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TITOLO TESI

# VALORIZATION OF POMACES FROM THE MECHANICAL EXTRACTION OF VIRGIN OLIVE OILS IN DAIRY ANIMAL FEEDING

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**Esame finale anno 2013**

*Ai miei figli*

*Gaia e Francesco*

## Table of Contents

# 1. INTRODUCTION

The olive (*Olea europaea* L.) is one of the earliest fruit trees to be cultivated and certainly one of the most important fruit trees in history. The evaluation of archeological remains and the examination of wild relatives suggests that olive tree was most likely domesticated in the Near East (Palestine, Israel, Jordan) in the third and fourth millennia B.C. (Galili *et al*., 1988; Zohary and Spiegel-Roy, 1975), from the wild oleaster through nine domestication times (Breton *et al*., 2009). Wild olives are distributed over the entire Mediterranean basin and have a small fruit size and low oil content. The main changes in olives under domestication include the increase of fruit production and growth ring enlargement; this development involves the fleshy and oil containing mesocarp (Terral, 1996). Olive production developed along the coastal and subcoastal areas of the eastern Mediterranean sea, including southern European and northern African countries, spreading later with the Romans to the northern areas of Italy, Spain, France and the Balkans (Blázquez Martínez, 1996). Olive production began in Italy in the first millennium B.C. thanks to, first, the Phoenicians, and later, the Greeks. The passage of the olive to the Roman world (Acerbo, 1937; Smartt and Simmonds, 1988) is reported, to be from Sicily, around the sixth century B.C., probably through Etruria (Boardman, 1977).Olives are long-living with a life expectancy of over 500 years. In the past seedling olive trees required many years before producing the first crop of fruit, hence, the olive tree is synonymous with peaceful endeavors in a stable society, important requirements for population growth. Therefore, since the beginning, the olive has always been considered a symbol of peace and abundance in the historical and in religious events which evolved over the centuries.

## 1.1. Olive oil and the Mediterranean diet

The olive is the most extensively cultivated fruit crop in the world counting 9.49 million hectares of harvested area in 2010 (FAOSTAT, 2012) and its cultivation area has tripled in the past 50 years. Italy ranks second in the world, after Spain, with 24% of the world

production of olive oil and 1,190,800.000 million hectares of harvested area in 2010 (FAOSTAT, 2012). The olive in Italy is very important for both economic and environmental purposes. Olive-groves contribute to prevent soil erosion in hilly areas, provide landscape related environmental benefits, and tourism related economic aspects. Italian olive biodiversity is a peculiar element of the Italian landscape with over 500 varieties, approximately 42% of world heritage, even though the Italian olive growing is based on approximately only 50 varieties (Santilli *et al*., 2011). The presence on the national territory of a large number of cultivars provides the peculiarity of the sensory profiles of Italian extra-virgin olive oils, as it consists of different characteristics depending on the regions, resulting from the interaction between genotype and territory.

Virgin olive oil plays an important and essential role in the Mediterranean diet. The term "Mediterranean diet" reflects food patterns typical of Crete, much of the rest of Greece and southern Italy in the early 1960s and is based mainly on the fact that in these areas the life expectancy of the populations is among the highest in the world and there is a lower incidence of coronary heart disease than that seen throughout the rest of the world (Willett, 1995). Keys *et al.*, (1984; 1986) conducted the 'Seven Countries Study' on this subject; this provided evidence that the reduced risk of coronary heart disease (CHD), degenerative diseases, cancer of the breast and colon were associated with the Mediterranean diet. In particular, it emerged that the number of deaths due to coronary heart disease was low in the trial groups using olive oil as the main fat (Keys *et al*., 1986). This study inspired much research on the combination pattern of the "Mediterranean diet", analysing the individual foods of the diet in relation to the health status of the population (Hu, 2002). Higher adherence to a Mediterranean diet, associated with other lifestyle factors, predicts increased longevity especially when consuming wholegrain cereals, foods rich in polyunsaturated fatty acids. Tognon *et al*., (2011) showed the beneficial role of this diet on the incidence of Parkinson's disease and Alzheimer's disease (Sofi *et al*., 2008).

