolate cream filling.

3.4. Susceptibility to oxidation of fortified cookies

Susceptibility to oxidation of the lipids extracted from the different cookies was determined by means of the induced FOX method prior to storage. Table 3 shows the different parameters calculated according to Tres et al. (2009) to better describe the time course of LHP evolution during incubation.

The initial LHP value was considered to measure the current amount of LHP present in the samples. The initial LHP content of cookies with added AP (AP and CAS 1:1 + AP treatments) was significantly lower than that of the samples without added antioxidants (control, control heme iron and CAS 1:1). The lowest initial LHP value was observed in the AP treatment, thus suggesting a protective effect of AP during preparation of the chocolate creams fortified with heme iron. This finding is in agreement with previous studies carried out on palm oil fortified with heme iron (Aleman et al., 2010; Alemán et al., 2014a, Alemán et al., 2014b). As discussed earlier, these results can be also related to the fact that AP effectively protected tocopherols and tocotrienols during sample preparation.

Similar conclusions can be drawn when assessing the other parameters (MAXLHP, TMAX and Final LHP, Table 3). The lowest MAXLHP corresponded to cookies with added AP (AP and CAS 1:1 + AP). Moreover, the AP treatment presented the highest TMAX (time to reach the MAXLHP), 29.5 hours, whereas the rest of the samples reached the MAXLHP in 0.5 hours. The Final LHP content of samples also followed a similar trend, with lowest values observed for the AP treatment.

Lastly, the AUC, which has been considered a good marker for susceptibility to oxidation in different matrixes (Alemán et al., 2014a; Tres et al., 2009), showed the lowest values for AP samples, which were even lower than those of control cookies (without added heme iron) or CAS 1:1 + AP samples. Thus, it can be concluded that the addition of AP to cookies reduced their susceptibility to oxidation.

3.5. Oxidation of fortified cookies during storage

The development of primary oxidation compounds during storage was monitored by means of PV and LHP content. The evolution of secondary oxidation of samples was assessed by means of hexanal content and the *p*-AnV (Table 4).

3.5.1. Primary oxidation: PV and LHP content

Comparing the different treatments, the PV of the control heme iron cookies was higher than that of the control cookie without heme iron, whereas the PV of the rest of the samples did not differ from either control cookies (Table 4).

With respect to storage time, both the PV and LHP content of cookies increased with storage time and reached maximum values at 360 days of storage (Table 4). Prior to storage (Time 0), cookies with added AP (AP and CAS 1:1 + AP) presented lower values than the control with heme iron. Indeed, they were similar to or lower than those for the control without added heme iron. At the end of storage, the most effective strategy for delaying primary oxidation was the AP treatment (Table 4). In between, cookies with CAS 1:1 and CAS 1:1 + AP displayed no differences in primary oxidation values and showed lower values than control heme samples.

In spite of some differences, the methods selected to determine primary oxidation compounds (PV and LHP content measured by means of the non-induced FOX method) provided similar results and thus were highly correlated ($r=0.978$; $p<0.0001$, $n=100$).

3.5.2. Secondary oxidation: hexanal content and *p*-AnV

There were no differences in hexanal content of cookies when considering the different treatments (Table 4). Over the course of storage, a lag phase (no differences over time) was observed in hexanal evolution till 180 days. However, hexanal content increased markedly after 360 days of storage. At this latter storage time, and only considering the heme iron-fortified cookies, those containing AP alone or in combination with CAS 1:1 showed the lowest hexanal content. However, control samples without heme iron showed by far the lowest hexanal content.

When analysing the treatment factor, we found that CAS 1:1 + AP cookies presented the lowest *p*-AnV value; however, this value was the same as that for the control heme iron samples. No differences were found between the other treatments (Table 4). When considering the storage time factor, the *p*-AnV for samples increased slightly till 180 days but thereafter decreased at 360 days (Table 4).

Contrary to results for the previous oxidation parameters, the *p*-AnV of control samples (with no added heme iron) was not lower than that of the control heme (with added heme iron). In this regard, it is important to note that the *p*-AnV was not correlated with any other oxidation parameter, whereas hexanal content, PV and LHP content were all correlated (hexanal vs. PV $r=0.699$; $p<0.0001$, $n=100$ and hexanal vs. LHP content $r=0.736$; $p<0.0001$, $n=100$ and PV vs. LHP content $r=0.978$; $p<0.0001$, $n=100$). These findings indicate that the *p*-AnV was not a suitable oxidation parameter for monitoring the evolution of secondary oxidation in this study. The most probable explanation for this is that (i) some of the secondary oxidation compounds measured by the *p*-AnV are volatile and thus may have been lost during the lipid extraction procedure (e.g. when vacuum was applied in rotary evaporation and in the desiccator) and (ii) it is also known that secondary oxidation products have the capacity to form adducts with proteins (Zamora & Hidalgo, 2011), the content of which was high in the matrix studied here.

In general, PV, LHP and hexanal content of heme iron-fortified cookies during storage was lower for samples with added AP alone or in combination with CAS 1:1 than for control heme

iron samples. The effectiveness of AP has previously been described in palm oil fortified with heme iron, used as model for iron fortification (Alemán et al., 2010; Alemán et al., 2014a). Therefore, the addition of AP either alone or in combination with CAS 1:1 seems to be suitable for the manufacture of sandwich-type cookies fortified with heme iron. *p*-AnV results indicated that this oxidation parameter was not a satisfactory method for assessing secondary oxidation in this case.

3.6. Colour of chocolate cream fillings prior to storage

Figure 1 shows the different CIE L*a*b* colour space parameters for experimental chocolate cream fillings, commercial chocolate cream fillings and chocolate bars with different percentages of cocoa. The lightness (L*) of chocolate creams fortified with heme iron did not differ from that of the control chocolate cream without heme iron (Figure 1.A). Only AP cream presented less lightness than the control cream with no added heme iron. All the chocolate fillings prepared for this study were less bright than the fillings of commercial cookies. However, the lightness of the experimental cookies was similar to that of chocolate bars with a high percentage of cocoa (72% and 85%).

In contrast to the L* values, a* values varied widely between the experimental cookie fillings. The control chocolate filling without added heme iron displayed higher redness (a*) than the other experimental fillings (Figure 1.B), meaning that the control filling tended towards a redder colour than the samples fortified with heme iron. Samples with AP (AP and CAS 1:1 + AP) presented higher a* values than the rest, thus indicating an AP effect on a* values. Similarly to L* values, commercial chocolate fillings had higher a* values than the experimental ones. Moreover, chocolate creams fortified with heme iron presented a* values similar to those of chocolate bars with a high cocoa content (72% and 85%).

The CAS 1:1 + AP chocolate filling displayed the lowest b* values of all samples (Figure 1.C). The b* value is related to a more yellowish colour when the values are positive and to a more bluish colour when the values are negative. Thus, it seems that the addition of AP had a slight effect on the colour of the chocolate cream. The differences between b* values of samples fortified with heme iron were minimal whereas the experimental chocolate cream without heme iron presented higher b* values, which were similar to those of the chocolate bars with a high cocoa content (85%). As for L* and a* values, those for the experimental chocolate creams presented b* values were quite different from those found in commercial cookies.

Overall, the colour of the chocolate cream fillings fortified with heme iron differed from the control non-fortified chocolate cream and also from the commercial chocolate creams. However, L* and a* values of heme iron-fortified chocolate creams were similar to those of chocolate bars with a high percentage of cocoa (72%-85%). The addition of AP had a slight effect on the colour of the cookie filling, which is difficult to explain as the amount of AP added was very low and the colour was measured prior to storage. In spite of these differences regarding the colour of the chocolate fillings of experimental and commercial samples, consumers did not dislike the overall appearance of the sandwich-type cookies fortified with

heme iron. The existence of various chocolate products (e.g. chocolate bars) with different colours may explain this response.

3.7. Sensory analysis

Consumer acceptability scores obtained for sandwich-type cookies at each storage time (0, 180 and 360 days) are presented in Table 5. At the initial time, the only cookies that were not accepted by consumers were those corresponding to the CAS 1:1 + AP treatment. This result is difficult to explain as consumers accepted those cookies with either AP or CAS 1:1 (acceptability scores > 5.0). However, it should be noted that cookies with CAS 1:1 + AP were accepted by consumers after 180 and 360 days of storage (acceptability scores > 5.0).

Moreover, after 180 and 360 days of storage, the acceptability of samples with CAS 1:1, with or without AP, did not differ from that of the control cookie without added heme iron. Conversely, consumers did not accept control heme iron cookies at these storage times. In addition, the acceptability of these cookies decreased with the storage time and was significantly lower than that of the control sandwich-type cookies without heme iron.

It is worth mentioning that consumers not only accepted control cookies without heme iron and cookies with CAS 1:1 at each storage time but also that the overall acceptability of these samples was found to be similar. These results seemed to be related to those regarding oxidation, but are not in complete agreement as the decreased overall acceptability of cookies with AP at the end of the storage period was not consistent with its higher oxidative stability. Therefore, the combination of AP and CAS 1:1 heme iron ingredient seems to be the best strategy as by the end of the storage time, these biscuits were accepted by consumers and showed lower oxidation values than control heme iron cookies.

4. Conclusions

The addition of AP to heme iron-fortified chocolate creams used to fill sandwich-type cookies prevented oxidation and the loss of tocopherols and tocotrienols during preparation of the chocolate creams.

During storage of heme iron-fortified sandwich-type cookies at room temperature, the formation of primary oxidation compounds and hexanal was minimised by means of the addition of AP, either alone or in combination with encapsulation of the heme iron ingredient by co-spray-drying.

The colour of freshly produced chocolate creams fortified with heme iron ingredients differed from that of commercial cookies creams. However, consumers accepted the experimental cookies probably because the colour of the heme iron-fortified chocolate fillings was similar to that of chocolate bars with a high percentage of cocoa (72%-85%).

After 360 days of storage, the heme iron-fortified cookies with CAS 1:1 alone or in combination with AP were accepted by consumers and, more importantly, their overall

acceptability scores did not differ from those obtained for control cookies without the addition of heme iron. Therefore, a combination of added AP and co-spray-drying of heme iron with caseinate (CAS 1:1 + AP) seems to be the most suitable strategy to achieve oxidative stability and consumer acceptance of sandwich-type cookies fortified with heme iron.

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7. Supplementary data

Producing heme iron ingredients: as described by González-Rosendo et al. (2010), the heme iron ingredient AproFER 1000™ used as control is a dark powder obtained from the blood of healthy pigs. After separating the blood cell fraction by centrifugation, a pressure pump was used to release the haemoglobin contained in the red blood cells. Then, the haemoglobin was enzymatically hydrolysed using a proteolytic enzyme (Alcalase®, Novo-Nordisk, Denmark) under controlled pH and temperature conditions. Following this, the heme group was concentrated by ultrafiltration and pH was adjusted to 8 before spray-drying.

To obtain the co-spray-dried ingredient with calcium caseinate at a ratio of 1:1 (w/w) (CAS 1:1), the heme iron concentrate obtained after ultrafiltration in the AproFER 1000™ ingredient production process was used as base. First, solid content was determined gravimetrically. Once the solid content was known, the same amount of calcium caseinate was added to the concentrate and mixed in a pilot plant mixer. The pH was adjusted to 8 before and after the addition of calcium caseinate. Mixing was performed at 40°C in order to facilitate dispersion of the caseinate in the liquid concentrate. Finally, all ingredients were spray-dried in an Anhydro (Copenhagen, DK) atomiser.

Physical properties of heme iron ingredients: Colour measurement: CIE (Commission International de L'Eclairage) lightness (L*), redness (a*), and yellowness (b*) values were determined from four different random surfaces of the heme iron ingredients. The instrument was set for illuminant D-65 and at a 2° observer angle, and standardised using a standard white plate.

In general, the heme iron ingredients could be described as dark. However, the co-spray-dried heme iron ingredient presented slightly higher L* values, meaning that it was whiter than the control heme iron ingredient (AproFER 1000™) (Table A). In all cases, the co-spray-dried heme iron ingredient displayed a more reddish and yellowish colour than the control heme as a result of higher a* and b* values. The colour of the heme iron ingredients was not stable over the course of storage at room temperature in the dark; however, the small changes observed did not follow a clear pattern over time.

Table A: Colour parameters (L*, a* and b*) determined in heme iron ingredients at the different storage times

Time	L*[1]	a*[1]	b*[1]
AproFER 1000™			
0 days	23.90 ± 0.05 a[2]	1.99 ± 0.02 a	-1.23 ± 0.03 a
90 days	24.04 ± 0.04 b	3.03 ± 0.14 d	-1.19 ± 0.03 a
180 days	23.85 ± 0.04 a	2.35 ± 0.07 b	-1.21 ± 0.05 a
360 days	24.00 ± 0.06 b	2.85 ± 0.08 c	-0.69 ± 0.04 b
CAS 1:1			

0 days	26.30 ± 0.04 a	4.04 ± 0.05 b	1.98 ± 0.04 b
90 days	26.41 ± 0.07 b	4.29 ± 0.04 d	1.72 ± 0.02 a
180 days	26.37 ± 0.02 ab	4.17 ± 0.04 c	1.79 ± 0.01 a
360 days	26.35 ± 0.02 ab	3.84 ± 0.03 a	2.11 ± 0.06 c

[1] L*: Lightness; a*: (+) red /(-) green; b* (+) yellow /(-) blue

[2] Mean ± SD; values in the same column corresponding to a specific ingredient and shown with different letters differed significantly with storage time ($P \leq 0.05$), n=4.

CAS 1:1: heme iron ingredient co-spray-dried with calcium caseinate at a 1:1 ratio

Water absorption and solubility indexes. The water absorption index (WAI) was calculated as follows: a screw-capped centrifuge tube (M, in g) was weighed, and approximately 0.5 g of heme iron ingredient (M_1, in g) was added. Next, water was added to disperse the ingredient with a powder:water ratio of 0.05:1 (w/w) at ambient temperature. The mixture was incubated in a water bath at 60°C for 30 min, immediately followed by cooling in an ice-water bath for 30 min. Then, the tube was centrifuged at 2800 *g* for 20 min. The resulting supernatant was removed and the centrifuge tube with sediment (M_3, in g) was weighed again. WAI was calculated in quintuplicate using the following formula: $WAI\ (g/g) = (M_3 - M)/M_1$

The water solubility index (WSI) of the different heme iron ingredients was determined as follows: heme iron ingredient (S_1, in g) was dispersed in a screw-capped centrifuge tube by adding water with a powder:water ratio of 0.02:1 (w/w) at ambient temperature. Then the mixture was incubated in a water bath at 80°C for 30 min, followed by centrifugation at 4000 *g* for 10 min. The supernatant was carefully collected in a pre-weighed evaporating dish (S_2, in g) and subjected to drying at 103 ± 2°C, and the evaporating dish with residue was weighed again (S_3, in g). WSI was calculated in quintuplicate using the following formula: $WSI(\%) = (S_3 - S_2)/S_1 \times 100$

The WAI of the control heme iron ingredient was 0.9 ± 0.1 g of hydrated sediment/g heme iron ingredient, whereas the WSI was 11.8% ± 1.9. The WAI of the co-spray-dried ingredient was 1.4 ± 0.1g of hydrated sediment/g heme iron ingredient, whereas the WSI was 16.2% ± 1.1. Therefore, the co-spray-dried ingredient (CAS 1:1) absorbed more water and dissolved more easily in water than the control heme iron ingredient. The heme iron ingredient is a peptidic material and these findings suggest that more lipophilic amino acids were exposed to the particle surface of the ingredient, whereas when the ingredient was co-spray-dried with calcium caseinate, the particle surface was probably more hydrophilic and hydratable.

Manufacture of sandwich-type cookies: We prepared the fortified heme iron chocolate filling in a food processor (Thermomix, Vorwerk, Germany). Briefly, we melted the palm oil in the food processor at 40°C, subsequently added lecithin and ascorbyl palmitate (when needed) followed by the icing sugar, and blended the ingredients until a homogenous white paste was obtained.

While the mixer was blending the palm oil, lecithin and icing sugar, the rest of the ingredients (cocoa powder, dry milk, vanilla extract and heme iron ingredient) were mixed in a container. When the colour of this powder mixture was homogeneous, it was carefully added to the

processor. Once all ingredients had been added, they were mixed for a further five minutes to ensure a homogeneous chocolate cream. Then, disposable pastry bags were filled with the cream and 7 g of cream was spread on top of a Marie biscuit. The cream was then carefully covered with another Marie biscuit and allowed to cool. When all biscuits of a given treatment were filled and cooled, the biscuits were packed in plastic aluminium-coated ziplock bags (3-10 cookies/bag), with very low permeability to water.

