Phenolic Compounds: Their Role During Olive Oil Extraction and in Flaxseed – Transfer and Antioxidant Function

Luz Stella Artajo Medina
Food Engineer

A thesis submitted to the Faculty of Agromical Engineer of the University of Lleida in partial fulfillment of the requirements of the degree of Doctorate of Philosophy

Supervisors:
María José Motilva Casado, Ph.D
María Paz Romero Fabregat, Ph.D

University of Lleida
Agronomical, Forestal and Food Systems Doctorate Program
Food Technology Department
Lleida, Spain, 2006

3. GENERAL RESULTS AND DISCUSSION

There is a rapidly growing area of research concerning the isolation and identification of plant secondary metabolites and their potential effects on human health. The general purpose of the projects collected in this doctoral thesis was to highlight the role of phenolic compounds during the olive oil extraction process at both plant pilot and industrial levels and to investigate their function as antioxidants in olive oil matrices. This project also investigated the role of the phenolic fraction of the flaxseed system and their antioxidant effects.

GENERAL CONTEXT

Previous works performed by the Fats & Oils laboratory group of the University of Lleida covered different aspects of study including changes in the HPLC phenolic profile of Arbequina olive oil from trees grown under different deficit irrigation strategies (Romero *et* al, 2002) and the activity of L-Phenylalanine ammonia-lyase (PAL) in developing olive fruit (Tovar *et al*, 2002)**.** At the same time, the composition of virgin olive oil with Protected Designation of Origin "Les Garrigues" in relation to the crop season (Romero *et al*, 2003) and the influence of regulated deficit irrigation strategies applied to olive tress on oil composition during fruit ripening were studied (Motilva *et al*, 2000). It was concluded that regulated deficit irrigation applied to Arbequina olive accelerated fruit ripening and affected fruit and oil composition during the early stages of ripening. Additionally, the content of secoiridoid derivatives 3,4-DHPEA-EDA, *p*-HPEA-EDA and 3,4-DHPEA-EA increased under water stress conditions, whereas vanillin concentration increased with the increase of the water applied. Lignans content was lower in the oils from the least irrigated treatments. Therefore, the sensorial characteristics and antioxidant capacity were also affected. PAL activity and the polyphenol and *ortho*-diphenol content of olive flesh decreased with fruit ripening and were affected by irrigation. The positive correlation observed between PAL activity and the phenolic content of olive flesh could evidence that

this enzyme conditioned the phenolics concentration in virgin olive oil. Subsequently, the effect of freeze injuries in olive fruit on virgin olive oil compositions and the effect of maturation process of the phenolic fraction of drupes and oils from Arbequina, Farga and Morrut cultivars were investigated (Morelló *et* al, 2003; Morelló *et* al, 2004a). Some conclusions from these studies showed an important decrease in the concentration of secoiridoid derivatives and 3,4-DHPEA-AC, which played a remarkable role in oil stability and sensorial attributes such as bitter index. Moreover, it can be noted that olive oil from the last stages olive fruit maturation presented lower levels of antioxidants such as α-tocopherol and phenol content. Significant differences have been shown in the phenolic content of olive drupes, mainly hydroytyrosol and oleuropein compounds decreased with the increase of the ripening degree. In addition, qualitative study of the minor fractions of virgin olive oil demonstrated a common pattern not depending on growing region and environmental conditions. Significant quantitative differences were observed in the composition of phenolic fraction. The most important differences were detected in hydroxytyrosol and tyrosol content with values higher in oils from Tarragona, probably due to growing region low altitude (Criado *et al*, 2004). Changes in commercial virgin olive oil (cv Arbequina) during 12 months of storage was also studied. There was a noticeable decrease in the secoiridoid derivatives and 3,4-DHPEA-AC, during 12 month storage after harvesting and lignans were the more stable phenolic compounds (Morelló *et a*, 2004b). A first study of the antioxidant activity of olive pulp and olive oil phenolic compounds of the Arbequina cultivar was performed. It was concluded that part of the antioxidative effects of phenolics present in olive pulp and olive oil can be explained by their radical scavenging activity. The radical scavenging properties of phenolic compounds assessed by the DPPH test seem to be explanatory factors, but not conclusive for the MeLo and liposome models (Morelló *et al*, 2005).

The flaxseed study completes the research projects performed by the Canadian Grain Commission referred to oilseeds. A method development of analysis of phenolic fraction in flaxseed and their potential function as antioxidants were the first approach for the Grain Research Laboratory in this area.

2.1 PHENOLIC COMPOUNDS DURING OLIVE OIL EXTRACTION ROCESS: EFFECT OF DIFFERENT TECHNOLOGICAL VARIABLES

Three important premises were considered in this part of study:

1. During the first steps of the oil extraction process the crushing of the olives produces a rupture of the cellular membranes releasing small drops of oil and obtaining a multiphasic system called olive paste phase (Ryan *et al*, 2001).