The Mediterranean diet of the early 1960s was characterized by high intake of a large amounts of plant food such as vegetables, legumes, fruits, nuts and seeds, cereals (unrefined in the past), minimally processed, seasonally fresh and locally grown. A moderately high intake of fish, a moderate intake of dairy products (mostly cheese and

yogurt), a low intake of meat and meat products, a regular and moderate intake of ethanol in the form of wine and generally during meals, and a high intake of olive oil as the principal source of fat (Kromhout *et al*., 1989; Willett *et al*., 1995).

## 1.2. Extra-virgin olive oil: chemical composition

Extra virgin olive oil (EVOO) is obtained exclusively by mechanical extraction from the olive fruit and can be consumed crude without any further physical-chemical treatments of refining. The chemical composition of EVOO is strongly affected by the agronomic and technological conditions of its production. Several parameters can modify its composition such as geographical and genetic origin, cultivar, fruit ripening, pedoclimatic conditions of production, some agronomic techniques, processing system and storage of the oil.

The chemical composition of VOO is characterized by the presence of two main groups: the major components (98-99% of the total oil weight), and the minor components (1-2% of the total oil weight). The major components represents more than 98% of the total oil weight include mainly triacylglycerols (Gunstone *et al*., 1994) and small concentrations of diacylglycerols, monoacylglycerols and some free fatty acids. The key aspect of this fatty fraction is the low concentration of saturated fatty acids (SFAs) and the high concentration of monounsaturated (MUFAs) and polyunsatured (PUFAs) fatty acids. Virgin olive oil is the main source of MUFAs in Mediterranean countries; the area of production is one of the parameters which affect the fatty acid composition of olive oil. The more concentrated fatty acids in olive oil are oleic acid (C18:1) a monounsaturated ω-9 fatty acid (Table 1), followed, in order of concentration, by linoleic acid, a polyunsaturated ω-6 fatty acid, palmitic acid a saturated fatty acid of olive oil, stearic acid (C18:0) a saturated fatty acid of olive oil and alpha-linolenic acid (C18:3), a polyunsaturated ω-3 fatty acid. Virgin olive oil is the main source of MUFAs in Mediterranean countries; the area of production is one of the parameters which affect the fatty acid composition of olive oil. The unsaponifiable fraction is low, about 2% of oil weight and includes more than 230 chemical compounds such as aliphatic and

triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants (Servili *et al*., 2004).

**Table 1.** Fatty acid composition (%) of virgin olive oil.

| Fatty acid | IOOC (a) |
|---|---|
| Myristic acid | <0,05 |
| Palmitic acid | 7.5-20.0 |
| Palmitoleic acid | 0.3-3.5 |
| Heptadecanoic acid | <0.3 |
| Staric acid | 0.5-5.0 |
| Oleic acid | 55.0-83.0 |
| Linoleic acid | 3.5-21.0 |
| Linolenic acid | <1.0 |
| Arachidic acid | <0.6 |
| Gadoleic acid | <0.4 |
| Behenic acid | <0.2 |
| Lignoceric acid | <0.2 |

(a: IOOC-2011)

### *1.2.1. Phenolic compounds in olive fruit and in virgin olive oil*

The healthy properties of olive oil in the past have been ascribed to its high oleic acid content as a source of monounsaturated fatty acid. However, in recent years converging evidence, reporting biological activities *in vitro*, *in vivo* and in clinical assays of phenolic compounds present in virgin olive oil (VOO), clarified that many of the beneficial effects of virgin olive oil intake, are mainly due to its minor compounds. Polyphenols contribute to the organoleptic properties of olive oil; in particular the phenolic constituents confer a bitter and pungent taste to the oil, both positive attributes (Gutiérrez-Rosales *et al.*, 1992; Montedoro *et al.*, 1992a). Moreover, acting as free radical scavengers, they are chiefly responsible for the defence against the auto-oxidation of unsaturated fatty acids, increasing the shelf life of the oil (Baldioli *et al.*, 1996). Phenolic compounds are present at levels between 200 and 1500 mg/kg,