Lipid extraction from cookies: Vacuum packed frozen cookies were allowed to thaw at room temperature overnight. The next morning, cookies were ground twice in a Retsch blender Grindomix GM 200 (Düseldorf, Germany) for 10 seconds at 6000 rpm. The first time, the grinder was set to discontinuous mode (1 second grinding, 1 second stopped) whereas the second time, they were ground in continuous mode. For storage times 0 and 90 days, three cookies were used for each replicate. For times 180 and 360 days, two cookies were used for each replicate. In order to avoid any trace of heme pigment in the fat extracted, the method followed for lipid extraction was adapted from that described by Rose & Oklander (1965), which uses a mixture of isopropanol/chloroform (3:2, v/v) as solvent for lipid extraction.

Five grams of the ground cookies were used for analysis of volatile content. The remaining amount of ground cookies was transferred to a special glass extraction vessel (GS 50, Kinematica, Lucerne, Switzerland) and 300 mL of the extracting mixture (isopropanol/chloroform, 3:2 v/v) was added. The sample was then homogenised in a Polytron PT 3100 at 19000 rpm for 40 seconds. Samples were allowed to settle and the upper liquid was decanted and filtered through a Whatman no. 1 filter paper at room temperature under vacuum into a Kitasato flask. Then, the solvent was transferred to a round bottom flask. This process was performed a further two times, the second time with 200 mL of the solvent mixture and the last time with 100 mL, to a total extraction volume of 600 mL. Thereafter, the Kitasato flask was rinsed with 20 mL of the solvent mixture, which was also poured into the round bottom flask. The extract was then dried in a rotary evaporator at 40°C. At storage times of 180 and 360 days, in which less amount of the sample was extracted, the volume of solvent mixture used was proportionally reduced, using a total of 400 mL divided into 200 mL, 120 mL and 80 mL, respectively.

Once the extract was dried, it was re-dissolved in 80 mL (60 mL for 180 and 360 days of storage) of a methanol/chloroform (2:1, v/v) mixture. The following steps were similar to those reported in Folch et al. (1957). The extract was sub-divided into two test tubes. 10 mL of 1% salt solution was added to each tube, tubes were manually shaken and samples were centrifuged at 540 *g* for 20 min. After phase separation, the chloroform phase was filtered through sodium sulphate anhydrous to remove any trace of water and the filtrate was collected in a round bottom flask. Thereafter, samples were evaporated in a rotary evaporator at 35°C. Next, the flasks were left overnight under vacuum in a desiccator. Finally, the fat in the flasks was melted at 60°C, transferred to small vials, blown with nitrogen and stored at -20°C until analyses were performed. The fat extraction yield was equal at all storage times.

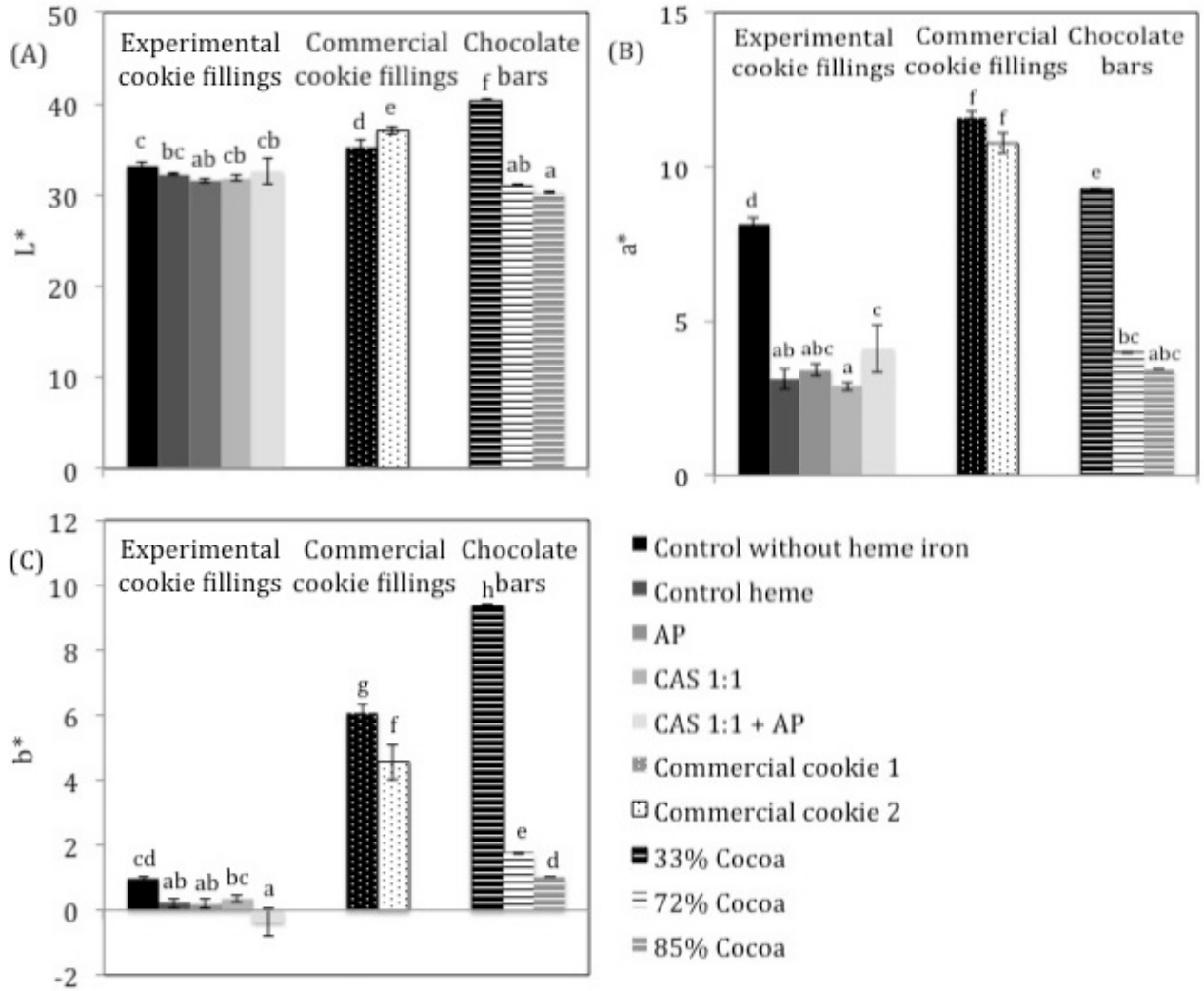
Fatty acid composition of the palm oil used to prepare the different treatments:

Fatty Acid[1]	Percentage[2]
10:0	0.02 ± <0.01
12:0	0.18 ± <0.01
14:0	1.04 ± <0.01
15:0	0.05 ± <0.01
16:0	43.27 ± 0.1
16:1n-9	0.03 ± <0.01
16:1n-7	0.16 ± <0.01
17:0	0.10 ± <0.01
17:1	0.02 ± <0.01
18:0	4.63 ± <0.01
18:1t	0.08 ±< 0.01
18:1n-9	38.53 ± 0.05
18:1n-7	0.77 ± 0.01
18:2n-6	10.21 ± 0.01
18:3n-3	0.17 ± <0.01
20:0	0.39 ± <0.01
20:1n-9	0.15 ± <0.01
20:2n-6	0.05 ± 0.02
20:3n-6	0.07 ± <0.01
24:0	0.07 ± <0.01
SFA	49.75 ± 0.06
MUFA	39.74 ± 0.06
PUFA	10.51 ± 0.02
PUFA n-6	10.34 ± 0.02
PUFA n-3	0.17 ± <0.01
Trans FA	0.08 ±< 0.01

[1] Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; *Trans* FA, *trans* fatty acids.

[2] Results are expressed as area normalisation of the different fatty acids in percentage. These values correspond to means ± standard deviation (n = 5)

Figure 1: Lightness (L*) (A), redness (a*) (B) and yellowness (b*) (C) values of the experimental chocolate cream fillings, commercial chocolate cream fillings and chocolate bars with different amounts of cocoa.



See Table 1 for treatment abbreviations

Table 1: Chocolate cream fillings for the preparation of sandwich-type cookies [1]

Treatments	Heme iron ingredient[2]	Ascorbyl palmitate [mg/kg palm oil]
Control	-	0
Control heme	AproFer 1000™	0
AP	AproFer 1000™	400
CAS 1:1	Heme iron concentrate : calcium caseinate at 1:1, w/w	0
CAS 1:1 + AP	Heme iron concentrate : calcium caseinate at 1:1, w/w	400

Abbreviations: AP, ascorbyl palmitate; CAS, calcium caseinate.

[1] All chocolate creams consisted of 54.3% icing sugar, 23.6% palm oil, 16.3% cocoa powder (10% fat), 5.6% dry milk (1.5% fat), 0.1% vanilla extract and 0.1% lecithin. Heme iron ingredients were added to provide 0.31 mg Fe/g cream (0.11 mg Fe/g cookie).

[2] Preparation of heme iron ingredients is described in the Supplementary data.

Table 2: Tocopherol and tocotrienol composition of the palm oil used to prepare sandwich-type cookies and that of the fat extracted from the different treatment cookies prior to storage[1]

	Palm oil	Control	Control heme	AP	CAS 1:1	CAS 1:1+AP
TOCOPHEROL [mg/kg]						
α	214.8±9c	169.6±18b	44.3±0a	157.7±6b	36.0±0.3a	168.2±5b
β	1.2±0.3	1.0±0.0	1.3±0.2	1.6±0.2	1.0±0.2	1.5±0.0
γ	2.6±0.3	2.1±0.4	2.9±0.3	2.4±0.5	1.8±0.7	2.0±0.5
δ	ND[2]	ND	ND	ND	ND	ND
TOTAL	218.6±9c	172.7±26b	48.5±0.5a	161.74±6b	38.9±0.6a	171.7±6b
TOCOTRIENOL [mg/kg]						
α	246.4±9c	156.1±16ab	185.6±15abc	173.0±43ab	149.9±8a	215.8±4bc
β	22.5±2	22.2±2	11.9±2	24.2±6	23.5±2	28.2±19
γ	176.5±9c	159.2±10bc	20.1±2.5a	154.0±5b	30.5±2a	153.9±3b
δ	9.9±1.2c	8.8±0.1bc	1.1±0.2a	8.8±0.4bc	0.8±0.2a	8.0±0.0b
TOTAL	455.3±20c	346.3±40b	218.7±20a	359.9±48b	204.6±4a	405.9±20bc
TOCOPHEROLS + TOCOTRIENOLS [mg/kg]						
TOTAL	673.9±29c	519.0±65b	267.2±20a	521.7±49b	243.5±3a	577.6±19bc

Results are expressed per weight of lipids.

[1] Please refer to Table 1 for the interpretation of sandwich-type cookies treatments. Values given in this Table correspond to means ± standard deviation (n = 3). Values in the same row with different letters present significant differences ($P \leq 0.05$).

[2] ND: not detected

Table 3: Susceptibility to oxidation of cookies (lipid extracts) measured by means of the FOX-induced method prior to storage [1]

Treatments	Initial LHP [mmol CHP eq/kg]	MAXLHP [mmol CHP eq/kg]	TMAX [h]	Final LHP [mmol CHP eq/kg]	AUC [(mmol CHP eq /kg) x h]
Control	0.94±0.04c	0.94±0.04c	0.50	0.14±0.02b	51.86±3.62b
Control heme	1.00±0.04cd	1.00±0.04cd	0.50	0.19±0.02c	63.80±4.47c
AP	0.03±0.01a	0.15±0.02a	29.50	0.06±<0.01a	18.23±0.67a
CAS 1:1	1.05±0.04d	1.05±0.04d	0.50	0.20±0.03c	62.66±5.08c
CAS 1:1 + AP	0.67±0.02b	0.67±0.02b	0.50	0.17±0.03bc	47.47±4.14b

[1] See Table 1 for treatment abbreviations. The parameters determined from the curve of formation of lipid hydroperoxides were: initial lipid hydroperoxide value (Initial LHP); Maximum lipid hydroperoxide value (MAXLHP); Time to reach the maximum lipid hydroperoxide value (TMAX); Final lipid hydroperoxide value (Final LHP) and the area under the curve (AUC). Results are expressed per weight of lipid extract.

Values given in this table correspond to means ± standard deviation (n=5). Means in the same column with different letters present significant differences ($P \leq 0.05$).

Table 4: Effect of treatment and storage time on primary (PV and LHP content) and secondary (hexanal content and *p*-AnV) oxidation parameters of sandwich-type cookies stored at room temperature in the dark for 360 days [1]

	PV [meq O_2/kg]	LHP content [mmol CHP eq/kg]	Hexanal content [µg/kg]	*p*-AnV
Treatment[1, 2]				
Control	8.32a	5.03	2.1	14.13b
Control heme	20.30b	11.05	48.9	13.57ab
AP	14.47ab	6.99	31.6	14.97b
CAS 1:1	18.33ab	10.00	44.5	16.10b
CAS 1:1 + AP	14.04ab	8.30	32.2	11.39a
SEM[3]	*3.33*	*1.97*	*16*	*0.85*
Storage time[2]				
0 days	1.14a	0.78a	1.3a	13.29a
90 days	12.32b	4.98b	7.0a	14.13ab
180 days	20.20c	11.46c	13.2a	15.94b
360 days	27.20d	15.86d	106.0b	12.78a
SEM[3]	*1.58*	*0.81*	*8.2*	*0.81*
Time 0[4]				
Control	1.05ab	0.94c	ND	13.75ab
Control heme	2.35b	1.00cd	1.20a	12.84ab
AP	0.28a	0.03a	TR	13.03ab
CAS 1:1	1.27ab	1.05d	1.53b	15.98b
CAS 1:1 + AP	0.73a	0.67b	1.78b	10.85a
SEM[5]	*0.42*	*0.08*	*0.1*	*1.24*
Time 90[4]				
Control	2.60a	1.35a	TR	11.64a
Control heme	19.76c	8.11c	14.84d	14.81ab
AP	14.52b	5.40b	6.43b	17.94b
CAS 1:1	19.77c	8.16c	10.75c	15.31ab
CAS 1:1 + AP	4.92a	1.88a	1.83a	10.93a
SEM[5]	*1.28*	*0.68*	*0.47*	*1.30*
Time 180[4]				
Control	9.02a	5.41a	1.14a	17.09bc
Control heme	26.23d	14.63c	20.65c	15.20b
AP	19.40b	9.49bc	13.69b	16.00b
CAS 1:1	23.52c	13.71c	15.06b	20.68c
CAS 1:1 + AP	22.82c	14.05c	15.37b	10.71a
SEM[5]	*0.78*	*0.40*	*1.02*	*1.22*
Time 360[4]				
Control	20.60a	12.42a	5.29a	14.04
Control heme	35.28d	20.39c	158.9c	11.43
AP	23.67b	13.03a	105.4b	12.90
CAS 1:1	28.76c	16.90b	150.5c	12.44
CAS 1:1 + AP	27.70c	16.58b	109.9b	13.07

SEM[5]	*0.74*	*0.44*	*8.5*	*0.94*

[1] See Table 1 for treatment abbreviations

[2] Values correspond to least-squares means obtained from multifactorial ANOVA (n=100)

[3] Standard error of the least-squares means

[4] Values correspond to means obtained from ANOVA (n=25)

[5] Standard error of the means

Other abbreviations: PV, peroxide value; LHP content, lipid hydroperoxide content measured by means of the non-induced FOX method; *p*-AnV, *p*-anisidine value. Means and least-squares means corresponding to a given factor with different letters present significant differences ($P \leq 0.05$).

PV, LHP content and *p*-AnV were determined in the lipid extracts. Hexanal content was determined in the ground cookies. Results are expressed per weight of lipid extract or whole cookie, accordingly.

Table 5: Panellists' overall acceptance of the different sandwich-type cookies after different storage times [1]

Treatments	0 days	180 days	360 days
Control	6.8 ± 2.0a	6.4 ± 1.8a	6.5 ± 2.1a
Control heme	5.3 ± 1.6ay	3.9 ± 2.3bx	3.3 ± 1.8bx
AP	5.4 ± 2.1ay	5.6 ± 2.2aby	3.8 ± 1.8bx
CAS 1:1	5.9 ± 1.7a	5.8 ± 2.3a	5.7 ± 1.7a
CAS 1:1 + AP	3.5 ± 2.4by	5.2 ± 2.0abx	5.4 ± 1.7ax

[1] See Table 1 for treatment abbreviations. Values given in this table correspond to means ± standard deviation (n=32). Means within the same column without a common letter (a-c) and means within the same row without a common letter (x-y) present significant differences ($P \leq 0.05$).