2. The antioxidant partition into phases is thermodynamically according to their affinities toward these phases. The proportion of phenolics in the three different phases (solids, oil, and water) depends on their relative polarities and the relative amounts of the phases (Rodis e*t al*, 2002).

3. The malaxation is the step in oil extraction that especially modifies their qualitative and quantitative composition (Montedoro *et al*, 1992).

It was also taken into account the reports of various authors about the effect of irrigation factor on virgin olive oil composition. Water stress during a specific period of the olive cycle, pit hardening and fruit growth, could influence the total amount of phenolic content as well as its profile. It is generally agreed that the level of phenolic compounds is higher in oils obtained from drought stressed crops than in those from irrigated crops, and that phenolic compounds in the oil are significantly affected by the irrigation regime. (Patumi *et al*, 2002; Tovar *et al*, 2001). Although, a little information is available in relation to transfer of phenolic compounds during olive oil processing in relation to different variables.

2.1.1. Transfer of phenolic compounds during olive oil extraction at pilot plant

The first studies of the doctoral thesis were related to the transfer of phenolic compounds at a pilot plant scale. Different variables during olive oil extraction were considered: ripening stage of the olive fruit, irrigation applied to olive trees (*Olea europaea* L), and addition of natural microtalc (NMT) during malaxation operation. Extraction of olive oil from olives of Arbequina variety was performed by using the Abencor system. The transfer and transformations of phenolics were quantitatively evaluated in all olive matrices. Analytical strategies dealing with the bioactive phenols in olive matrices were optimized in a first approach

for the recovery of these compounds. For most phenolics, the general analytical strategy involved recovery of the phenolic from the sample matrix followed by separation, identification and quantification. The phenolic fraction of olive paste and pomace phases were analyzed with a modified extracting method based on an ethanolic sample preparation followed by a methanolic purification. (Artajo *et al*, 2006) and separation by reversed-phase HPCL.

Effect of Ripening Index. Many modifications took place in olive compounds as a consequence of the maturation process. Further cellular destruction and mixing of the cellular content during olive oil extraction (crushing and malaxation) also produced significant changes in the composition of olive matrices. These transformations included hydrolysis of glycerides by lipases, hydrolysis of glycosides and oligosaccharides by glucosidases, oxidation of phenolic compounds by phenoloxidases, and polymerisation of free phenols as reported by Ryan and Robards (1998). Crushing implied the release of secoiridoid aglycons such as 3,4-DHPEA-EDA, *p*-HPEA-EDA and 3,4-DHPEA-EA due to the hydrolysis of oleuropein, demethyloleuropein and ligstroside. In addition to secoiridoid aglycons. Phenolic acids (caffeic, vanillic, and *p*-coumaric), phenolic alcohols (3,4-DHPEA and *p*-HPEA), lignans (acetoxypinoresinol and pinoresinol) and flavonoids (luteolin and apigenin) were detected in olive paste, pomace, wastewater and oil.

The transfer of phenolic compounds during the extracting process was evaluated using olive fruits at three different stages, from green to black stage, corresponding to ripening indexes of 2, 5 and 6. The concentration of hydroxytyrosol and derivatives showed a clear increase trend while increasing ripening index in olive paste and pomace phases. At the same time, a slightly decrease in the transfer from olive paste to pomace was observed for the majority of these phenolic compounds. Detection of an unknown high polarity compound was observed, probably a degradation product of the hydrolysis of secoiridoids, which increased its concentration in pomace with the increase of ripening index. The partition of vanillic and homovanillic acids between solid phases remained almost constant throughout the three different ripening indexes.

Oleuropein, the major phenolic compound in the pulp of many olive cultivars, was detected at low concentrations in the olive paste phases, suggesting an early degradation of this compound during crushing leading to the formation of new derivative compounds. Oleuropein concentration did not follow the expected decrease with the physiological development of the fruit during the green maturation phase. Even though, an increased activity of the hydrolytic enzymes with maturation could have happened. (Ryan *et al*, 2002; Amiot *et al,* 1989). Luteolin was the main flavonoid found in the olive paste, showing an increase with the raise of maturation index. The content of apigenin ant its glucosilated form was similar in the solid matrices.

Simple phenolics had comparable behaviours when their partition in liquid phases (oil and wastewater) was analyzed. A slight increase of hydroxytyrosol and tyrosol concentrations was observed in the oil phase with the increase of ripening degree. The transfer from olive paste (raw material) to liquid phases also increased between sampling. Vanillic remained constant in oil during all analyses, however, its transfer to liquid phases reached a maximum value in wastewater at the ripening index of 6. Secoiridoid derivatives, 3,4-DHPEA-EDA, *P*-HPEA-EDA, *p*-HPEA-EA, and, 3,4-DHPEA-AC were detected in both oil and wasterwater matrices, whereas 3,4-DHPEA-EA was only identified in oil. A great affinity of 3,4-DHPEA-EDA to wastewater should be remarked. Conversely, luteolin and apigenin were not transferred to wastewater.