depending on the olive tree variety, climatic and agronomic conditions, degree of maturation at harvest, and the manufacturing process (Servili *et al.*, 2004) and up to now the methods commonly used to evaluate the phenolic content of extra virgin olive oil are the Folin-Ciocalteau colorimetric assay (Visioli *et al.*, 1995b) and the HPLC (Montedoro *et al.*, 1992b).

Tyrosol [(*p*-hydroxyphenyl)ethanol] or *p*-HPEA and hydroxytyrosol [(3,4-dihydroxyphenyl)ethanol] or 3,4-DHPEA are the main phenolic alcohols in the olive fruit and in virgin olive oil (Montedoro *et al.*, 1992b). It has been proven that the latter is the most interesting phenolic alcohol, because of its pharmacological and antioxidant activity. The secoiridoids, are present exclusively in the *Oleaceae* family that includes *Olea europea* (Servili *et al.*, 2004) and are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure (Montedoro *et al.*, 1992c, 1993), in VOO are present in aglycon forms and are derivatives of oleuropein, demethyloleuropein and ligstrostide, the seicoiridoid glucosides contained in all the constitutive part of olive fruit (Figure 1). Another seicoridoid, detected in the olive seed is the nüzhenide (Servili *et al.*, 1999).

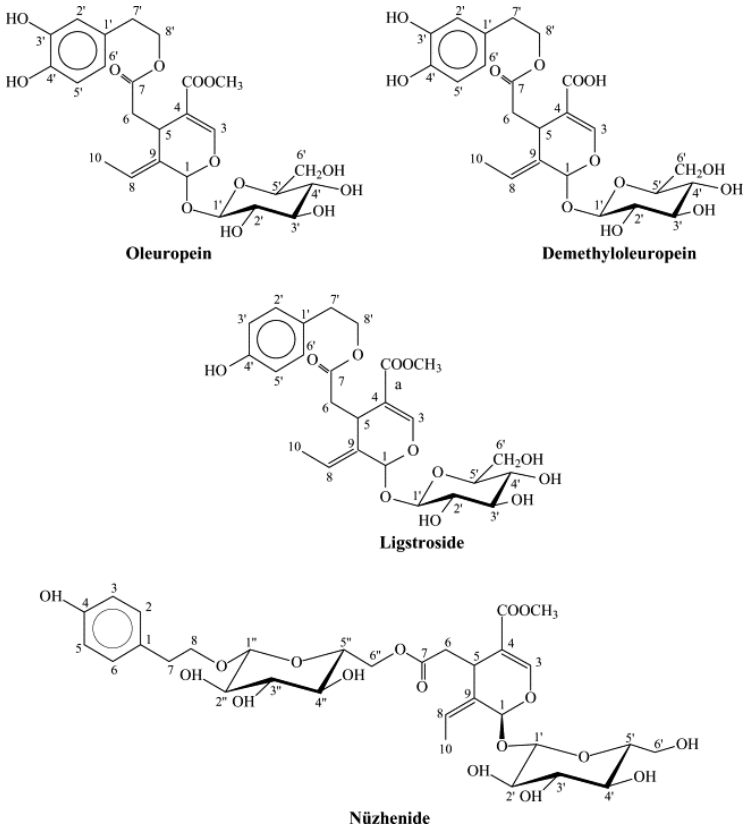


**Figure 1**. Chemical structures of the secoiridoids glucosides in the olive fruit.

The olive fruit contains a large amount of phenols, between 1% and 3% of the fresh pulp weight. In addition to secoiridoids, the main phenolic compounds in the olive fruit are phenolic acids, phenolic alcohols, and flavonoids (Table 2).