6. RESULTS AND DISCUSSION

6.1. Addition of antioxidants as strategy for preventing oxidation in a model for bakery products fortified with heme iron (studies 1, 2 and 4)

The first two studies of this thesis focused on the selection of the best antioxidant and its concentration for delaying the onset of oxidation of a palm oil matrix fortified with heme iron as model for bakery products. In the first study, three antioxidants, namely, ascorbyl palmitate (AP), citric acid (CA) and tocopherol extract (TE), having different mechanism of action were combined at high concentrations. In the second study, the two most effective antioxidants of the precedent work were selected for optimizing their concentrations. In the fourth study, the encapsulation of the heme iron, which is co-spray-dried together with a coating agent, is compared and combined with the addition of AP in a palm oil matrix fortified with heme iron.

In each study, the susceptibility towards oxidation of palm oil samples fortified with heme iron was assessed by means of the induced ferrous oxidation-xylenol orange (FOX) method. In order to better describe susceptibility of samples to oxidation, different parameters were calculated from the time course of lipid hydroperoxides (LHP) formation during its incubation at room temperature. These parameters were: initial LHP, which represents the actual LHP content of samples; MAXLHP, which is the maximum LHP concentration reached during the incubation time; TMAX, which is the time taken to reach MAXLHP; Final LHP, which is the concentration of LHP at the end of the incubation time; and AUC, which is the area under the curve of LHP formation. This last parameter has been reported as a good descriptor for measuring the susceptibility to oxidation in some food matrixes (Tres et al., 2009). Given the fact that AUC provided similar information to the other useful parameters, this will be the main parameter that will be considered in this section in order to simplify the overall discussion.

In the first study, the susceptibility to oxidation of TE samples was increased compared to control samples with no antioxidants added (Figure 6.1).

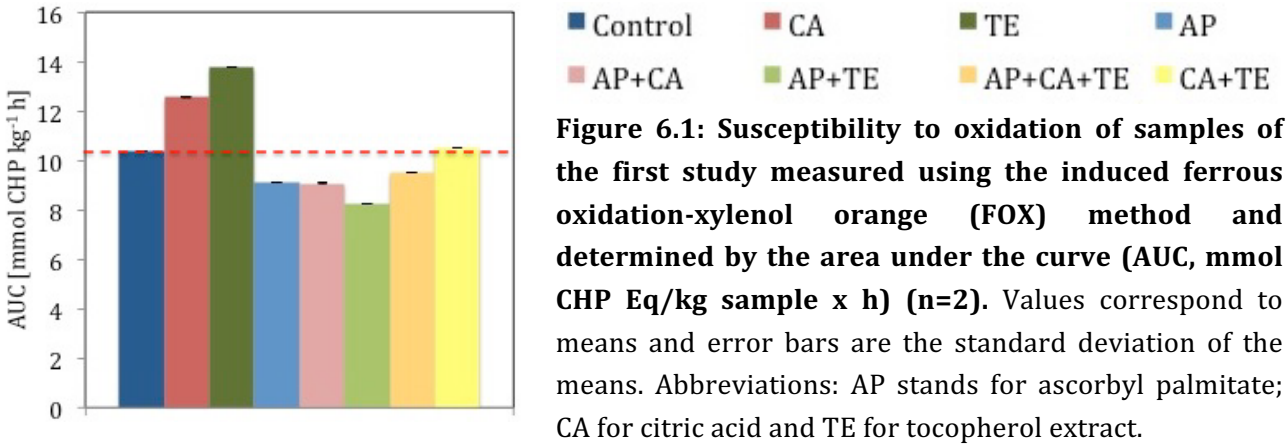


Figure 6.1: Susceptibility to oxidation of samples of the first study measured using the induced ferrous oxidation-xylenol orange (FOX) method and determined by the area under the curve (AUC, mmol CHP Eq/kg sample x h) (n=2). Values correspond to means and error bars are the standard deviation of the means. Abbreviations: AP stands for ascorbyl palmitate; CA for citric acid and TE for tocopherol extract.

In addition, regarding primary and secondary oxidation compounds, there were no differences between the control samples and those samples with added TE when comparing the formation of oxidation compounds (Figure 6.2). Therefore, in this case TE was not suitable

as antioxidant and, consequently, it was discarded for further studies. The high tocopherol and tocotrienol content present in the palm oil may explain the lack of effect of exogenous addition of tocopherols.

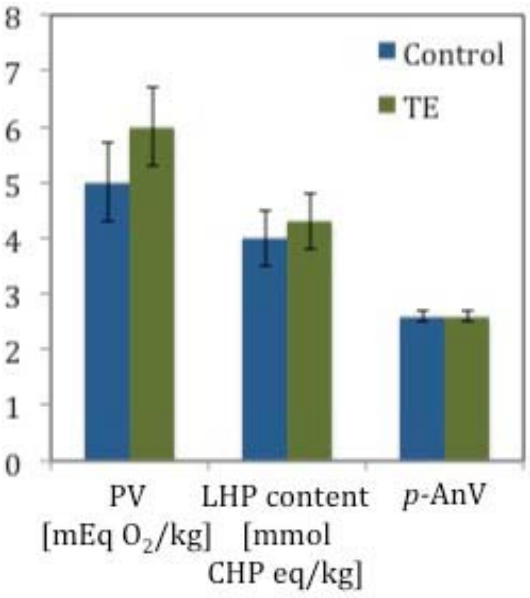


Figure 6.2: Effect of tocopherol extract (TE) on peroxide value (PV), lipid hydroperoxide (LHP) content and *p*-anisidine value (*p*-AnV). Values correspond to least-squares means (n=7) of the oxidation parameters at all storage times and were obtained from multifactorial ANOVA. Error bars are the standard error of the least-squares means.

With respect to the addition of AP, in the first study the samples with added AP (alone or in combination with other antioxidant) presented lower AUC values (Figure 6.1). Therefore, the addition of AP to samples and irrespective of its mode of addition, alone or in combination with CA or TE, caused the decrease of the susceptibility towards oxidation of palm oil fortified with heme iron. In addition, the lower initial LHP values of samples with AP compared to the control (0.01 vs. 0.14 mmol CHP eq/kg, respectively) suggested that the addition of AP could have a protective effect during the heating treatment to which samples were subjected to mimic baking conditions (220 °C for 10 min).

Figure 6.3 shows the evolution of the peroxide value (PV), lipid hydroperoxide (LHP) content and *p*-anisidine value (*p*-AnV) over storage time in samples form the first study. PV of the treatments with no AP added was higher than the rest. In addition, samples with AP showed a delayed oxidation onset (Figure 6.3.A and B).

The treatments that combined AP and CA showed lower PV after 80 days of storage than those containing AP without CA. Thus, these results are suggesting a synergism between both antioxidants. After 200 days of storage, the addition of AP (an oxygen scavenger) alone and its combination with CA (a chelating agent) inhibited the formation of primary oxidation compounds (as measured by PV) when compared with the control.

Similarly, the evolution of sample's LHP content shows a protective effect of the AP when added either alone or in combination with CA, also showing a synergism between both antioxidants at 80 and 120 days of storage (Figure 6.3.B).

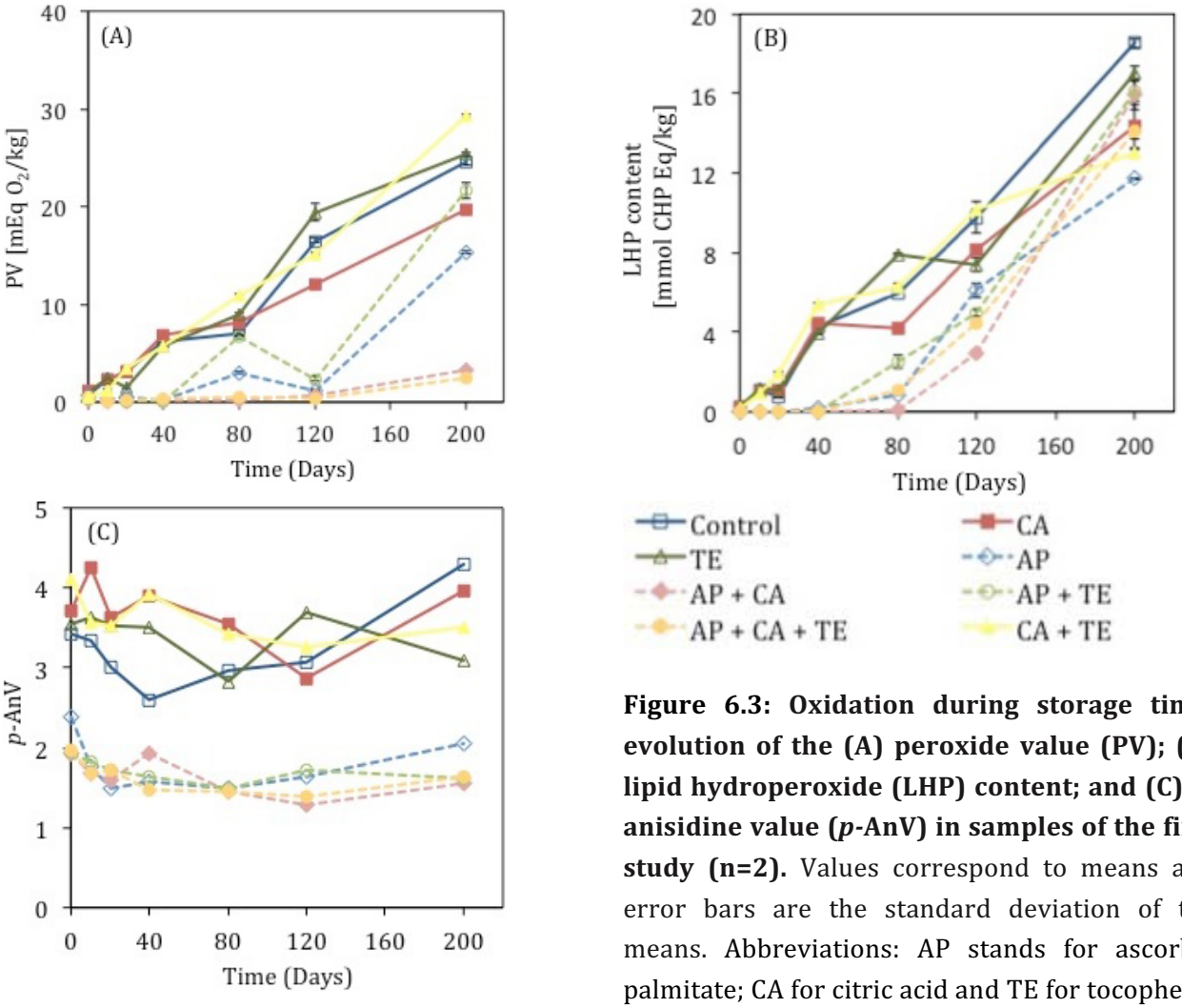


Figure 6.3: Oxidation during storage time: evolution of the (A) peroxide value (PV); (B) lipid hydroperoxide (LHP) content; and (C) *p*-anisidine value (*p*-AnV) in samples of the first study (n=2). Values correspond to means and error bars are the standard deviation of the means. Abbreviations: AP stands for ascorbyl palmitate; CA for citric acid and TE for tocopherol extract.

In the first study, the secondary oxidation evolution was measured by the *p*-anisidine value (*p*-AnV) (Figure 6.3.C). Similarly to the primary oxidation results, samples containing AP had a lower *p*-AnV than the other samples thus indicating the effectiveness of AP as an antioxidant. However, the synergistic effect of AP and CA recorded in this study when primary oxidation products were monitored was not observed when *p*-AnV was studied. In addition, no increase in the *p*-AnV was observed during 200 days of storage.

Taking the results of the first study into account, the aim of the second study was to optimize the dose of AP and CA, and to study the possible synergistic effect between these antioxidants.

Continuing with the AP effect on the different studies, the addition of AP (alone or in combination with CA) was found to be the most effective strategy in decreasing AUC in the second study (Figure 6.4). However, it is important to note that AUC of samples with AP at either 200 or 400 mg/kg were lower than those with 100 mg/kg. In addition, the lower initial LHP values of samples with AP, when added at concentrations higher than 100 mg/kg, confirmed that AP has a protective effect during the heating of samples at 220 °C for 10 min (0.01 vs. 0.17 mmol CHP eq/kg for either 200 or 400 mg/kg and control sample, respectively).

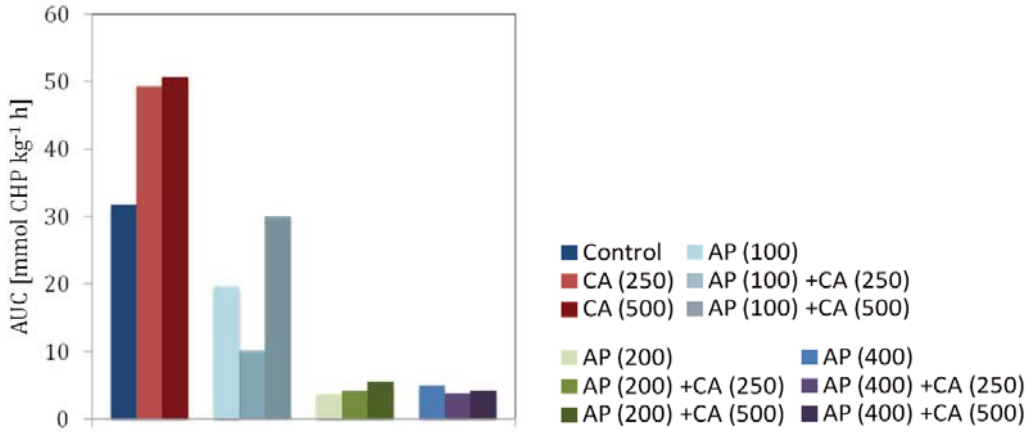


Figure 6.4: Susceptibility to oxidation of samples of the second study measured using the induced ferrous oxidation-xylenol orange (FOX) method and determined by the area under the curve (AUC, mmol CHP Eq/kg sample x h) (n=2). Values correspond to means and error bars are the standard deviation of the means. Abbreviations: AP stands for ascorbyl palmitate; and CA for citric acid. In the legend, numbers in between brackets correspond to the antioxidant concentration expressed in mg/kg.

The effectiveness of AP was also assessed in the fourth study. However, in this study the AUC of the control (without added AP) was not different from samples in which AP was added at 400 mg/kg (AUC 14.26 vs. 14.42, respectively). Similarly, the other parameters calculated from the FOX induced method showed no differences when compared with the control. Although these results were opposite from those obtained in the first two studies, those treatments with added AP showed a protective effect on tocopherol and tocotrienol loss produced by processing conditions and heating at 220 °C for 10 min to which samples were submitted (total tocopherol + tocotrienol content of the control sample was 537.3 mg/kg whereas this amount in samples containing AP were of 642.7 mg/kg which in turn was equal to its content in fresh palm oil). Therefore, AP protected samples from oxidation during the heating treatment, which is in agreement with the results obtained in the previous studies regarding the decreased initial LHP content of AP samples.

Related with that, in each study, the oxidation status of samples was assessed over the storage time by monitoring the formation of primary and secondary oxidation compounds. Figure 6.5 shows the effect of AP on the assessed oxidation parameters that have been followed-up in the studies with palm oil fortified with heme iron (studies 1, 2 and 4). As pointed out before in the first study, the addition of AP at 500 mg/kg was effective avoiding both primary and secondary oxidation in a palm oil matrix fortified with heme iron (Figures 6.3 and 6.5).

The effectiveness of AP on reducing the onset of oxidation was previously described by Masson et al. (2002). These authors described the reduction of PV levels in potato chips fried in palm oil containing 500 mg of AP/kg compared with chips fried with no AP. As AP is an oxygen scavenger, therefore, it is reasonable to think that the main source of oxidation in our matrix was caused by the presence of oxygen dissolved in the fat matrix rather than the heme iron or the free iron present in samples.