These results emphasise the importance of the ripening index factor to produce virgin oil of different compositional characteristics. An increase in phenolic fraction could be obtained when olive fruit with high ripening index (RI of 6) is processed. It is important to highlight the hydroxytyrosol (3,4-DHPEA) content in all phases due to its potential antioxidant properties. 3,4-DHPEA-EDA concentration was also significant in the virgin olive oil obtained from olive fruit with RI equal to 6. In view of the results of this study, the next step was to analyze the effect of irrigation treatment on olive oil processing.

Effect of irrigation treatment.

The moisture and fat content in samples from olive fruits with a Ripening Index

value equal to 5 were analyzed. The differences between moisture and fat content in the solid phases obtained during olive oil extraction were considered to observe the behaviour of the phenolic compounds in each phase because of their hydro-soluble nature and their affinity toward aqueous or oil phases. The moisture content of olive paste phase in non- irrigation treatment was close to 47.1%, while the irrigated samples had moisture of 61.7%. The pomace phase presented values of moisture content around 62.5%, higher at irrigated regime. Fat content of 26.9% was found for olive paste phase in non-irrigated samples. The pomace obtained from non-irrigated samples reached 13.3% in fat content, three units higher than the results from samples issued of irrigated strategy.

The olive paste, pomace, olive oil and wastewater extracts obtained from Arbequina variety during olive oil extraction process presented different phenolic chromatographic profile. These compounds were classified in groups such as simple phenols, phenolic acids, aldehydes, secoiridoid, hydrocinnamic derivatives, secoiridoid derivatives, and flavonoids. The partition of phenolic compounds between the phases obtained during olive oil extraction process responded to a transfer and transformation that was observable through all groups of phenolic compounds found in olive paste, pomace, olive oil and wastewater.

A high reduction in polyphenolic content was observed in each phase of olive oil extraction (raw material and all by-products) under irrigation regime compared to non-irrigated. This is in agreement with results reported by Salas *et al* (1997) who found that phenolic concentration is reduced because of the irrigation, probably as a consequence of water stress produced by water reduction. Phenolic content from samples of non-irrigated olive fruits were higher than that of irrigated, with the exception of volatile compound vanillin, that remained constant in both non-irrigated and irrigated sampling.

Phenolic compounds found in solid phases in non-irrigation and irrigation sampling included simple phenols, phenolic acids, aldehydes, secoiridoids and flavonoids. 3,4-DHPEA specie found in olive paste phase in both non-irrigated and irrigated samples, was probably related to crushing operation, which allowed the bio-transformation of hydroxytyrosol compound. An unknown peak

with a 15.75 min of retention time was found. It showed spectral characteristics similar of those of oleuropein, suggesting a secoiridoid derivative compound. The origin of this product for catabolic or anabolic processes could be attributed to the activation of the endogenous β-glycosidases during crushing operation (Servilli *et al*, 2004; Ryan *et al*, 2002). Phenolic derivatives of secoiridoids compounds have not been identified in olive. Similarly, these compounds were not found in olive paste during this study. Irrigation treatment affects the occurrence of hydroxytytosol specie (simple phenolic), oleuropein, dimethyloleuropein, and verbascoside in paste. Water stress suffered by the trees could influences the synthesis of phenolic compounds in the fruit and in the subsequent olive paste obtained from it during crushing operation.

The prevalent phenolic compound found in the Arbequina olive paste phase was a flavonoid, luteolin, in both non-irrigated and irrigated sampling, followed by rutin (non-irrigated regime) and luteolin-7-glucoside (irrigated regime). Second most important group quantitatively was simple phenols with hydroxytyrosol and hydroxytyrosol specie. This was the first comparative study dealing with phenolic compounds in olive paste phase from irrigation and non-irrigation treatments.

There were significant differences between antioxidants compounds found in pomace phase obtained from olive fruits grown under non-irrigated and irrigated regimes. Luteolin was the major phenolic found in pomace phase. Simple phenols and flavonoids were the compounds of major interest in this part of the study. It was important to take into account the vanillin since its concentration increased in this phase relative to olive paste but remained relatively constant in non-irrigated and irrigated samples.

Many studies reported the presence of simple phenols such as hydroxytyrosol 3,4-DHPEA) and tyrosol (*p*-HPEA) in olive fruit. Vanillic acid, vanillin and *p*-coumaric acid, 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC) and lignans have also been found in different olive cultivars. Dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA), and oleuropein aglycon (3,4-DHPEA-EA) were found in previous investigations. Tovar *et al* (2001) showed that with an irrigation treatment of Kc=0.85 (crop coefficient), all phenols

occurring in olive oil at harvest time had lower concentrations as compared with those in irrigation treatment of Kc=0.25. However, in this study the phenols concentrations in the irrigation treatment did not reach similar values as those reported by Tovar *et* al (2001) likely due to the sampling period.