**Table 2.** Phenolic compounds in olive fruit.

| | |
|---|---|
| **Phenolic acid** | **Phenolic alcohols** |
| Chlorogenic acid | (3,4-Dihydroxyphenyl) ethanol (3,4-DHPEA) |
| Caffeic acid | *(p* -Hydroxyphenyl) ethanol (*p* -HPEA) |
| P-Hydroxybenzoic acid | |
| Protocatechuic acid | **Flavonols** |
| Vanillic acid | Quercitin-3-rutiniside |
| Syringic acid | |
| *p* -Coumaric acid | **Flavones** |
| *o* -Coumaric acid | Luteolin-7-glucoside |
| Ferulic acid | Luteolin-5-glucoside |
| Sinaptic acid | Apigenin-7-glucoside |
| Benzoic acid | |
| Cinnamic acid | **Antocyanins** |
| Gallic acid | Cyanidin-3-glucoside |
| | Cyanidin-3-rutinoside |
| **Seicoridoids** | Cyanidin-3-caffeygucoside |
| Oleuropein | Cyanidin-3-caffeylatinoside |
| Demethyloleuropein | Delphinidin 3-rhamosylglucoside-7-xyloside |
| Ligstroside | |
| Nuzhenide | **Hydroxycinnamic acid derivatives** |
| | Verbascoside |

Oleuropein and demethyloleuropein are the main secoiridoids of the olive fruit, which also contains verbascoside, a derivative of hydroxicinnamic acid (Figure 2).

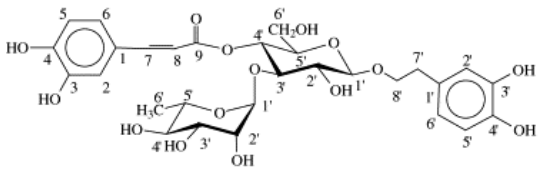

**Figure 2.** Chemical structure of verbascoside.

The most important antioxidants in virgin olive oil are polyphenols that include lipophilic and hydrophilic phenols. The first group includes tocotrienols and tocopherols, among which, α-tocopherol is the most abundant in virgin olive oil, about 90% of total concentration. The second group is shown in Table 3 and consists of hydrophilic phenols: phenolic acids, phenolic alcohols, hydroxy-isocromans, flavonoids, secoiridoids and lignans.

**Table 3.** Phenolic composition of virgin olive oil (VOO).

| **Phenolic acids and derivatives** | **Phenolic alcohols** |
|---|---|
| Vanillic acid | (3,4-Dihydroxyphenyl) ethanol (3,4-DHPEA) |
| Syringic acid | (*p* -Hydroxyphenyl) ethanol (*p* -HPEA) |
| *p* -Coumaric acid | (3,4-Dihydroxyphenyl) ethanol-glucoside |
| *o* -Coumaric acid | |
| Gallic acid | **Lignans** |
| Caffeic acid | (+)-1-Acetoxypinoresinol |
| Protocatechuic acid | (+)-Pinoresinol |
| *p* -Hydroxybenzoic acid | |
| Ferulic acid | **Flavones** |
| Cinnamic acid | Apigenin |
| 4-(Acetoxyethyl)-1,2-dihydroxybenzene | Luteolin |
| Benzoic acid | |
| Hydroxy-isocromans | **Hydroxy-isochromans** |
| | 1-(3'-methoxy-4'-hydroxyphenyl)-6,7-dihydroxyisochroman |
| | 1-phenyl-6,7-dihydroxyisochroman |
| **Secoiridoids** | |
| Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA) | |
| Dialdehydic form of decarboxymethyl elenolic acid linked to *p* -HPEA (p-HPEA-EDA) | |
| Oleuropein aglycon (3,4-DHPEA-EA) | |
| Ligstroside aglycon | |
| Oleuropein | |
| *p* -HPEA-derivative | |
| Dialdehydic form of oleuropein aglycon | |
| Dialdehydic form of ligstroside aglycon | |

The phenolic acids (Figure 3) were the first group of phenols described in virgin olive oil (Mannino *et al*., 1993; Montedoro *et al*., 1992b; Servili *et al*., 2004; Tsimidou *et al*., 1996).