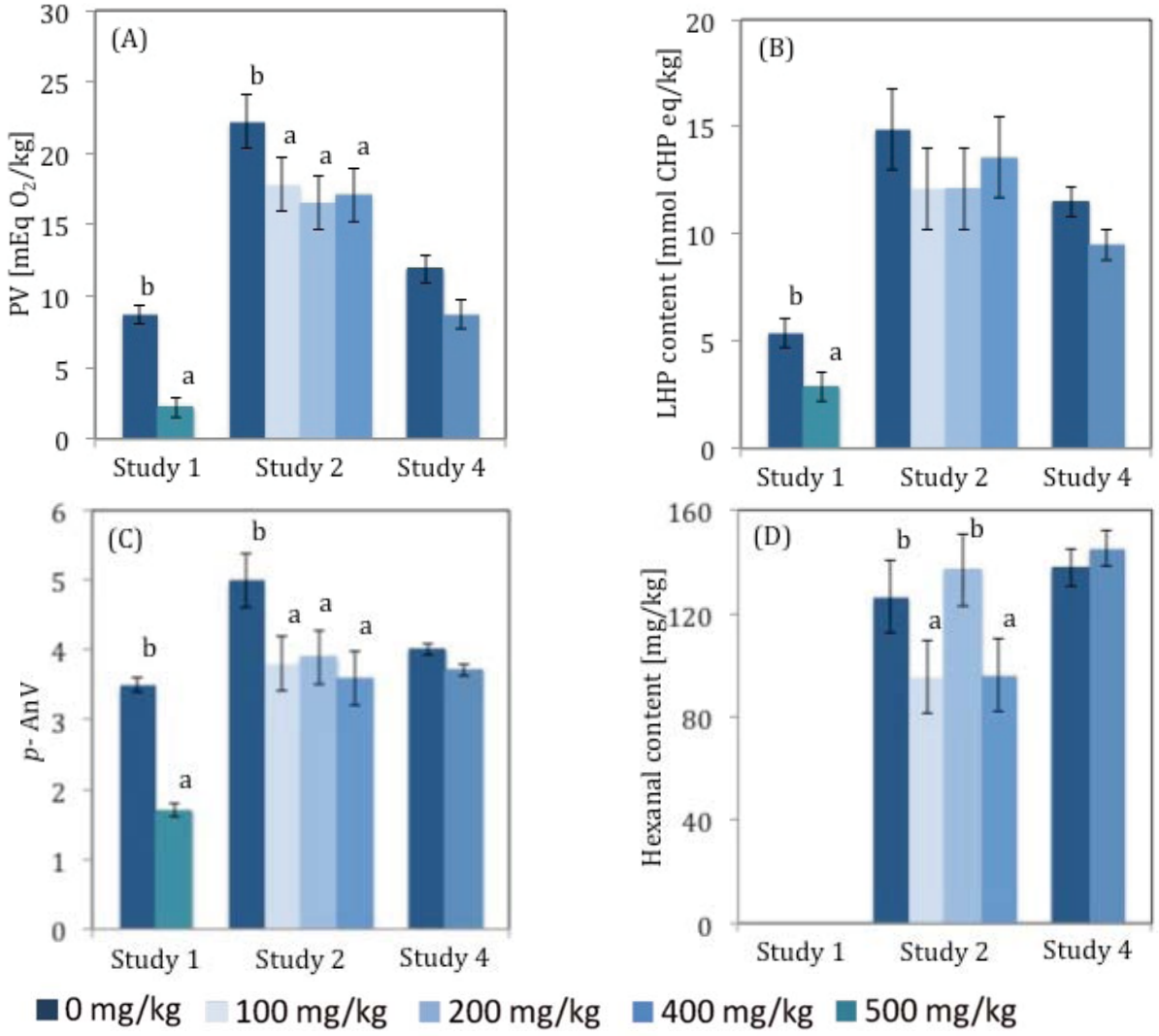


Figure 6.5: Effect of the concentration of ascorbyl palmitate added at different concentrations on (A) peroxide value (PV); (B) lipid hydroperoxide (LHP) content; (C) *p*-anisidine value (*p*-AnV) and (D) hexanal content of samples from different studies. Values correspond to least-square means (study 1, n=7; study 2, n=18 for PV, LHP content and *p*-AnV and n=8 for hexanal content, study 4, n=20) of the oxidation parameters at all storage times obtained from multifactorial ANOVA. Error bars are the standard error of the least-square means. Values with different letters differ significantly ($p \leq 0.05$).

In the second study, there is a similar trend in preventing both primary and secondary oxidation with the addition of AP alone. In this study the peroxide value was reduced by the AP addition whereas no differences were found for the LHP content of samples according to the treatment. For secondary oxidation parameters, AP decreased *p*-AnV (Figure 6.5).

In study 4, the addition of AP at 400 mg/kg did not show a protective effect for any of the studied oxidation parameters (Figure 6.5). However, as commented above, it is important to note that AP protected from tocopherol plus tocotrienol loss during the heating treatment in this study (220 °C for 10 min). This overall AP ineffectiveness over the storage time in study 4 can be attributed to differences among the matrixes in studies 1, 2 and 4, which will be commented further in this section.

The addition of CA in the first study of the thesis neither reduced the susceptibility to oxidation of samples (Figure 6.1) nor protected from oxidation (Figures 6.3 and 6.6). Nevertheless, as explained before, in the first study a possible synergistic effect between AP and CA on PV was found after 80 days of storage at room temperature in the dark (Figure 6.3.A and B).

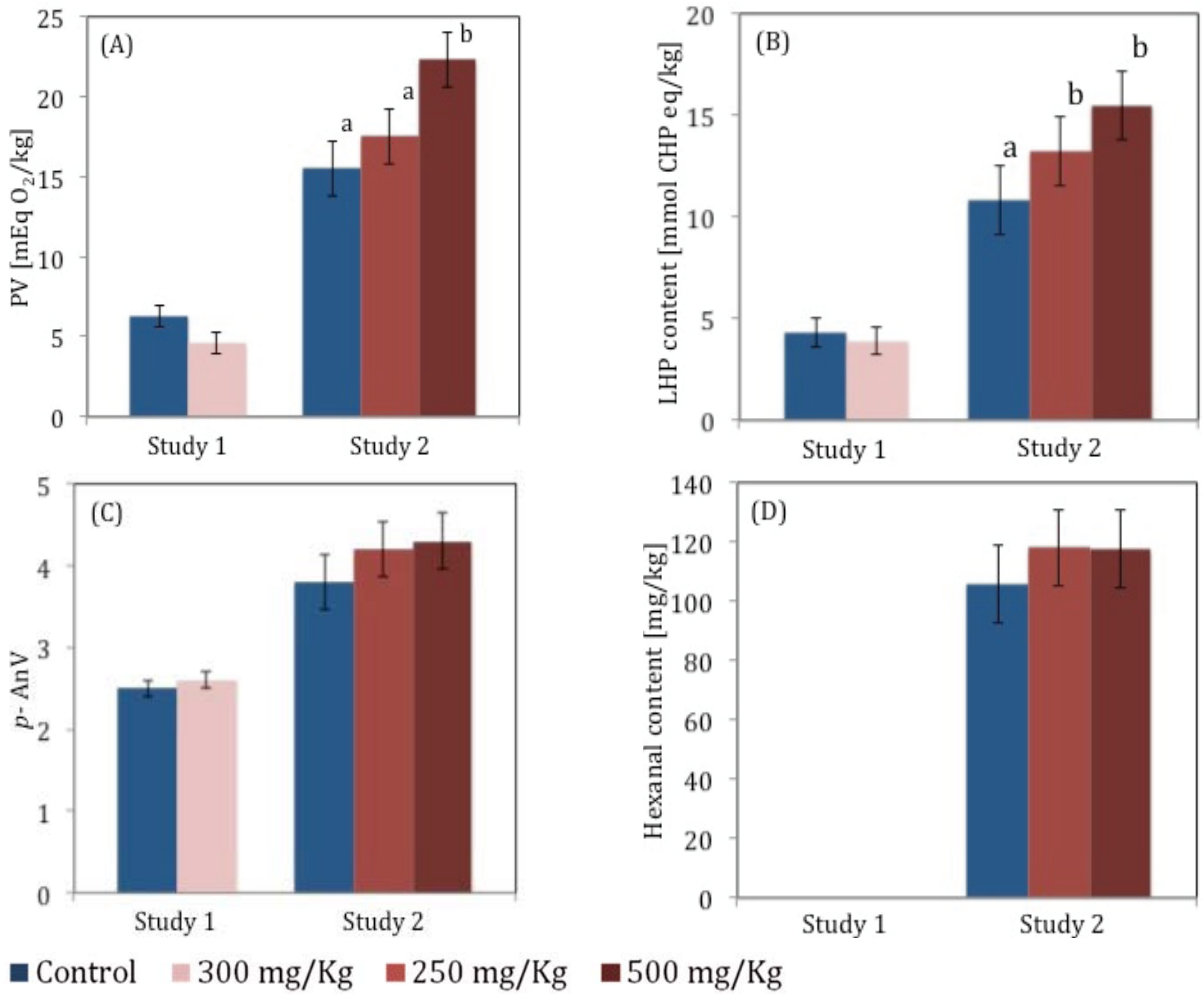


Figure 6.6: Effect of the concentration of citric acid added at different concentrations on (A) peroxide value (PV); (B) lipid hydroperoxide (LHP) content; (C) *p*-anisidine value (*p*-AnV) and (D) hexanal content in the different studies. Values correspond to least-square means (study 1, n=7; study 2, n=18 for PV, LHP content and *p*-AnV and n=8 for hexanal content, study 4, n=20) of the oxidation parameters at all storage times obtained from a multifactorial ANOVA. Error bars are the standard error of the least-squares means. Values with different letters differ significantly ($p \leq 0.05$).

The addition of CA alone to samples clearly increased their AUC in the second study (Figure 6.4) thus suggesting a pro-oxidant behaviour of CA. With respect to the formation of oxidation compounds in this latter study, CA showed either no effect or a tendency towards a pro-oxidant effect when added alone (Figure 6.6). This pro-oxidant behaviour of CA was not counteracted by its combination with AP (data not shown in figures 6.5 and 6.6) and, therefore, the synergistic effect between these two antioxidants was discarded. However, it is important to note that a significant interaction between AP and CA was found for both

primary oxidation parameters (PV and LHP content, $p \leq 0.001$) and *p*-AnV ($p=0.001$). Therefore, when no CA was added to samples, the antioxidant effect of the AP was clear; the PV, LHP content and *p*-AnV decreased as AP concentration was increased (Figure 6.7).

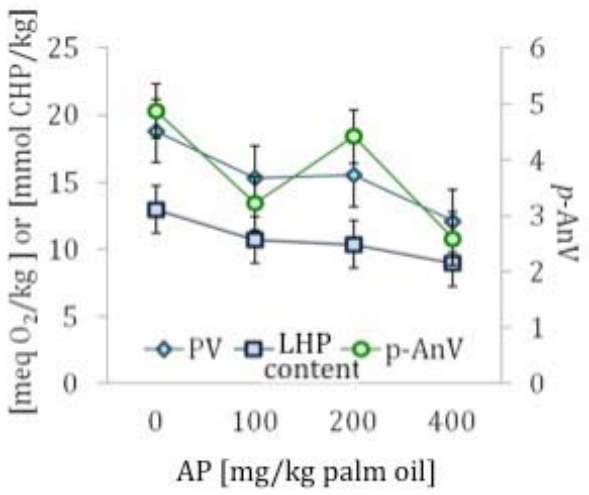


Figure 6.7: Effect of ascorbyl palmitate (AP) concentration, without citric acid addition, on the oxidation parameters of the second study (peroxide value (PV), lipid hydroperoxide content (LHP content), and *p*-Anisidine Value (*p*-AnV). Values correspond to means at all storage times and error bars are the standard deviation of the means (n=18).

The overall decrease in the formation of oxidation compounds with AP addition and the observed effects of CA confirmed that the presence of oxygen was the main cause of oxidation in these matrixes.

The pro-oxidant trend of CA in this second study was not found in the first study. These results are difficult to explain, as the used models, consisting in palm oil fortified with heme iron, are very similar in both studies. Thus, the existence of minor differences between both studies may be responsible for the different CA behaviour. In this regard, the amount of free iron in the first study was 4%, whereas in the second study was 2%. In these latter conditions, CA acted as pro-oxidant while AP (an oxygen scavenger) was effective in preventing lipid oxidation in both studies. It is also important to consider that the CA employed in both studies was the same. Hence, the CA added in the second study was one year older than in the first study. In spite of this, it is worth to mention that CA was stored accordingly to the conditions suggested by the supplier (hermetically sealed in the dark) and used 3 years before its expiration date.

In the literature we can find reports on the iron-citrate complexes intricacy. Under concrete conditions (Fe:citric molar ratio, pH and temperature) there exist an influence on the formation of ferric citrate species in aqueous solution (Gautier-Luneau et al., 2005). Moreover, depending on these conditions the formation of hydroxyl radical may be favoured in the presence of iron and CA in cold drinks and aqueous solutions (Gautier-Luneau et al., 2007; Gutteridge, 1991). The CA pro-oxidant effect has been reported in systems with heme iron. The addition of CA (1 mM) promoted lipid oxidation (conjugated dienes and TBARS) mediated by fish haemoglobin (3 mM) in liposomes (Maestre et al., 2009). Also, the addition of CA (1%, w/w) to salmon pâté fortified with cod liver oil favoured the formation of volatile compounds (Olsen et al., 2006). However, it is important to bear in mind that all the systems previously described are different from our model.

Apart from these considerations, it should be taken into account that in the first study *p*-AnV did not increased over the storage time (Figure 6.3.C), whereas it did markedly in the second study and slightly in the fourth. Differences in the matrixes used in each storage experiment were most probably responsible of this fact. The same hypothesis applies to the different AP behaviour observed in study 4. In this regard, the fatty acid composition and tocopherol and tocotrienol content of the palm oils used in each storage experiment are presented in annexes 9.6 and 9.7. These compositions are typical of this kind of oil (Tres et al., 2011). Overall, the slight differences found on FA composition (MUFA, PUFA, linoleic acid and linolenic acid) do not help to explain the differences between studies in AP effect and in *p*-AnV evolution over storage time.

Nevertheless, significant differences were found on the total amount of tocopherols plus tocotrienols of the fresh palm oils used in the different studies. The total tocopherol and tocotrienol content of the 4th study palm oil is higher than that of the previous studies (632.4 mg/kg vs. 530.9 and 469.1 mg/kg in the first and second study, respectively). The higher tocopherol and tocotrienol content could modify the susceptibility to oxidation of the matrix. This matter has been studied further by comparing the AUC of control samples and the tocopherol plus tocotrienol losses after the heating treatment of the control samples at 220 °C during 10 min (Figure 6.8).

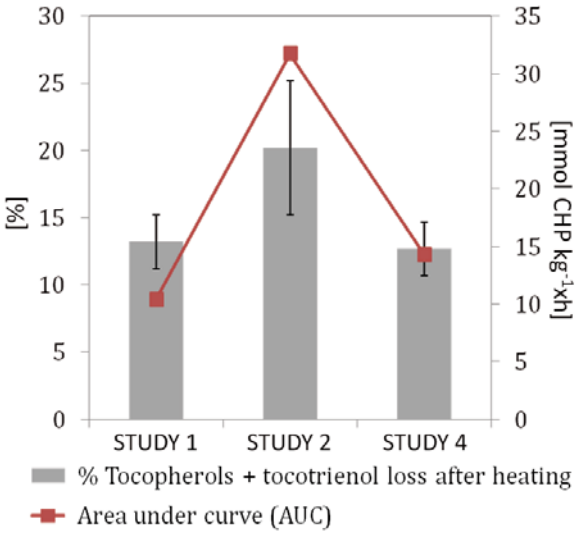


Figure 6.8: Differences on the susceptibility toward oxidation and tocopherol plus tocotrienol loss of control samples after heating treatment in each storage experiment with palm oil fortified with heme iron.
Values correspond to means and error bars are the standard deviation of the means. Regarding the tocopherol and tocotrienol loss n=4 in study 1, n=6 in study 2 and n=5 in study 4; as for the susceptibility to oxidation n=2 in studies 1 and 2 and n=5 in study 4.

AUC and tocopherol plus tocotrienol losses after heating of control sample in study 4 are similar to those of the first study, and at the same time lower than those of the second study. These findings point out that both control samples of study 1 and 4 were less susceptible to oxidation than those samples from study 2 and this could explain that in study 2 the increase of the *p*-AnV during storage was higher. In order to explain the differences between studies in AP effect and *p*-AnV evolution it is also important to compare the iron content of samples (Table 6.1).

Table 6.1: Total iron content and percentages of free iron on the different heme iron fortified palm oil studies.

Average iron content of samples at time 0 of storage	*Study 1*	*Study 2*	*Study 4*
Total iron content [mg Fe/g sample]	1.61 ±0.22[1]	0.95±0.51	1.20±0.16
% Free iron	4	2	27

[1]Mean ± SD (Study 1, n= 2; study 2 and 4, n= 5)

As explained in the experimental design section, samples from study 1 contained more iron in order to ensure the selection of the best antioxidant. However, the higher iron content did not cause a different susceptibility towards oxidation of samples, as it is equal in studies 1 and 4 (Figure 6.7). Here, it is important to remark that the palm oil used on study 4 contained higher tocopherol plus tocotrienol content than the other palm oils used in studies 1 and 2, and thus it should be less prone to oxidation than the others. However, the higher free iron content in this study may have masked the protective effect of the higher tocopherol and tocotrienol content on the oxidation of the samples.