Oil composition was affected by irrigation treatment, especially polyphenol content. Oil stability increased with decreased water supply. As it is also known, phenylalanine ammonialyase (PAL, EC 4.3.1.5) catalyses the reductive deamination of L-phenylalanine into *trans*-cinnamic acid. This is the first step of the biosynthesis of plant phenylpropanoid compounds, which lead to the formation of lignin, flavonoids and hydroxycinnamic acids. It has been reported by Tovar *et al* (2002) that there is a probable correlation between changes in enzyme activities and changes in levels of phenolic compounds in the oil, the activity of this enzyme in the fruit varied as a result of changes in water status.

The total phenolic compounds between the phases were negatively affected by irrigation. The hypothesis that the different water content of the pastes could imply a different solubilisation of phenols, which are more soluble in water than in oil (Romero *et al*, 2002) could be confirmed at the present study due to the results obtained. This behaviour could not be attributed to a different degree of Ripening Index since the olive fruit presented the same Ripening Index as other authors reported (Patumi *et al*, 2002). Additionally of the results reported by Romero *et al* (2002), this study showed that irrigation strategy, with a fixed percentage of water applied during the whole growth cycle of the olive tree, affected the transfer from the olive paste to the olive oil during extraction process.

The results confirm the direct effect of agronomical practices on quality of virgin olive oil. At this point, it can be concluded that olive fruits from non-irrigated olive trees with RI equal to 5 have good quality characteristics to obtain virgin olive oil containing significant amounts of important phenolic compounds such as simple phenols and secoiridoid derivatives.

To complete the study about partition of phenolic compounds during olive oil extraction at pilot plant scale, the addition of Natural Micro-Talc was studied as

an important technological variable.

Effect of the addition of NMT. A similar profile of phenolic compounds in olive paste and pomace was obtained, including simple phenols, phenolic acids, aldehydes, secoiridoids and flavonoids.

The addition of the NMT directly influenced the phenolic compound concentration in oil. Significant increases in the solid phases, olive paste and pomace, was observed when the co-adjuvant was used. However, tyrosol reached its highest concentration in pomace obtained without addition of NMT during the extraction process. Olive oil and wastewater presented a high concentration of secoiridoid derivatives produced especially during malaxation operation. 3,4-DHPEA-AC was found in a greater amount in olive oil phase, while hydroxytyrosol, tyrosol and 3,4-DHPEA-EDA concentrations were higher in wastewater. Flavonoids glycosilated in 7 position were extracted only in wastewater, which was in agreement with results reported by García *et al* (2001) in Arbequina and Picual varieties. In general, phenolic compound concentration in olive oil increased with the use of NMT, this fact could confirm its positive effect on the phenolic extraction. However, vanillic acid and vanillin levels remained relatively constant in this phase. This results suggested that phenolic compounds present in the wastewater occurred in a higher concentration when the co-adjuvant was added during process.

In summary, it is possible to affirm that a control of some agronomical practices on olive trees growing areas such as irrigation treatment and selected picking could contribute to an enhancement of the phenolic content in all phases during olive oil processing. Irrigation appeared to be the most significant variable with effect on simple phenols and secoiridoid derivatives concentrations when compared with ripening index of olive fruit. In processing, NMT has a remarkable influence in 3,4-DHPEA-EDA released in the wastewater phase.

2.1.2 Partition of phenolic compounds during industrial olive oil process

The second study dealing with the partition of phenolic compounds was achieved

with samples from industrial olive oil process. Because of the complexity to scale the pilot plant conditions to industrial level, a direct study of the phenolic transfer was performed with a two-phase processing line. In this case, production period was considered a dependent agronomical variable to assess the main differences in the phenolic profile as well as in the quantification of the individual phenolics. In processing, phenolic compounds were evaluated at three times during malaxation operation.

Hydroxytyrosol and tyrosol, the most important phenolic alcohols occurring in olives, were detected in significant amounts in the olive paste. Hydroxytyrosol derivatives and secoiridoid compounds were also found in high quantities. Oleuropein and ligstroside were the most significant oleosides (secoiridoids characterised by an exocyclic 8,9-olefinic functionality) occurring in olive paste. This agreed with the information reported by Ryan *et al* (2002), who showed that these two compounds were the most important phenolics found in olive fruit. Previous studies of extraction systems showed that phenolic content varied in relation to the extraction methodology (Angerosa & Di Giovacchino, 1996; Ranalli & Angerosa, 1996). During this first operation, the crushing of the olives resulted in a breaking down of the cellular membranes releasing small drops of oil results in a multi-phased system. Crushing is a softer operation in which olive break determine the release of the oil. It is generally accepted that the crushing operation is the first step leading to the activation of the endogenous β-glucosidases present in the olive fruit. Oleuropein and demethyloleuropein act as substrates for this enzyme to give asolid stable aglycone product is then formed. In aqueous system, however, this product could be further degradated into other products (Limiroli *et* al, 1995).