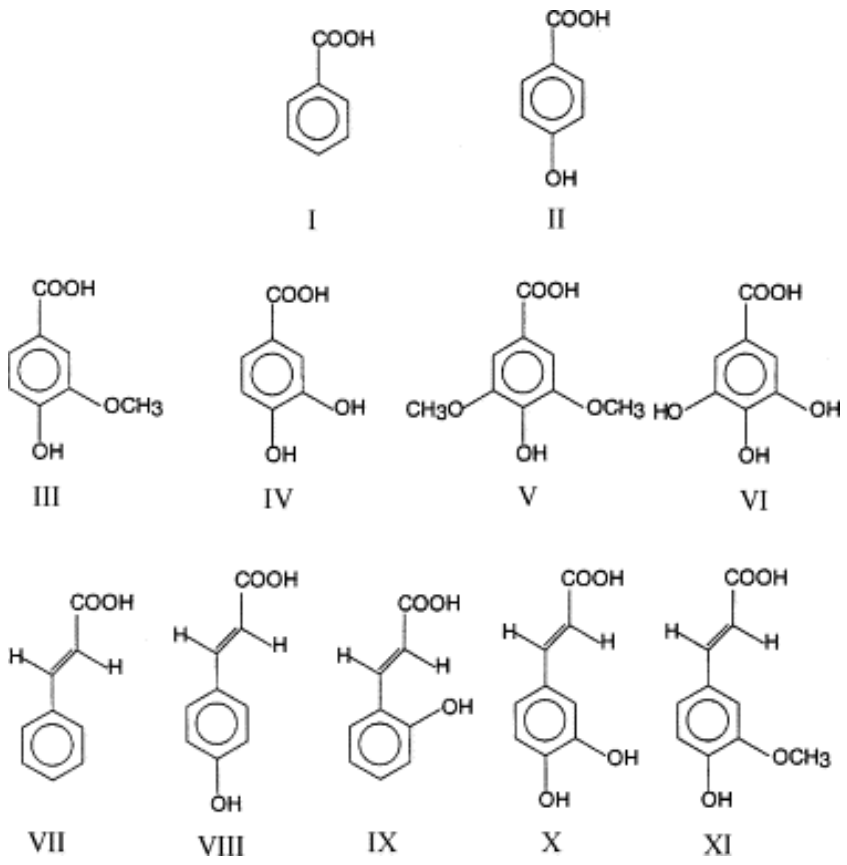


**Figure 3.** Chemical structure of the main phenolic acids of VOO: benzoic acid [I], p-hydroxybenzoic acid [II], vanillic acid [III], protocatechuic acid [IV], syringic acid [V], gallic acid [VI], cinnamic acid [VII], p-coumaric acid [VIII], o-coumaric acid [IX], caffeic acid [X], ferulic acid [XI].

These compounds are present in small amounts in virgin olive oil, together with phenyl-alcohols, hydroxy-isocromans and flavones. Hydroxy-isochromans were detected in VOO by Bianco *et al*., (2001), and are formed by the reaction between hydroxytyrosol and vanillin [1-(3'-methoxy-4'-hydroxyphenyl)-6,7-dihydroxyisochroman] and between hydroxytyrosol and benzaldehyde [1- phenyl-6,7-dihydroxyisochroman].

Secoiridoids and lignans, are the most abundant hydrophilic phenols in virgin olive oil; in particular Owen *et al.,* (2000a) reported 27.72 mg/kg of total seicoridoids and 41.53 mg/kg of total lignans in virgin olive oil. The lignans were isolated and characterized by Owen *et al*., (2000b) who indentified (+)-1-Acetoxypinoresinol and (+)-Pinoresinol as the most concentrated in virgin olive oil (Figure 4).