Thus, the other main difference between the studies is the amount of free iron, as samples from study 4 had 27% of free iron whereas samples of the other studies had 2-4% of free iron. Therefore, the higher free iron amounts in study 4 changed the course of oxidation and caused that AP, an oxygen scavenger, to be ineffective over the storage time. Also, the less susceptibility to oxidation of study 1 matrix, compared with study 2, plus the low free iron amounts, compared with study 4, lead *p*-AnV to be a non-suitable parameter for assessing the evolution of secondary oxidation during storage in the first study of the thesis. This matter points out the importance of measuring the oxidation evolution with different parameters.

Taking into account all these considerations, AP was the best antioxidant for avoiding the oxidation during storage of palm oil matrixes fortified with heme iron with low free iron content. Moreover, the concentration that proved to be the most effective was 400 mg of AP/kg of palm oil. On top of that, AP at 400 mg/kg was also able to avoid the tocopherol plus tocotrienol loss during the heating treatment (at 220°C, during 10 min). However, it is important to remark that AP effectiveness is affected by the amount of free iron present in the heme iron fortified palm oil. When the free iron content is low (2-4%) AP is very effective but resulted to be ineffective when the free iron is high (27%).

Opposite to AP effectiveness when there is low free iron content, neither the addition of TE nor CA avoided the oxidation development in the heme iron fortified palm oil. Under certain conditions, CA acted as a pro-oxidant.

6.2. Co-spray-drying as strategy for preventing oxidation in a model for bakery products fortified with heme iron (studies 3 and 4)

As it has been pointed out in the review of the literature, an alternative approach for delaying the onset of oxidation of an iron fortified food product is the encapsulation of the iron compound. By means of this strategy, the contact between the pro-oxidant heme iron and the fat matrix is avoided and thus oxidative reactions are prevented.

There are different encapsulation techniques and among them the co-spray-drying of the heme iron ingredient with coating materials offers good possibilities for its simplicity. The co-spray-drying of the heme iron ingredient as strategy for preventing oxidation was used in studies 3, 4 and 5. Different quantities of the heme iron ingredients were dispersed in the lipid matrixes in order to maintain constant the iron content in all samples of each study.

The aim of the study 3 was to select a co-spray-drying agent, and thus two different coatings were assessed: calcium caseinate and a mixture of modified starch (octenyl succinate) plus maltodextrin (50:50, w/w). These coating materials were assessed at two different ratios (heme iron concentrate : coating material at 1:1 and 2:1, w/w). In order to quickly select the appropriate coating material, the evolution of the oxidation was followed in a sunflower oil matrix (more prone to oxidation than palm oil) fortified with the different co-spray-dried heme iron ingredients upon storage at 60 °C. Despite the fact that both coating materials were able to delay oxidation, the heme iron co-spray-dried with calcium caseinate at a higher concentration (1:1 ratio) showed to be the most effective practice (Table 6.2).

Table 6.2: Percentage of reduction of peroxide value (PV) and *p*-anisidine value (*p*-AnV) after 331 h of storage at 60 °C in samples of study 3 (n=2)

Treatment	*Reduction of PV (%)*	*Reduction of p-AnV (%)*
CAS 2:1	63	28
CAS 1:1	79	45
ST-MD 2:1	37	26
ST-MD 1:1	57	35

Percentages are calculated in comparison with the control sample, with AproFER 1000 ™, oxidation status at 331 hours of storage.
CAS 2:1 stands for heme iron co-spray-dried with calcium caseinate at 2:1 ratio; CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio; ST-MD 2:1 stands for heme iron co-spray-dried with a mixture of modified starch and maltodextrin (50:50, w/w) at 2:1 ratio; ST-MD 1:1 stands for heme iron co-spray-dried with a mixture of modified starch and maltodextrin (50:50, w/w) at 1:1 ratio.

In study 3, heme iron co-spray-dried with calcium caseinate at different ratios efficiently reduced both PV and *p*-AnV when compared with the control sample in which the heme iron

ingredient (AproFER 1000™) was added (Table 6.2. and Figure 6.9). Therefore, calcium caseinate was selected to be tested on a palm oil matrix fortified with heme iron (4th study). However, in this latter study the heme iron co-spray-dried with calcium caseinate was not effective on delaying the oxidation. With respect to PV in palm oil samples, this oxidation parameter was not reduced when comparing the co-spray-dried heme iron with the control heme iron ingredient. Conversely, the *p*-AnV was reduced when the co-spray-dried heme iron ingredient was added in 2:1 ratio (Figure 6.9).

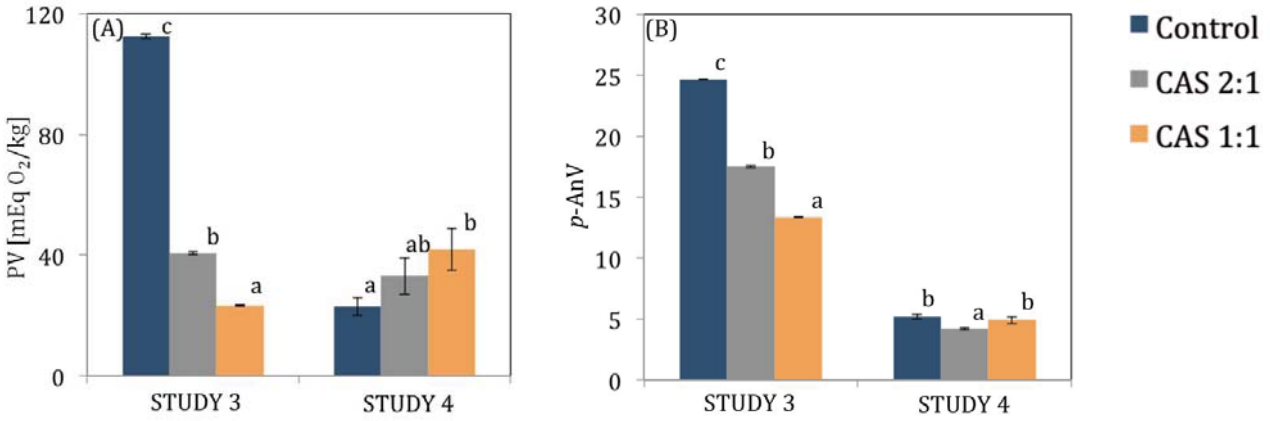


Figure 6.9: Effect of co-spray-drying of the heme iron ingredient with calcium caseinate on (A) Peroxide Value (PV) and (B) *p*-anisidine index (*p*-AnV) in studies 3 and 4 at the end of the storage time (331 hours and 360 days, respectively).

Values correspond to means and error bars are the standard deviation of the means. Values within a study with different letters differ significantly ($p \leq 0.05$). Study 3, n=2; study 4, n= 5.

CAS 2:1 stands for heme iron co-spray-dried with calcium caseinate at 2:1 ratio (heme iron concentrate:calcium caseinate, w/w); CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio (heme iron concentrate:calcium caseinate, w/w).

With respect to the effectiveness of the different co-spray-dried heme iron ingredients, the different behaviour observed in these studies may be attributed to those differences between experiments. In this regard one of the differences between them is the lipid source. In the third experiment, sunflower oil was used as the lipid matrix whereas palm oil was used in the fourth study. In addition, we found differences between the percentages of heme iron on each heme iron ingredient used in both studies. In study 3, the free iron was 8% of the ingredient's total iron, whereas in the 4th study was 17% (Table 6.3). This last free iron content was also higher than that found in the previous studies (1 and 2) where the free iron accounted for the 4% of the total iron.

Table 6.3: Percentage of heme iron in the different heme ingredients and in samples after the heating treatment (220°C, 10 min) in studies 3 and 4

	% Heme iron of ingredients	*% Heme iron in samples after heating*
Study 3(sunflower oil)	92±1[1]	104±4
Study 4 (in palm oil)	83±3	73±2

[1] Mean ± SD (Study 3 n=3, Study 4 n=5)

Although heme iron was the main form of iron in studies 3 and 4; the heating treatment of samples (10 min at 220°C) caused the release of free iron in study 4. The heme iron percentage with respect to total iron was reduced down to 73%, resulting in 27% of free iron in samples. This result differs from study 3, where the maximum amount of free iron in samples was 8% (coming from the heme ingredients), since the iron liberation during the heating treatment was negligible (Table 6.3).

According to these results it is reasonable to think that co-spray-drying of heme iron is efficient while iron is not markedly liberated from the heme moiety. The use of calcium caseinate as a coating material seemed to have little effect when free iron content is high. Here, it is important to remark that the heme iron co-spray-dried ingredients, as stated in the methodology section, were prepared in the same way, starting from the heme iron concentrate obtained in each study. In consequence, the differences in iron liberation and free iron content between both studies could be due to small changes in the production of the heme iron concentrate. In this regard, the heme iron producer assessed new conditions (e.g., ultrafiltration pressures and temperatures) in the production of these two batches of heme iron concentrates.

In conclusion, the co-spray-drying of heme iron, either with calcium caseinate or the mixture of modified starch and maltodextrin, was able to delay the onset of both primary and secondary oxidation in the sunflower oil matrix fortified with heme iron stored at 60°C, being the most effective the calcium caseinate coating. However, this effectiveness was not observed when heme iron co-spray-dried with calcium caseinate was assessed in the palm oil model. The palm oil matrix contained a much higher free iron amount (27%) than the sunflower oil matrix (8%) and thus the most probable cause for this co-spray-drying ineffectiveness is the higher free iron amount in the palm oil matrix.

6.3. Combination of strategies for preventing oxidation in a model for bakery products fortified with heme iron (study 4)

According to our previous findings two strategies, namely the addition of AP (400 mg/kg) and the co-spray-drying of the heme iron with calcium caseinate, were shown to be effective on delaying the oxidation onset (studies 1, 2 and 3). Therefore, in the 4th study, we studied if the combination of these two approaches can result in an improved oxidative stability of palm oil samples.

Regarding the susceptibility to oxidation of samples, the AUC of samples that combined the addition of both AP and heme iron co-spray-dried with calcium caseinate did not differ from the control samples (Figure 6.10.A). The addition of calcium caseinate in a ratio 1:1 with the heme iron, either co-spray-dried or not, favours the formation of LHP. In addition, those treatments that combined AP with the co-spray-dried ingredients presented much higher TMAX (48-43.5 h) than the rest, indicating that this combination could be effective to prevent oxidation (Figure 6.10.B). In agreement with AUC results, those samples with calcium caseinate at ratio 1:1, co-spray-dried or not, reached the maximum LHP concentration at the lowest times (2.5 hours).

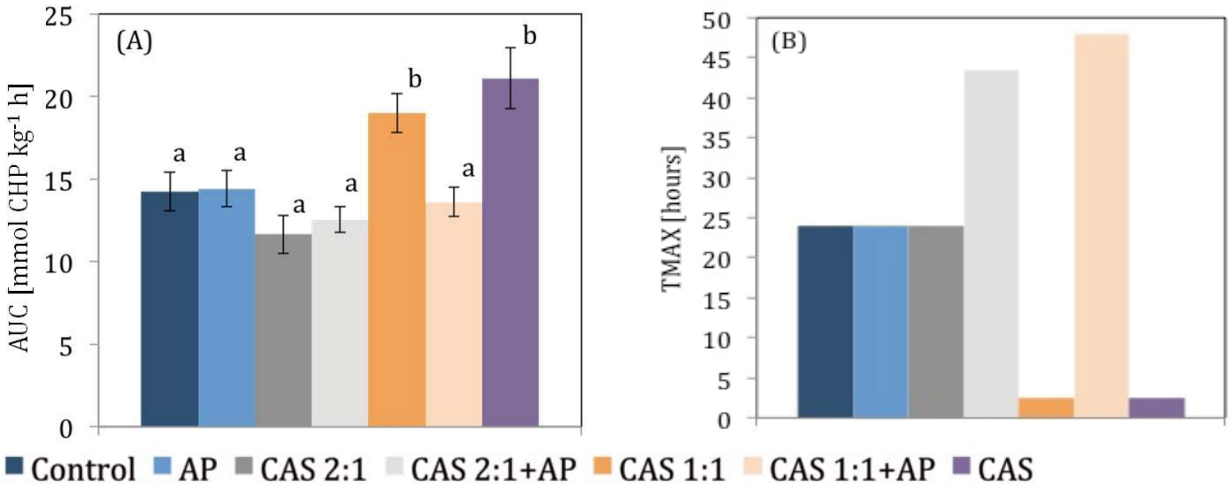


Figure 6.10: Susceptibility to oxidation of samples of the fourth study measured using the induced ferrous oxidation-xylenol orange method (FOX) and determined by (A) the area under the curve (AUC, mmol CHP Eq/kg palm oil x h) and (B) the time to reach the maximum lipid hydroperoxide content (TMAX, hours).

Values correspond to means (n=5) and error bars are the standard deviation of the means. Values with different letters differ significantly ($p \leq 0.05$).

AP stands for ascorbyl palmitate; CAS 2:1 stands for heme iron co-spray-dried with calcium caseinate at 2:1 ratio; CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio and CAS stands for calcium caseinate added with heme iron at 1:1 ratio without co-spray-drying.

In study 4, the loss of tocopherols plus tocotrienols after the heating (220 °C for 10 min) was measured in each treatment. The percentage of tocopherol plus tocotrienol loss was lower in those samples containing AP (Figure 6.11). Thus, AP prevented the loss of tocopherol and

tocotrienols during heating, especially in the case of α-tocopherol, which is the most abundant tocopherol analogue (data not shown in figure 6.11). Similar results were found for the total tocotrienol content, which indicate the protective effect of AP during heating.

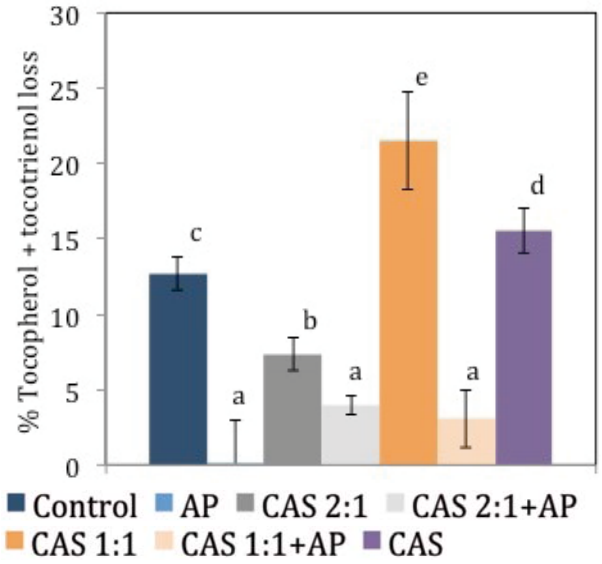
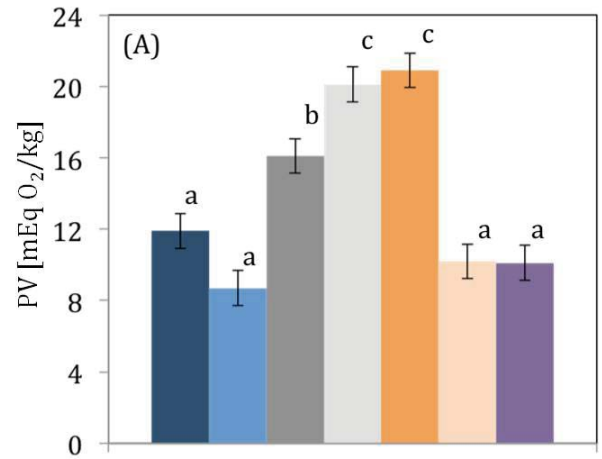


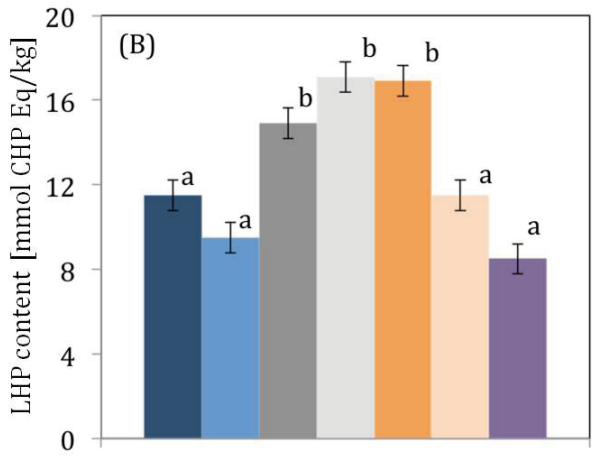
Figure 6.11: Percentage of tocopherol plus tocotrienol loss after heating samples at 220ºC for 10 minutes in study 4.

Values correspond to means and error bars are the standard deviation of the means. Values with different letters differ significantly ($p \leq 0.05$).

AP stands for ascorbyl palmitate; CAS 2:1 stands for heme iron co-spray-dried with calcium caseinate at 2:1 ratio; CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio and CAS stands for calcium caseinate added with heme iron at 1:1 ratio without co-spray-drying; n=5.