After crushing operation some of the native oleosides of olive fruit were present in olive paste; one of the most important secoiridoid derivatives, 3,4-DHPEA-EDA, was detected in a significant concentration, suggesting the beginning of enzymatic activity. Hydrocinnamic derivative, verbascoside, was also found in olive paste as expected; its presence has been reported in peel, pulp and seed matrices (Ryan *et al*, 1999; Servilli *et al*, 1999; Brenes *et al*, 1995; Amiot *et al*, 1986; Gariboldi *et al*, 1986). Flavonoids such as luteolin 7-O-G and rutin have been reported only in olive peel. It was likely that mechanical operation resulted in the transfer of these compounds to both olive paste and wet pomace phases.

A lot of studies have shown the presence of the secoiridoid derivatives in olive oil matrix and wastewater. The presence of these compounds was an indicator of the degradation pathways of the phenolic oleosides. The degradation of oleuropein could be performed by two main routes: enzymatic cleavage by specific esterases or activation of the β-glucosidades. During hydrolysis the lypophilic compounds (secoiridoid aglycons) are transformed into hydrophilic substances (hydroxytyrosol and tyrosol) (Brenes *et al*, 2001). It has been assumed that some phenolic compounds found in olive oil are naturally occurring as a result of processing. In the two-phase extraction system water was added (ranging from 14%-26%) at the end of malaxation step. This could contribute to a degradation of the oleosides compounds, although the mechanism for the bio-transformations in terms of quantity is far from being elucidated completely. The final products of the different mechanisms proposed in previous works (De Nino *et al*, 2000; Bianco *et* al, 1999) were occurring at a lipidic/water interface, which results in complicated isomerisations and equilibrium. The antioxidant partition in the three different phases (wet pomace, oil and wastewater) depended on their relative affinity toward solid and liquid phases (Artajo *et al*, 2006; Rodis *et al*, 2002).

Differences found between samples infer that the production season time affects the total phenolic compound content and the phenolic profile of olive paste and virgin olive oil. 3,4-DHPEA-EDA content, by the far, presented the most important decrease with the increase of the production period. This could have implied the decrease in the substrates compounds of olive fruit responsible to the biosynthesis of secoiridoid derivatives.

In conclusion, it seemed to be relevant to control the production period as a crucial factor affecting the phenolic composition of all phases during olive oil extraction. This could help to obtain products with added value, in terms of their phenolic fraction.

3.2 FUNCTION OF PHENOLIC COMPOUNDS IN OLIVE OIL

Enrichment of Olive Oil with Phenolic Compounds.

In view of the nature of the analytical challenge to measure the antioxidant function of natural compounds, several methods can be used. Screening of bioextracts from matrices played a strategic role in the phytochemical investigation of olive extracts.

The next affirmation was considered the scientific support for this study.

The chemical properties of phenolic compounds in terms of the availability of the phenolic hydrogens as hydrogen-donating radical scavengers predicts their antioxidant activity (Rice-Evans *et al*, 1996).

The first approach involved the determination of previously identified phenolic compounds. This study was simplified to the preparation of an extract, HPLC analysis followed by a determination of the retention time, UV spectral characteristics, and mass spectral analysis of the phenolic compounds using a liquid chromatographic-mass spectrometry system when needed, and finally, the quantification of the phenolic compound. The second step included the preparation of an extract, fractionation, isolation and purification. The confirmation of structure elucidation of a new compound occurring in olive oil was also performed.

The addition of phenolics (standards and isolated) to food matrices was the base of the antioxidant research. Refined (ROO) and two extra virgin olive oils (EVOO) with different composition profile were used in the study. Individual and combined phenolic compounds were added to the lipid matrices and the antioxidant activity was determined trough the oxidative stability using a Rancimat equipment. This measure of the oxidative stability is a common practice used at industrial level that report reproducible data and could be established as quality parameter in oil processing companies.

Results showed that the antioxidative activity depended on the phenolic concentration and it was closely related to the chemical structure. Higher antioxidant activities were found with phenolic compounds that posess 3,4 dihydroxyl and 3,4,5-tryhydroxy structures linked to an aromatic ring (oleuropein, 3,4-DHPEA-EDA, and the methylated form of 3,4-DHPEA-EA. The difference found between the effect of the same phenolic on the ROO and EVOO matrices suggested that some interactions could take place with other components of the oils such as the initial phenolic and pigment content.

At this point with this first approach in the research on enrichment of food matrices, it is important to see if the food industry could be capable of produced olive oil-enriched products aimed to those seeking healthier food products.

3.3 PHENOLIC COMPOUNDS IN FLAXSEED

It is widely known the occurrence of phenolic compounds in plant foods is extremely variable, ranging from simple phenolic molecules to highly polymerized compounds. Majority of these compounds have relative low molecular weights and are soluble in water depending on their polarity and chemical structure: degree of hydroxylation, glycosylation or acylation.