The effect of the treatments on the different oxidation parameters is shown in Figure 6.12. For PV and LHP content, we found no differences between the addition of AP alone or in combination with the heme iron co-spray-dried at 1:1 ratio. Hence, this combination resulted in no further advantages regarding primary oxidation parameters. However, when AP was combined with the heme iron co-spray-dried in proportion 2:1 there is an increment in primary oxidation measured by means of the PV and LHP content (Figure 6.12.A and B). Therefore, opposite to what was found in the preliminary study (study 3), the co-spray-drying of the heme iron ingredient with calcium caseinate at any ratio did not protected samples against primary oxidation (Figure 6.12.A and B).





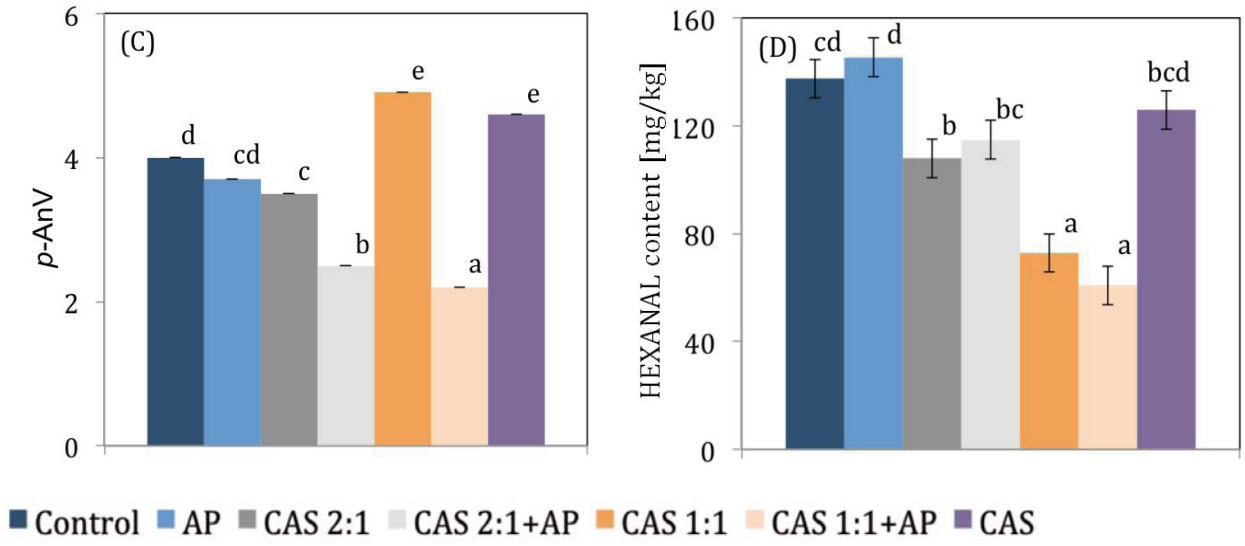


Figure 6.12: Effect of the combination between co-spray-drying the heme iron and the addition of AP on the different oxidation parameters: (A) peroxide value (PV); (B) LHP content; (C) *p*-AnV; (D) and hexanal content at all storage times.

Values correspond to least-squares means obtained from multifactor ANOVAs. Error bars are standard error of the least-squares means. Values with different letters differ significantly ($p \leq 0.05$). AP stands for ascorbyl palmitate; CAS 2:1 stands for heme iron co-spray-dried with calcium caseinate at 2:1 ratio; CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio; and CAS stands for calcium caseinate added with heme iron at 1:1 ratio without co-spray-drying; n=20.

However, the combination of AP and co-spray-dried heme iron ingredients, effectively protected from secondary oxidation when compared to the control (Figure 6.12.C and D). Moreover, the *p*-AnV of samples that combined both strategies was lower than the rest of treatments (Figure 6.12.C). Similarly, the hexanal content of samples with the co-spray-died heme iron ingredient at 1:1 ratio in combination with AP was lower than the rest samples (Figure 6.12.D).

To conclude, the reduction on the secondary oxidation was higher when AP was combined with the heme iron co-spray-dried with calcium caseinate at 1:1 ratio than when was combined with the heme iron co-spray-dried at 2:1 ratio. The combination between AP and the heme iron co-spray-dried at 1:1 ratio was not only the most effective in delaying secondary oxidation of the palm oil matrix fortified with heme iron but also showed a synergistic to protect against increase of *p*-AnV (Figure 6.12.C). Therefore, the combination of the addition of AP and the heme iron co-spray-dried at 1:1 ratio showed to be the most efficient strategy to minimize oxidation in a palm oil matrix fortified with heme iron with high free iron content.

6.4. Oxidative stability, cookie filling colour and overall acceptability of sandwich-type cookies fortified with heme iron (study 5)

Different strategies were shown to be effective in delaying the oxidation in several models for bakery products fortified with heme iron. Therefore, the best strategies were compared in a real food sample. Accordingly to the previous studies, in the 5th study of this project thesis, we studied the effectiveness of the addition of AP and the co-spray-dried heme iron on the oxidative stability, cookie filling colour and overall acceptability of sandwich-type cookies filled with chocolate creams fortified with heme iron.

6.4.1. OXIDATIVE STABILITY OF SANDWICH-TYPE COOKIES FORTIFIED WITH HEME IRON

Regarding the susceptibility to oxidation of the different samples, the AUC of cookies with AP added alone or in combination with the heme iron co-spray-dried with calcium caseinate at 1:1 ratio (CAS 1:1) presented the same or lower AUC values than the control cookies without added heme iron (Table 6.4).

Table 6.4: Susceptibility towards oxidation of the cookies measured by determining the following parameters in the lipid fractions extracted from the different sandwich-type cookies prior to storage: the area under the curve (AUC) obtained by the induced ferrous oxidation-xylenol orange method (FOX), the tocopherol plus tocotrienol content and its relative loss.

Treatments	*AUC [mmol CHP Eq/kg x h]*	*Tocopherol + tocotrienol content [mg/kg fat]*	*Tocopherol + tocotrienol loss [%]*[1]
Control without heme	52±4b[2]	519±65b	-
Control heme	64±4c	267±20a	48.5
AP	18±1a	522±49b	0
CAS 1:1	63±5c	244±3a	53.1
CAS 1:1 + AP	47±4b	578±19bc	0

[1] Compared with cookie with no heme iron added (Control without heme)

[2] Mean ±SD (AUC, n=5; tocopherol plus tocotrienol content, n=3)

Values with different letters differ significantly ($p \leq 0.05$). AP stands for ascorbyl palmitate and CAS 1:1 for heme iron co-spray-dried with calcium caseinate at 1:1 ratio.

Similarly, just after cookie's manufacturing, samples fortified with heme iron without AP added (control heme iron and CAS 1:1) presented lower tocopherol and tocotrienol content than fortified samples with AP (AP and CAS 1:1 + AP cookies). Additionally, tocopherol plus tocotrienol content of cookies with AP added did not differ from control cookies without heme iron added. Therefore, the addition of AP protected from the degradation of both tocopherols

and tocotrienols that occurred during the preparation of the cookie filling. This involves the heating of the fortified chocolate cream at 40 °C for approximately 40 minutes and, therefore, these results are in accordance with the previous studies where the addition of AP reduced the tocopherol and tocotrienol loss in palm oil samples fortified with heme iron that were heated for 10 minutes at 220 °C to mimic typical baking conditions.

The evolution of primary and secondary oxidation compounds was monitored during the storage time of fortified cookies. With respect to the different treatments, some minor differences were observed in the different oxidation parameters (Table 6.5). Most remarkably, the highest PV was found in the control sample containing the heme iron ingredient although no differences were seen regarding the LHP content. As for the hexanal content, no differences were observed between the studied treatments. The *p*-AnV of samples that combined AP and CAS 1:1 was lower than in samples from control without heme iron, AP and CAS 1:1 treatments but equal to those of control heme iron samples.

Table 6.5: Effect of treatment and storage time on primary (PV and LHP content) and secondary (hexanal content and *p*-AnV) oxidation parameters of sandwich-type cookies stored in the dark for 360 days at room temperature.

	PV [meq O_2/kg]	*LHP content [mmol CHP/kg]*	*Hexanal content [mg/kg]*	*p-AnV*
Treatment[1]				
Control without heme	8.32a	5.03	2.1	14.13b
Control heme	20.30b	11.05	48.9	13.57ab
AP	14.47ab	6.99	31.6	14.97b
CAS 1:1	18.33ab	10.00	44.5	16.10b
CAS 1:1 + AP	14.04ab	8.30	32.2	11.39a
SEM[2]	*3.33*	*1.97*	*16*	*0.85*
Storage time[1]				
0 days	1.14a	0.78a	1.3a	13.29a
90 days	12.32b	4.98b	7.0a	14.13ab
180 days	20.20c	11.46c	13.2a	15.94b
360 days	27.20d	15.86d	106.0b	12.78a
SEM	*1.58*	*0.81*	*8.2*	*0.81*

[1] Values correspond to least-squares means obtained from multifactor ANOVA (n =100)

[2] Standard error of the least-squares means.

Values corresponding to a certain factor with different letters differ significantly ($p \leq 0.05$). AP stands for ascorbyl palmitate; CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio; PV stands for peroxide value and *p*-AnV stands for *p*-anisidine value.

With respect to the storage time factor, it is interesting to observe the overall increase of PV and LHP content of samples with time (Table 6.5). Conversely, secondary oxidation parameters did not follow the same trend. Hexanal content was maintained constant till 180

days but at 360 days this content was increased. In relation to *p*-AnV, this oxidation parameter slightly increased till 180 days and slightly decreased thereafter.

In Figure 6.13, it is presented the oxidation status of the different sandwich-type cookies at the end of the storage time (360 days). At this time the treatments that presented lower primary oxidation values (Figure 6.13.A and B) were those control cookies with no heme iron added and the ones with AP. Control cookies with heme iron presented the highest primary oxidation values. There were no differences between CAS 1:1 cookies and those with CAS 1:1 + AP at this storage time. It is worth to note that the results found for PV and LHP content are similar and thus highly correlated ($r=0.978$; $p<0.0001$, $n=100$).

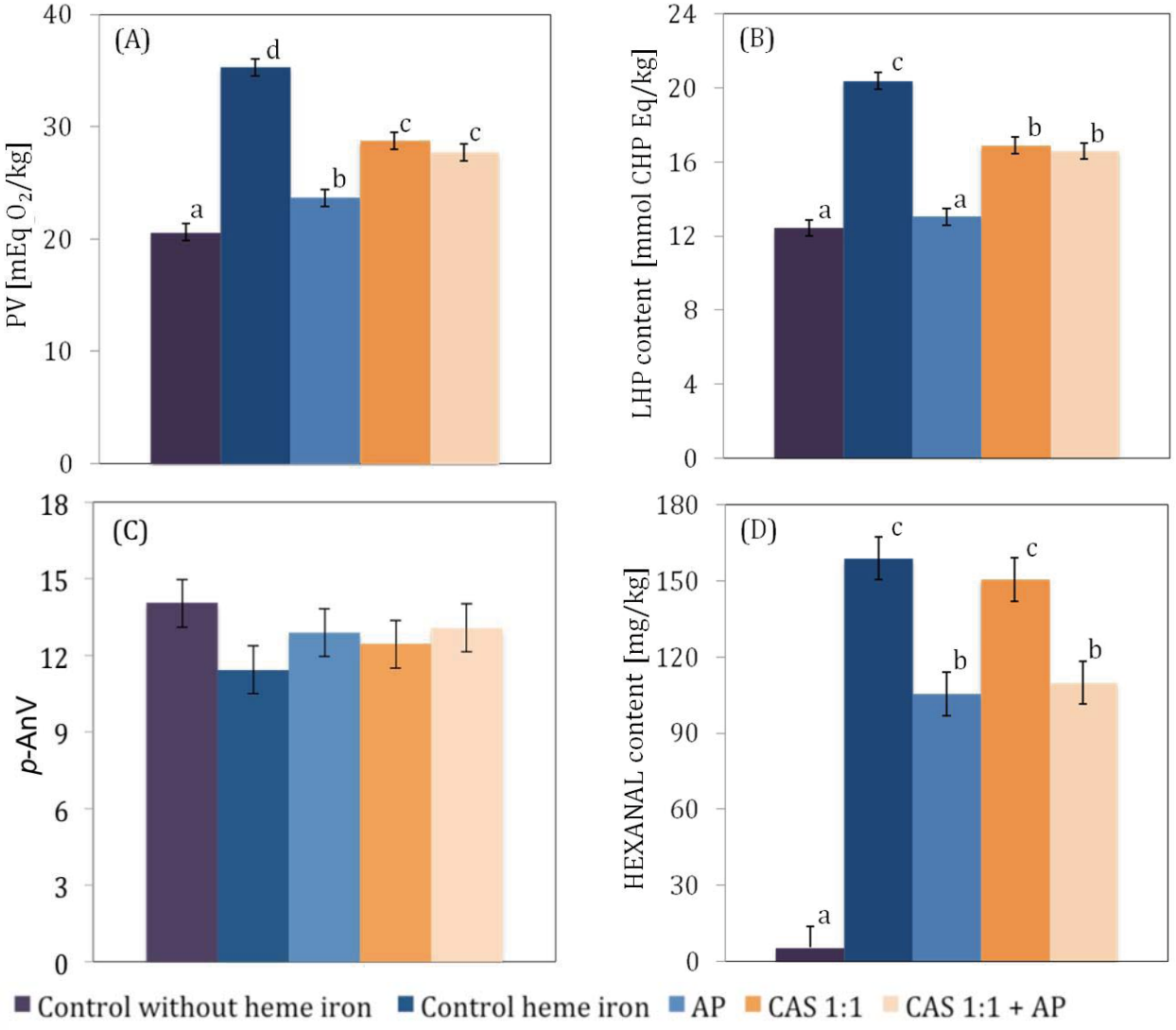


Figure 6.13: Effect of the combination of strategies on the different oxidation parameters at the end of the storage time (360 days): (A) Peroxide value (PV); (B) lipid hydroperoxide (LHP) content; (C) *p*-anisidine content (*p*-AnV) and (D) hexanal content. Values correspond to least-squares means obtained from multifactor ANOVAs. Error bars are standard error of the least-squares means. AP stands for ascorbyl palmitate and CAS 1:1 for heme iron co-spray-dried with calcium caseinate at 1:1 ratio, n=5. Values with different letters differ significantly ($p \leq 0.05$).

Regarding the secondary oxidation, at the end of the storage time, those samples fortified with heme iron containing AP recorded lower hexanal content (Figure 6.12.D). However, at the end of the storage time *p*-AnVs (Figure 6.12.C) were not different between the treatments. This fact is remarkably because for all the other oxidation parameters, control samples with no heme iron recorded the lowest oxidation values at the end of the storage time and the highest were recorded for control samples with heme iron. Also, opposite to the primary oxidation parameters, *p*-AnV and hexanal content were not correlated. Moreover, *p*-AnV was the only oxidation parameter that was not correlated with any other oxidation parameter, whereas PV, LHP content and hexanal content where correlated between them (PV vs. LHP content $r=0.978$; $p<0.0001$, $n=100$; PV vs. hexanal content $r=0.699$, $p<0.0001$, $n=100$ and LHP content vs. hexanal content $r=0.736$; $p<0.0001$, $n=100$).

Taking all this into account, it seems that *p*-AnV was not a suitable oxidation parameter in this study. Most probably this is due to (i) during the lipid extraction procedure the more volatile secondary oxidation compounds may have been evaporated (e.g. when vacuum was applied in rotary evaporation and in the dessicator) and (ii) because it is known that secondary oxidation products are able to form adducts with proteins (Zamora & Hidalgo, 2011), which content in this matrix is much higher than in palm oil.

Overall, the addition of AP alone or its combination with the co-spray-dried heme iron leaded to decreased PV, LHP content and hexanal content compared with samples with the control heme iron. Therefore, this strategy showed its effectiveness on preventing oxidation in heme iron fortified sandwich-type cookies.

6.4.2. COMPARISON OF THE CHOCOLATE FILLING COLOUR OF HEME IRON FORTIFIED COOKIES WITH COMMERCIAL COOKIES AND CHOCOLATE BARS CONTAINING DIFFERENT COCOA PERCENTAGES

The heme iron ingredients produced presented different colours depending on how the heme iron concentrate was spray-dried (alone or in combination with a co-spray-drying agent). Therefore, chocolate creams prepared with these heme ingredients to fill the *Marie biscuits* also presented different colours. For this reason, in this section, we decided to compare the differences among the chocolate creams prepared in this study and, as well, to compare them with other chocolate creams from commercial cookies. Besides, we compared the colour of the chocolate fillings with chocolate bars with different amounts of added cocoa (33, 72 and 85% of cocoa).