The next affirmations were considered in this study:

1. Some phenolic compounds can be linked to cell wall components (polysaccharides, lignin). Owing to the nature of the ester linkages, these compounds can be solubilized in alkaline conditions or are otherwise retained in the fiber matrix. Phenolic compounds in flaxseed occur in association with fibre in plant cell walls.

2. Extraction of phenolic compounds in flaxseed is usually performed using organic solvents, acid or alkaline hydrolysis, or combination of these. Alkaline hydrolysis is mainly used to release secoisolariciresinol diglucoside (Milder *et al,* 2004).

3.2.1 Method for extracting phenolics

Two forces drove the application of alkaline hydrolysis treatments. Initially, many phenolics and mainly the phenolic acids exits in a wide range of conjugated forms and the free phenolic compounds are liberated following alkaline hydrolysis. Therefore, alkaline conditions have been employed in the isolation of phenolic acids from different samples including cereals (Andreasen *et al*, 2000) and oilseeds (Xu *et al*, 1997) and medicinal plants (Andrade *et al*,

1998) in order to determine bound phenolics. The loss of o-diphenols by oxidation via the corresponding quinones is a concern under alkaline conditions. In many cases, an inert atmosphere and addition of an antioxidant stabilizer was used as a routine precaution due to the poor stability of some phenolic acids in alkaline room conditions.

The research dealing with the phenolic compounds in flaxseed implied different analytical strategies. Studies on non-defatted and defatted seeds from flax and solin were performed to see the differences between samples. The experimental work showed that using non-defatted samples the total phenolic level (expressed as g caffeic acid/Kg seed) was higher when compared to deffated samples. Phenolic content of the oil from seed samples were also analyzed. Results demonstrated that phenolics are present in low quantities in the oil phase.

Diverse methods are reported for extracting phenolic in flaxseed. The most widely used extraction method in the literature was a single 15h stirred extraction in aqueous ethanol or dioxane. Results demonstrated that rapid triple extraction for 2 min with an electric homogenizer (Polytron type) yielded twice the phenolics obtained from a single stirred extraction. Triple extraction was found to have a much higher effect on yield than the additional particle size reduction achieved with the polytron. The literature reports that flaxseed phenolics are incorporated in a complex matrix and must be hydrolyzed for analysis. HPLC analysis and total phenolic assays showed that alkaline hydrolysis was an important step of flaxseed phenolic compound analysis.

It was of interest to study the effect of acid hydrolysis on flaxseed. Acid hydrolysis decreased the total phenolic compound content by about 50%. HPLC analysis of the acid hydrolyzed extracts showed a decrease in the lignan SDG without a corresponding increase in AHNSEC as reported in literature (Jonhson *et* al, 2002). Furthermore, acid hydrolysis of standard SDG resulted in a complex pattern of hydrolysis products that could not be easily quantified when following methods reported in the literature. HPLC analyses showed that *p*-coumaric acid glucoside and ferulic acid glucoside were the main phenolic compounds of solin seed whereas ferulic acid glucoside and SDG were the main phenolic compounds of flaxseed. Therefore, there was no a completely satisfactory procedure that was suitable for extraction of all phenolics compounds occurring in flaxseed.

In conclusion, it appears affirm that solubility of phenolic compounds is governed by the type of solvent (polarity) used, degree of polymerization, as well as interaction of phenolics with other food components and formation of insoluble complexes.

3.2.2 Flaxseed as an antioxidant system

The knowledge that flaxseed contains SDG and flaxseed oil contains the highest level of α-linolenic acid (ALA) when compare to the other oilseeds, was the guideline to perform the second study.

Solin (form of linum) contain less than 5% ALA compared to more than 50% in flaxseed oil; fatty acid composition has been modified to obtain a light and suitable oil for cooking. Lipid oxidation is responsible most for flavors deterioration in high fat content foods. Diverse parameters are involved in deterioration of seeds (mould, bacteria); in oilseeds this usually translated as an increase in the oxidation of the seed lipids.

Whole flax and ground flax were stable a room temperature for several days (Malcolmson *et al,* 2000). Therefore, the study involving the flaxseed system responded to a lately concern about the mechanism of the stability of samples of both whole and ground flax

The effect of dehulling on chemical composition and physical properties of flaxseed has been studied (Oomah & Mazza, 1997). Hull and dehulled samples were analyzed. The SDG was mainly found in the hull whereas *p*-coumaric acid and ferulic acid glycosides were found both in the hull and the dehulled meal. The meal was not completely dehulled, intact seeds and part of the hull was found in the sample, this might explain its relatively high content in SDG. A water extract containing phenolic compounds was prepared from defatted flaxseed to see its antioxidant effect, even if they were not implicated in the main flaxseed antioxidant system. The water-extracted meal and the water extract showed different total phenolic content and composition. SDG, *p*-coumaric acid glycoside and ferulic acid glycoside were mainly found in the water-extracted meal with little of these found in the water extract. The peroxide and aldehyde contents of the oil from the water-extracted meal increased. Hence, the results suggested that water extract strongly reduced the antioxidant properties of the flax meals.