The lightness (L*) of the chocolate cream fortified with heme iron was not different from that of the control chocolate cream without heme iron (Figure 6.14.A). Only the filling with the control heme iron and AP added presented less lightness than the control cookie with no heme iron added. It is important to note, that all the chocolate fillings of this study were less bright than the fillings of commercial cookies. However, the lightness of the experimental cookies was similar to chocolate bars with high cocoa percentages (72 and 85%).

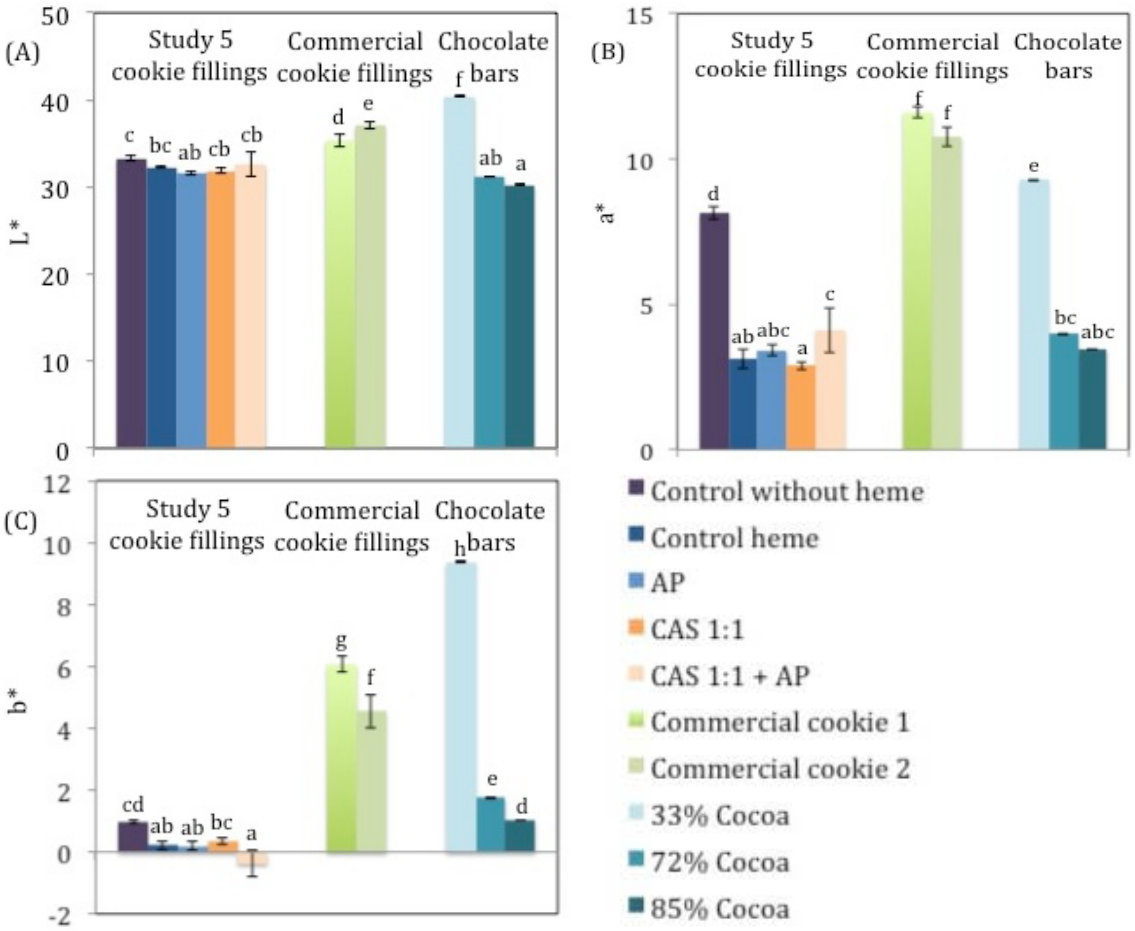


Figure 6.14: Lightness (L*) (A), redness (a*) (B) and yellowness (b*) (C) values of the experimental chocolate cream fillings, commercial chocolate cream fillings and chocolate bars with different amounts of cocoa.

Values correspond to means and error bars are the standard deviation of the means. AP stands for ascorbyl palmitate; CAS 1:1 stands for heme iron co-spray-dried with calcium caseinate at 1:1 ratio; n=4.

Opposite to the L* values, a* values greatly differed between the experimental cookie fillings. The control chocolate filling without heme iron added displayed higher redness (a*) than the other experimental samples (Figure 6.14.B). This means that the control sample tended to a more reddish colour than the samples fortified with heme iron. In this case, it seems that the addition of AP could have an effect on a* values as the samples with the co-spray-dried heme iron and this antioxidant added presented higher a* values than control samples with heme iron and CAS 1:1 samples. Equally to what was found for L* values, commercial chocolate fillings had higher a* values than the experimental ones. Also, chocolate creams fortified with heme iron presented a* values similar to that of chocolate bars with high cocoa content (72 and 85%).

The b* value is related to more yellowish colour when the values are positive and to a more bluish colour when the values are negative. The chocolate fillings with the co-spray-dried heme iron and the AP added displayed the lowest b* values of all samples. Again, it seems that

the addition of the antioxidant could slightly affect the colour of the chocolate cream. The differences between b* values of samples fortified with heme iron are minimum whereas the experimental chocolate cream without heme iron presented higher b* values which are similar to that of the chocolate bars with high cocoa content (85%) (Figure 6.14.C). As for L* and a* values, the experimental chocolate creams presented b* values that are quite different from those found in commercial cookies.

Overall, the colour of the chocolate cream fillings fortified with heme iron differed from the control non-fortified chocolate cream and also from that of the commercial chocolate creams. However, L* and a* values of heme iron fortified chocolate creams were similar to those of chocolate bars with high cocoa percentages (72-85%). On the other hand, the addition of AP had a slight effect on the cookie-filling colour, which is difficult to explain as the amount added of this antioxidant is so little and the colour was measured prior to storage. In spite of these differences regarding the colour of the chocolate fillings of experimental and commercial samples, the consumer panel did not dislike the overall appearance of the sandwich-type cookies fortified with heme iron. The existence of different chocolate products (e.g. chocolate bars) with different colours may explain this fact.

6.4.3. OVERALL ACCEPTABILITY OF SANDWICH-TYPE COOKIES FORTIFIED WITH HEME IRON

Apart from the colour of the chocolate fillings and the oxidation status of samples, the overall acceptability of cookies over the storage time was assessed by means of a consumer panel. The results obtained by means of the sensory analysis of fortified sandwich-type chocolate cream filled cookies on 0, 180 and 360 days of storage are presented in figure 6.15.

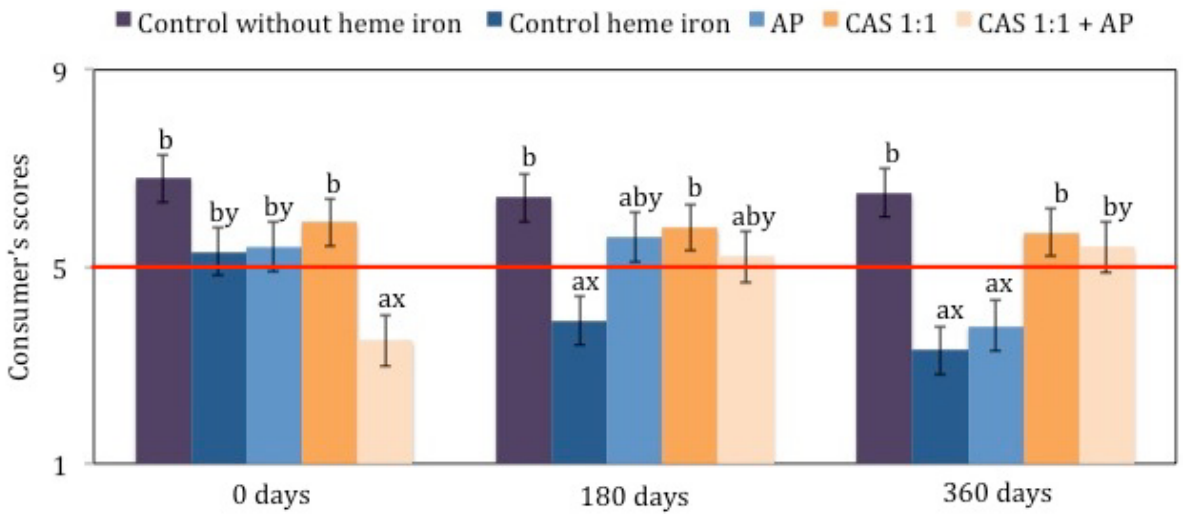


Figure 6.15: Consumers' overall acceptability scores at different storage times.
Overall acceptability of cookies was assessed using a 9-points hedonic scale (9 excellent, 1 very poor). Values correspond to least squares means (n =32) obtained from multifactor ANOVA. Error bars are standard error of the least-squares means. At each storage time, values with different letters differ

significantly ($p \leq 0.05$). "a" and "b" represent differences between treatments at the same storage time, whereas "x" and "y" represent differences over the storage time of each treatment.
AP stands for ascorbyl palmitate and CAS 1:1 for heme iron co-spray-dried with calcium caseinate at 1:1 ratio.

At the initial time, consumers approved (average score > 5) all the treatments with the exception of the one that combined the addition of AP and CAS 1:1. This fact is difficult to explain because consumers approved those treatments that involved the addition of AP or CAS 1:1, separately. However, consumers that did not approved CAS 1:1 + AP cookies at the initial storage time pointed out that they considered those samples "too sweet" and therefore their score was lower than the rest.

Overall acceptability of the control cookies with the heme iron ingredient, AproFER 1000™, clearly decreased with the storage time. However, when this heme iron ingredient was combined with the addition of AP, consumers approved cookies till 180 days of storage. At the end of the storage time, those samples with the heme iron ingredient with or without AP added recorded scores below 5, and thus considered as not acceptable. Control cookies without heme iron and cookies with CAS 1:1 (without AP) were accepted at all storage times and showed no differences between them. Despite the fact that cookies with CAS 1:1 and AP were not accepted at the initial storage time, its acceptability increased at 180 and 360 days of storage. Moreover, at the end of the storage period, those cookies without heme iron and those with CAS 1:1 (with and without AP) showed no acceptability differences. Therefore, those cookies involving the CAS 1:1 approach showed a good acceptability after one year of storage that is also similar to that of the non-fortified cookies.

These results are related with those regarding oxidation but not in complete agreement as AP cookies overall acceptability at the end of the storage period was in disagreement with its higher oxidative stability (Figure 6.13). Thus, the encapsulation of heme iron combined with the addition of AP is likely the most appropriate approach as these cookies at the end of the storage are accepted by consumers and show lower oxidation values than control heme iron cookies.

6.5. Characterization of the heme iron ingredients: physicochemical properties

The manufactured co-spray-dried heme iron presented several differences in comparison with control heme iron ingredient. These differences included different colour and different ability of the powders to disperse in water and lipid matrixes. Therefore, we decided to measure the different colour of the heme iron ingredient powders after their manufacture. Also, the evolution of the colour of the ingredients with storage time at room temperature in the dark was measured in the last study. In addition, the water absorption and water solubility indexes of the heme iron ingredients used in the last study were measured.

In table 6.6, are shown the values of L*, a* and b* of the different heme iron ingredients used in studies 3, 4 and 5. In each study, the colour of each heme iron ingredient was different from the others. Also, we find small differences when comparing the colour of the ingredients, including the AproFER 1000™ (control heme iron), from different studies.

Table 6.6: Lightness (L*), redness (a*) and yellowness (b*) of heme iron ingredients used in studies 3, 4 and 5 at the initial storage time.

Ingredient	*L**	*a**	*b**
Study 3			
Control AproFER 1000™	23.52 ± 0.09 ax[1]	3.08 ± 0.09 ay	-1.18 ± 0.05 ax
CAS1:1	29.32 ± 0.06 ey	4.75 ± 0.03 ez	3.72 ± 0.07 ey
CAS 2:1	27.38 ± 0.03 dx	3.96 ± 0.04 cx	2.14 ± 0.01 dx
ST-MD 1:1	25.21 ± 0.14 c	4.22 ± 0.02 d	1.23 ± 0.04 c
ST-MD 2:1	23.81 ± 0.15 b	3.53 ± 0.12 b	0.12 ± 0.10 b
Study 4			
Control AproFER 1000™	24.70 ± 0.10 az	3.37 ± 0.05 bz	-0.21 ± 0.08 az
CAS 1:1	30.37 ± 0.14 cz	4.44 ± 0.04 cy	4.94 ± 0.07 cz
CAS 2:1	28.97 ± 0.06 by	4.38 ± 0.01 cy	3.65 ± 0.03 by
Calcium caseinate	95.63 ± 0.08 d	-1.04 ± 0.01 a	5.30 ± 0.03 d
Study 5			
Control AproFER 1000™	23.90 ± 0.05ay	1.99 ± 0.02 ax	-1.23 ± 0.03 ay
CAS 1:1[9]	26.30 ± 0.04 bx	4.04 ± 0.05 bx	1.98 ± 0.04 bx

[1] Mean ± SD, n=4.

Values corresponding to a certain factor within a study bearing different letters differ significantly ($p \leq 0.05$). "a, b c and d" denote differences between heme iron ingredients from the same study. "x, y and z" denote differences of the heme iron ingredients from the different studies.

CAS 1:1 stands for heme iron ingredient co-spray-dried with calcium caseinate at 1:1 ratio; CAS 2:1 stands for heme iron ingredient co-spray-dried with calcium caseinate at 2:1 ratio; ST-MD 1:1 stands for heme iron ingredient co-spray-dried with a mixture of starch and maltodextrin (50:50, w/w) at 1:1 ratio; ST-MD 2:1 stands for heme iron ingredient co-spray-dried with a mixture of starch and maltodextrin (50:50, w/w) at 2:1 ratio.

In general, we can describe the heme iron ingredients as dark (L* values close to 0 are black whereas values close to 100 are white). In order to illustrate this issue further, in Figure 6.16 are presented images corresponding of each heme iron ingredient.

Figure 6.16: Colour differences of heme iron ingredients: (A) Control heme iron ingredient, AproFER 1000™; (B) heme iron co-spray-dried with calcium caseinate at 1:1 ratio (w/w) and (C) heme iron co-spray-dried with calcium caseinate at 2:1 ratio (w/w).

However, the co-spray-dried heme iron ingredients present slightly higher values of L*, thus confirming that they are whither than the used control heme iron. As for a* values, these provide information about the redness of the product (positive values tend to be red whereas negative values tend to be green), whereas b* values provide information about the yellowness of the product (positive values tend to be yellow and negative tend to be blue). In all cases, the co-spray-dried ingredients presented higher a* and b* values than the control heme iron ingredients, thus indicating its tendency to more reddish and yellowish colour than the control heme iron ingredient (AproFER 1000™).

Finally, regarding the colour evolution of the heme iron ingredients along the storage time, from results of study 5 we can affirm that the colour of the ingredients slightly changes along the storage time at room temperature in the dark. However, these changes do not follow a clear pattern over storage time (Table 6.7).

Table 6.7: Lightness (L*), redness (a*) and yellowness (b*) determined on heme iron ingredients at the different storage times

Time	*L**	*a**	*b**
AproFER 1000™			
0 days	23.90 ± 0.05 a[1]	1.99 ± 0.02 a	-1.23 ± 0.03 a
90 days	24.04 ± 0.04 b	3.03 ± 0.14 d	-1.19 ± 0.03 a
180 days	23.85 ± 0.04 a	2.35 ± 0.07 b	-1.21 ± 0.05 a
360 days	24.00 ± 0.06 b	2.85 ± 0.08 c	-0.69 ± 0.04 b
CAS 1:1			
0 days	26.30 ± 0.04 a	4.04 ± 0.05 b	1.98 ± 0.04 b
90 days	26.41 ± 0.07 b	4.29 ± 0.04 d	1.72 ± 0.02 a
180 days	26.37 ± 0.02 ab	4.17 ± 0.04 c	1.79 ± 0.01 a
360 days	26.35 ± 0.02 ab	3.84 ± 0.03 a	2.11 ± 0.06 c

[1] Mean ± SD; Values in the same column, corresponding to a certain ingredient, with different letters differ significantly with storage time ($p \leq 0.05$), n=4.
CAS 1:1 stands for heme iron ingredient co-spray-dried with calcium caseinate at 1:1 ratio

With respect to the water absorption and water solubility indexes of both ingredients (AproFER 1000™ and the heme iron co-spray-dried with calcium caseinate at 1:1 ratio), the co-spray-dried heme iron presented both higher water absorption and solubility indexes than the control heme iron ingredient (Table 6.8). Therefore, the co-spray-dried heme iron was more soluble and absorbed more water than the control heme iron ingredient AproFER 1000 ™. As explained in the experimental design and methodology section, the AproFER 1000 ™ ingredient is a peptidic compound and these findings suggest a likely exposure of more lipophilic amino acids towards the surface, while when the ingredient is co-spray dried with calcium caseinate the particle surface may be more hydrophilic and hydratable.