Although the water extracts contained low level of phenolic compounds, an antioxidant effect was still observed. The peroxide and aldehyde content of the commercial flax oil stored in the same condition were doubled the peroxide and aldehyde contents of the oils supplemented with the water extracts. There was no relationship, however between the amount of extract and the antioxidant effect.

In summary, more studies involving flaxseed components and its relationships with oxidation are needed. It could lead to elucidate how non-phenolic compounds could be responsible for the antioxidant effect.

REFERENCES

1. Amiot, M.J.; Fleuriet, A.; Macheix, J.J. Accumulation of oleuropein derivates during maturation. *Phytochem* **1989,** *28*, 67-69.

2. Amiot, M.J.; Fleuriet, A.; Macheix, J.J. Importance and evolution of phenolic compounds in olive during growth and maturation *J Agric Food Chem* **1986,** *34*, 823-826.

3. Andrade, P.B.; Seabra, R.M.; Valentao, P.; Areias, F. Simultaneous determination of flavonoids, phenolic acids and coumarins in seven medicinal plants by HPLC/diode array deterctor. *J Liq Chrom & Rel Technol* **1998,** *21*, 2813-2817.

4. Andreasen, M.F.; Christensen, L.P.; Meyer, A.S.; Monsen, A. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye varieties. *J Agric Food Chem* **2000,** *48*, 2837-2842.

5. Angerosa, F. and Di Giovacchino, L. Natural antioxidants of virgin olive oil obtained by two and tri-phase centrifugal decanters. *Grasas y Aceites* **1996,** *47*, 247-254.

6. Artajo, L.S.; Romero, M.P; Motilva, M.J. Transfer of phenolic compounds during olive oil extraction in relation to ripening stage of fruit. *J Sci Food Agric,* **2006,** *86*, 518-527.

7. Bianco, A.D.; Muzzalupo, I.; Piperno, A.; Romeo, G.; Uccella, N. Bioactive derivatives of oleuropein from olive fruits. *J Agric Food Chem* **1999,** *47,* 3531-3534.

8. Brenes, M.; García, A.; García, P.; Garrido, A. Acid hydrolysis of secoiridoid aglycones during storing of virgin olive oil. *J Agric Food Chem* **2001,** *49*, 5609-5614.

9. Brenes, M.; Rejano, L.; Garcia, P.; Sanchez, A.H.; Garrido, A. Biochemical Changes in Phenolic Compounds during Spanish-Style Green Olive Processing. *J Agric Food Chem* **1995,** *43,* 2702-2706.

10. Criado, M.N.; Morelló, J.R.; Motilva, M.J.; Romero, M.P. Effect of growing area on pigment and phenolic fractions of virgin olive oils of the Arbequina variety in Spain. *JAOCS, J Am Oil Chem Soc* **2004,** *81,* 633-640.

11. De Nino, A.; Mazzotti, F.; Perri, E. Procopio, A.; Rafaeeli, A.; Sindona, G. Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from *Carolea* and *Cortina* cultivars by ionspray ionization tandem mass spectrometry. *J Mass Spectrom* **2000,** *35,* 461-467.

12. García, A.; Brenes,M.; Martínez, F.; Alba, J.; García, P.; Garrido, A. High-performance liquid chromatography evaluation of phenols in virgen olive oil during extraction at laboratory. *JAOCS, J Am Oil Chem Soc* **2001**, *78,* 625-629.

13. Gariboldi, P.; Jommi, G.; Vrotta, L. Secoiridoids from *Olea europaea*. *Phytochem* **1986,** *25,* 865-869.

14. Johnsson, P.; Peerlkamp, N.; Kamal-Eldin, A.; Andersson, R.E.; Andersson, R.; Lundgren, L.N.; Aman, P. Polymeric fractions containing phenol glucosides in flaxseed. *Food Chem* **2002,** *76,* 207-212

15. Limiroli, R.; Consonni, R.; Ottolina, G.; Marsilio, V.; Bianchi, G.; Zetta, L. ^{1}H and ^{13}C NMR characterization of new oleuropein aglycones. *J Chem Soc* **1995,** *1,* 1519-1523.

16. Malcolmson, L.J.; Pryblski, R.; Daun, J.K. Storage stability of milled flaxseed. *JAOCS, J Am Oil Chem Soc* **2000**, *77,* 235-238.

17. Milder, I.E.J.; Arts, I.C.W.; Venema, D.P.; Lasaroms, J.J.P.; Wähälä, K.; Hollman, P.C.H. Optimization of a liquid chromatography-tandem mass spectrometry method for quantification of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in foods. *J Agric Food Chem* **2004,** *52*, 4643-4651.

18. Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. Simple and hydrolyzable compounds in virgen olive oil. II Initial characterization of the hydrolyzable fraction. *J Agric Food Chem* **1992,** *40*, 1577-1580.