Table 6.8: Water absorption index (WAI) and water solubility index (WSI) of both ingredients used in study 5

	WAI (g water/g ingredient)	*WSI (%)*
Control AproFER 1000™	0.89 ± 0.12[1]	11.82 ± 1.87
CAS 1:1	1.43 ± 0.16	16.16 ± 1.10

[1] Mean ± SD, n=5.
CAS 1:1 stands for heme iron ingredient co-spray-dried with calcium caseinate at 1:1 ratio

7. CONCLUSIONS

The overall conclusions that can be drawn from the obtained results have been summarized as follows:

- **With respect to the antioxidant addition** strategy in a palm oil model for bakery products (heated at 220 °C for 10 min and thereafter stored at room temperature in the dark for 200 or 360 days) (studies 1, 2 and 4).

  - The addition of tocopherol extract was ineffective in delaying the onset of oxidation in a heme iron fortified palm oil model with low free iron content (4%); neither when it was added alone nor when it was combined with other antioxidants (citric acid and ascorbyl palmitate) (study 1).

  - The addition of ascorbyl palmitate delayed the onset of both primary and secondary oxidation during the storage of a heme iron fortified palm oil matrix with low free iron content (2-4%). Under these conditions, ascorbyl palmitate at high concentrations (400-500 mg/kg of palm oil) delayed the oxidation onset more effectively than at low concentrations (100 or 200 mg/kg) (studies 1 and 2).

  - Ascorbyl palmitate was ineffective in delaying the onset of oxidation during the storage of a heme iron fortified palm oil matrix with high free iron content (27%)(study 4).

  - Regardless of the free iron content, ascorbyl palmitate was able to protect the heme iron fortified palm oil from oxidation during the heating treatments applied prior to storage for sample preparation and to mimic baking conditions (studies 1, 2 and 4).

  - The addition of citric acid to a heme iron fortified palm oil with low free iron content (2-4%) did not delay the oxidation onset of the matrix, even showing a pro-oxidant behaviour in some cases (studies 1 and 2).

  - The synergistic effect between ascorbyl palmitate and citric acid was finally discarded in heme iron fortified palm oil, when the free iron content was low (2%)(study 2).

- **With respect to the co-spray-drying of heme iron strategy** in lipid models (heated at 220 °C for 10 min and stored thereafter) (studies 3 and 4).

  - The addition of heme iron co-spray-dried with calcium caseinate or with a mixture of modified starch and maltodextrin (50:50, w/w) at 1:1 and 2:1 ratios (heme iron concentrate : co-spray-drying agent, w/w) in sunflower oil delayed the onset of both primary and secondary oxidation when this lipid mixture was stored at 60 °C (free iron content, 6-10%). Moreover, the most effective co-spray-drying agent under these conditions was calcium caseinate (study 3).

  - The co-spray-drying of the heme iron with calcium caseinate at any ratio (2:1 or 1:1, heme iron concentrate : calcium caseinate, w/w) did not delay the onset of oxidation

during the storage at room temperature in the dark of a heme iron fortified palm oil matrix with high free iron content (27%)(study 4).

- **With respect to the combination of strategies** in a palm oil model for bakery products (heated at 220 °C for 10 min and stored thereafter) (study 4).

  - The combination of the addition of ascorbyl palmitate (400 mg/kg of palm oil) and heme iron co-spray-dried with calcium caseinate at 1:1 ratio protected from oxidation in a palm oil model stored in the dark for one year at room temperature (free iron content, 27%); which indicates synergism between ascorbyl palmitate and the co-spray-dried heme iron ingredient.

- **With respect to the combination of strategies in sandwich-type cookies filled with a chocolate cream fortified with heme iron** (study 5).

  - The addition of ascorbyl palmitate (400 mg/kg of palm oil) to heme iron fortified chocolate creams used to fill sandwich-type cookies prevented the loss of tocopherols and tocotrienols during the preparation of these cookies.

  - During storage at room temperature in the dark of heme iron fortified sandwich-type cookies (with 14% free iron content), the formation of primary oxidation compounds and hexanal is minimized by the addition of ascorbyl palmitate, either alone or in combination with the heme iron co-spray-dried with calcium.

  - Consumers accepted those cookies containing co-spray-dried heme iron alone or in combination with ascorbyl palmitate after 360 days of storage at room temperature in the dark and, in addition, they found no differences with non-fortified heme iron cookies. Therefore, those cookies that combined the addition of ascorbyl palmitate and the co-spray-drying of heme iron not only recorded lower oxidation values than control heme cookies but also showed better acceptability scores after storage.

  - The colour of freshly produced chocolate creams fortified with heme iron ingredients differed from creams of commercial cookies. However, the freshly produced experimental cookies were accepted by the consumers probably because the colour of the heme iron fortified chocolate fillings was similar to that of chocolate bars with high cocoa percentages (72-85%).

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9. ANNEXES

9.1. List of abbreviations

^{1}Sen*: Singlet state molecule
^{3}Sen*: Triplet state molecule
AH: Chain breaking antioxidant
ANOVA: Variance Analysis
AOAC: Association of Official Analytical Chemists
AOCS: American Oil Chemistry Society
AP: Ascorbyl Palmitate
AUC: area under the curve of LHP formation
BC: Before Christ
BHA: Butylated hydroxyanisole
BHT: Butylated hydroxytoluene
BMI: Body Mass Index
CA: Citric Acid
CAS: Calcium Caseinate
CHP: Cummene Hydroperoxide
CIE: Commission International de L'Eclairage (Illumination)
COFEPRIS: Mexican Federal Commission for the Prevention of Health Risks
CVD: Cardiovascular Disease
DALY: Disability-Adjusted Life Years
DMSO: Dimethyl Sulfoxide
DRI: Daily Recommended Intake
EDTA: Ethylenediaminetetraacetic acid
EFSA: European Food Safety Authority
EU: European Union
FAME: Fatty Acid Methyl Esters
FAO: Food and Agriculture Organization
Final LHP: final LHP value measured achieved when the absorbance at 560 nm was stable
FOSHU: Food for Specified Health Uses
FOX: Ferrous Oxidation Xylenol-Orange
HIP: Heme Iron Polypeptide
Hb: Haemoglobin
HPLC: High Pressure Liquid Chromatography
HPMC: Hydroxi Propyl Methyl Cellulose
ID: Iron deficiency
IDA: Iron deficiency anaemia
Initial LHP: initial LHP value measured after 30 min of incubation
LHP: Lipid Hydroperoxide
MAXLHP: maximum LHP value
MI: Micronutrient Initiative
***p*-AnV:** *p*-Anisidine Value
PG: Propyl gallate

PV: Peroxide Value
RSD: Relative Standard Deviation
SEM: Standard Error of the Mean
SHS: Static Head Space
ST-MD: Mixture modified starch (octenyl succinate) and maltodextrin, 1:1, w/w.
TBHQ: Tertiary-butylhydroquinone
TE: Tocopherol extract
TMAX: time until the MAXLHP was achieved
UIL: Upper Intake Levels
UNICEF: United Nations Children's Fund
USA: United States of America
WAI: Water Absorption Index
WHO: World Health Organization
WSI: Water Solubility Index

9.2. List of tables

9.2.1. TABLES IN SECTION 2

9.2.2. TABLES IN SECTION 4

9.2.3. TABLES IN SECTION 6

9.3. List of figures

9.3.1. FIGURES IN SECTION 2

9.3.2. FIGURES IN SECTION 4

9.3.3. FIGURES IN SECTION 6

9.4. Answer sheet given to the panel in the sensory analyses

9.4.1. ANSWER SHEET TRANSLATED TO ENGLISH

TEST 1

NAME:
DATE:
DATE OF BIRTH:

Taste the samples in the same order as they are presented in this answer sheet.
Write down the number of the samples in the boxes from 1 to 9 according to your preference.
You may write down the difference (if any) in the colour, flavour or texture in the comments section.

173	529	358	693	788

Overall marks

I don't like it at all			Neither like nor dislike					I like it very much
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Comments

- **Colour**

- **Flavour**

- **Texture**

9.4.2. ORIGINAL FORMAT GIVEN TO PARTICIPANTS

TEST 1

NOMBRE:
FECHA:
FECHA DE NACIMIENTO:

Probar las muestras en el mismo orden como se presentan en la hoja de respuestas.
Anotar el número de las siguientes muestras en las casillas del 1 al 9 según su grado de preferencia. Una vez anotada la preferencia, si observas alguna diferencia de color aroma o textura la puedes anotar en la sección de comentarios.

173	*529*	*358*	*693*	*788*

Valoración global

No me gusta nada				*Ni me gusta ni me disgusta*				*Me gusta mucho*
1	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***

Comentarios

- ***Color***

- ***Aroma/Sabor/Flavor***

- ***Textura***

9.5. Rules given to the panel before attending to the sensory analyses

9.5.1. RULES TRANLATED TO ENLISH

Once the panel leader got in contact with the consumers for attending to the sensory analysis test the participant is selected in case of consuming chocolate products at least 12 times per year, must be in good conditions for doing the test at the agreed time and following the next rules:

- Do not smoke one hour before the settled time
- Do not have eaten any food product at least two hours before the analyses, especially coffee.
- Do not wear any perfume, soap or any other cosmetic with strong odour. Soap with no perfume or few perfumed must be used for washing hands. If not, hands should be dried as many times as needed for eliminating any odour.
- If any physiological disability, especially if either sense of smell or taste is affected, or the consumer is under any psychological effect that may impair the concentration in the sensory analyses, the consumer should communicate it to the panel leader. The panel leader must then decide to dismiss the consumer from the analyses or allow him/she to keep on the duty, taking into account the possibility of bias of the affected consumer from the average marks of the rest of the consumer.
- Once the consumer accomplishes the previous rules, he/she will take his/her place with order and silence.
- Once the consumer is seated, he/she must examine if the material needed is in the correct order, checking if the codes of the samples are the same as in the answer sheet.
- Read the instructions in the answer sheet, being aware of not starting the analyses before he/she has understood what is being asked. In case of doubt, the consumer must ask to the panel leader privately.

9.5.2. ORIGINAL FORMAT GIVEN TO PARTICIPANTS

Rebuda la comunicació per part del cap del panel per intervenir en un anàlisi sensorial, el participant serà seleccionat si consumeix productes similar amb xocolata 12 cops a l'any coma mínim, haurà d'estar en condicions de realitzar-lo a l'hora prèviament senyalada i atenir-se a les següents normes:
- S'abstindrà de fumar com a mínim una hora abans de l'hora fixada.
- No haver pres cap aliment al menys dues hores abans de realitzar la cata, i tampoc cafè.
- No utilitzarà cap perfum, sabó o altres cosmètics amb olor persistent. Pel rentat de les mans s'usarà un sabó no perfumat o poc perfumat procedint a esbandir les mans i assecar-les tants cops com sigui necessari per eliminar qualsevol olor.

- De trobar-se en condicions d'inferioritat fisiològica, particularment si té afectat el sentit de l'olfacte o del gust, o sota algun efecte psicològic que l'impedeixi concentrar-se en el seu treball, haurà de comunicar-ho al cap del panel per què l'aparti del treball, o bé per què prengui les decisions oportunes, tenint en compte la seva possible desviació dels valor mitjos de la resta del panel.

- El catador un cop complides les normes precedents, procedirà a ocupar el lloc que se li indicarà amb el major ordre i silenci possible.

- Un cop assegut procedirà a examinar si el material que necessita està en l'ordre correcte, comprovant si els codis de les mostres es corresponen amb els fulls de resultats.

-Llegirà detingudament les instruccions contingudes en el full de resultats, no començant l'examen de la mostra fins que estigui totalment assabentat del treball que ha de realitzar. En cas de dubte, ha de consultar privadament les dificultats trobades amb el cap del panel.

9.6. Fatty acid composition of palm oils

Fatty acid	*Study 1*	*Study 2*	*Study 4*	*Study 5*
10:0	0.05 ± 0.02[1]	0.03 ± 0.01	0.03 ± <0.01	0.02 ± <0.01
12:0	0.31 ± <0.01	0.28 ± <0.01	0.42 ± <0.01	0.18 ± <0.01
14:0	1.06 ± <0.01	1.12 ± 0.01	1.14 ± 0.01	1.04 ± <0.01
15:0	0.05 ± <0.01	0.05 ± <0.01	0.05 ± <0.01	0.05 ± <0.01
16:0	43.64 ± <0.01	44.11 ± 0.30	42.84 ± 0.1	43.27 ± 0.1
16:1n-9	0.03 ± <0.01	0.03 ± <0.01	0.03 ± <0.01	0.03 ± <0.01
16:1n-7	0.16 ± <0.01	0.17 ± <0.01	0.16 ± <0.01	0.16 ± <0.01
17:0	0.11 ± <0.01	0.10 ± <0.01	0.10 ± <0.01	0.10 ± <0.01
17:1	0.03 ± <0.01	0.03 ± <0.01	0.02 ± <0.01	0.02 ± <0.01
18:0	4.42 ± <0.01	4.36 ± 0.03	4.76 ± 0.02	4.63 ± <0.01
18:1t	0.12 ± <0.01	0.09 ± 0.01	0.11 ± 0.01	0.08 ±< 0.01
18:1n-9	37.90 ± <0.01	38.74 ± 0.20	38.71 ± 0.09	38.53 ± 0.05
18:1n-7	0.69 ± <0.01	0.66 ± 0.02	0.75 ± 0.06	0.77 ± 0.01
18:2n-6	10.52 ± <0.01	9.37 ± 0.05	9.99 ± 0.02	10.21 ± 0.01
18:3n-3	0.19 ± <0.01	0.14 ± <0.01	0.15 ± <0.01	0.17 ± <0.01
20:0	0.39 ± <0.01	0.38 ± <0.01	0.41 ± <0.01	0.39 ± <0.01
20:1n-9	0.14 ± <0.01	0.15 ± <0.01	0.15 ± <0.01	0.15 ± <0.01
20:2n-6	0.05 ± <0.01	0.03 ± <0.01	0.03 ± <0.01	0.05 ± 0.02
20:3n-6	0.08 ± <0.01	0.07 ± <0.01	0.08 ± <0.01	0.07 ± <0.01
24:0	0.08 ± <0.01	0.08 ± <0.01	0.08 ± <0.01	0.07 ± <0.01
SFA[2]	50.10 ± 0.01	50.52 ± 0.30	49.83 ± 0.08	49.75 ± 0.06
MUFA	38.94 ± <0.01	39.87 ± 0.20	39.93 ± 0.06	39.74 ± 0.06
PUFA	10.83 ± <0.01	9.62 ± 0.05	10.24 ± 0.01	10.51 ± 0.02
PUFA n-6	10.65 ± <0.01	9.47 ± 0.05	10.09 ± 0.01	10.34 ± 0.02
PUFA n-3	0.19 ± <0.01	0.14 ± <0.01	0.15 ± <0.01	0.17 ± <0.01
Trans FA	0.12 ± <0.01	0.09 ± 0.01	0.11 ± 0.01	0.08 ±< 0.01

[1] Mean (%)± SD, Study 1, n = 2, Study 2, n=4; Studies 4 and 5, n=5.

[2] SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, *Trans* FA = *trans* fatty acids

9.7. Tocopherol and tocotrienol composition of palm oils

	Study 1	Study 2	Study4	Study 5
Tocopherol content [mg/kg]				
Alpha	124.1±5[1]	141.0±5.6	188.8±3	214.8±9
Beta	2.2±0.1	2.4±0.07	1.4±0.2	1.2±0.3
Gamma	6.4±0.3	3.0±0.3	3.0±0.1	2.6±0.3
Delta	1.10±0.09	ND[2]	ND	ND
Total tocopherol	133.8 ±6	146.4±5.6	193.2±3	218.6±9
Tocotrienol content [mg/kg]				
Alpha	173.3±7	155.2±5.5	213.2±8	246.4±9
Beta	24.4±0.8	9.8±0.8	21.4±0.2	22.5±2
Gamma	182.1±6	148.5±7.3	192.4±5	176.5±9
Delta	17.3±0.7	9.2±0.3	12.1±0.2	9.9±1.2
Total tocotrienol	397.1±14	322.7±11.5	439.2±13	455.3±20
Total tocopherol + tocotrienol	530.9±14	469.1±17	632.4±14	673.9±29

[1] Mean ± SD; Study 1, n=4; Study 2, n=6; Study 4, n=5; Study 5, n=3.

[2] ND: not detected