19. Morelló, J.R.; Motilva, M.J.; Ramo, T.; Romero, M.P. Effect of freeze injuries in olive fruit on virgen olive oil composition. *Food Chem* **2003,** 81, 547-553.

20. Morelló, J.R.; Romero, M.P.; Motilva, M.J. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from Arbequina, Farga, and Morrut Cultivars. *J Agric Food Chem*, **2004a,** *52,* 6002-6009.

21. Morelló, J.R.; Motilva, M.J.; Tovar, M.J.; Romero, M.P. Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chem*, **2004b**, *85*, 357-364.

22. Morelló, J.R. Vuorela, S.; Romero, M.P.; Motilva, M.J.; Heinonen, M. Antioxidant activity of olive pulp and olive oil phenolic compounds of the Arbequina cultivar. *J Agric Food Chem,* **2005,** *53*, 2002-2008.

23. Motilva, M.J.; Tovar, M.J.; Romero, M.P.; Alegre, S.; Girona, J.G. Influence of regulated deficit irrigation strategies applied to olive trees (*Arbequina* cultivar) on oil yield and oil composition during the fruit ripening period. *J Sci Food Agric* **2000,** *80*, 2037-2043.

24. Oomah, B.D. and Mazza, G. Effect of dehulling on chemical composition and physical properties of flaxseed. *Food Sci Technol* **1997,** *30,* 135-140.

25. Patumi, M.; D'Andria, R.; Marsilio, V.; Fontanazza, G.; Morelli, G.; Lanza, B. Olive and olive oil quality after intensive monocone olive growing (Olea europaea L., cv. Kalamata) in different irrigation regimes. *Food Chem*, **2002,** *77*, 27-34.

26. Ranalli, A. and Angerosa, F. Integral centrifuges for olive oil extraction. The qualitative characteristics of products. *JAOCS, J Am Oil Chem Soc* **1996**, *73,* 417-422.

27. Rice-Evans, C.; Miller, N.J.; Paganga, G. Structural antioxidant activity relationships of flavonoids and phenolics acids. *Free Rad Bio & Med* **1998,** *20,* 933-956.

28. Rods, P.S.; Karathanos, V.T.; Mantzavinou, A. Partition of olive oil antioxidants between oil and water phases. *J Agric Food Chem* **2002,** *50,* 596-601.

29. Romero, M.P.; Tovar, M.J.; Girona, J.; Motilva, M.J. Changes in the HPLC phenolic profile of virgin olive oil from young trees (Olea europaea L. cv. Arbequina) grown under different deficit irrigation strategies. *J Agric Food Chem* **2002,** *50,* 5349-5354.

30. Romero, M.P.; Tovar, M.J.; Ramo, T.; Motilva, M.J. Effect of crop season on the composition of virgin olive oil with protected designation of origin "Les Garrigues". *JAOCS, J Am Oil Chem Soc* **2003,** *80,* 423-430.

31. Ryan, D. and Robards, K. Phenolic compounds in olives. *Analyst* **1998,** *123,* 31R-44R.

32. Ryan, D.; Antolovich, M.; Prenzler, P.; Robards, K.; Lavee, S. Biotransformations of phenolic compounds in *Olea europaea* L. *Sci Hort* **2002,** *92,* 147-176.

33. Ryan, D.; Robards, K.; Lavee, S. Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. *J Chromatogr A* **1999,** *832,* 87-96.

34. Salas, J.M.; Castor, P.J.; Vega, V. Influencia del riego sobre la composición y caracaterísticas organolépticas del aceite de oliva. *Grasas y Aceites*, **1997,** *48*, 74-82.

35. Servili, M.; Baldioli, M.; Selvaggini, R.; Macchioni, A.; Montedoro, G. Phenolic compounds of olive fruit: One- and two-dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit. *J Agric Food Chem* **1999,** *47,* 12-18.

36. Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. Health and sensory properties of virgin olive oil hydrophilic phenols: Agronomic and technological aspects of production that affect their occurrence in the oil. *J Chromatogr A* **2004,** *1054,* 113-127.

37. Tovar, M.J.; Motilva, M.J.; Luna, M.; Girona, J.; Romero, M.P. Analytical characteristics of virgen olive oil from young trees (*Arbequina* cultivar) growing under linear irrigation strategies. JAOCS*, J Am Oil Chem Soc* **2001,** *78,* 1-7.

38. Tovar, M.J.; Romero, M.P.; Girona, J.; Motilva, M.J. L-Phenylalanine ammonia-lyase activity and concentration of phenolics in developing olive (*Olea europaea* L cv Arbequina) fruit grown under different irrigation regimes. *J Sci Food Agric* **2002b,** *82*, 892-898.

39. Xu, L.; Diosady, L.L. Rapid method for total phenolic acid determination in rapeseed/canola meals. *Food Research Int* **1997,** *30*, 571-574.