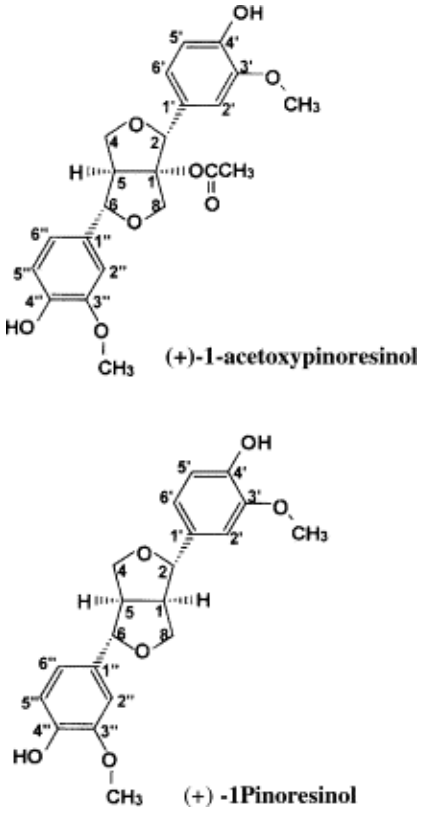


**Figure 4.** Chemical structures of the lignans occurring in VOO.

The aglyconic form of secoridoids (Figure 5) in VOO arises from the hydrolytic process, catalyzed by endogenous *β*-glucosidases, according to the proposed mechanism reported in Figure 6 (Servili *et al.*, 2004). In this way the secoiridoids derivatives are released into the oil and in by-products.

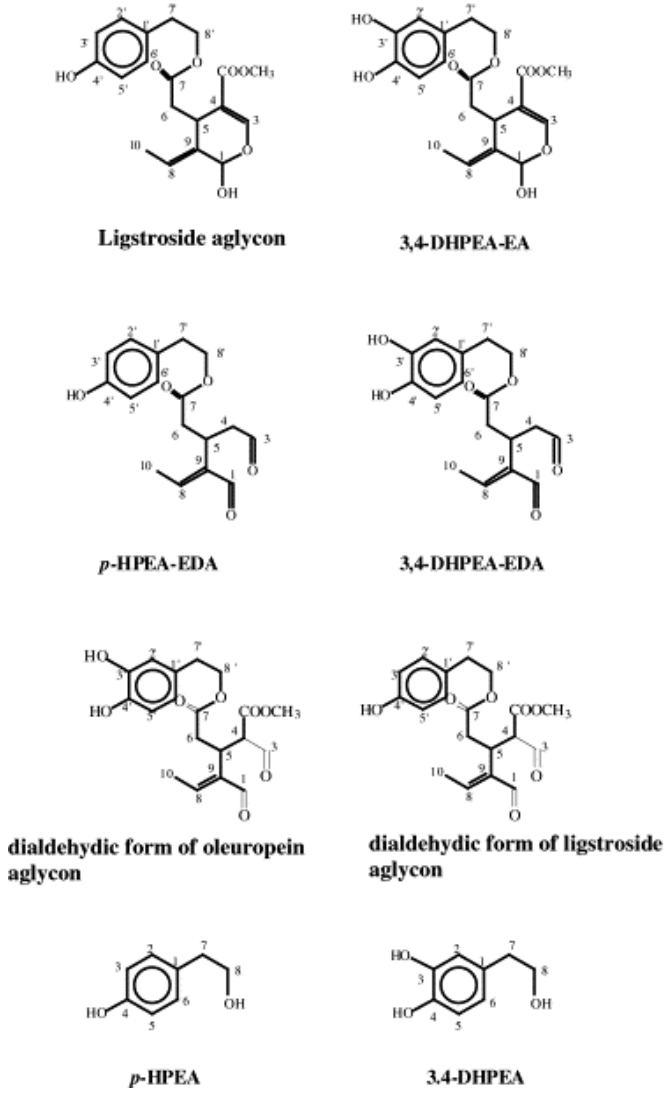


**Figure 5.** Chemical structures of the secoiridoids derivatives and phenolic alcohols of VOO.

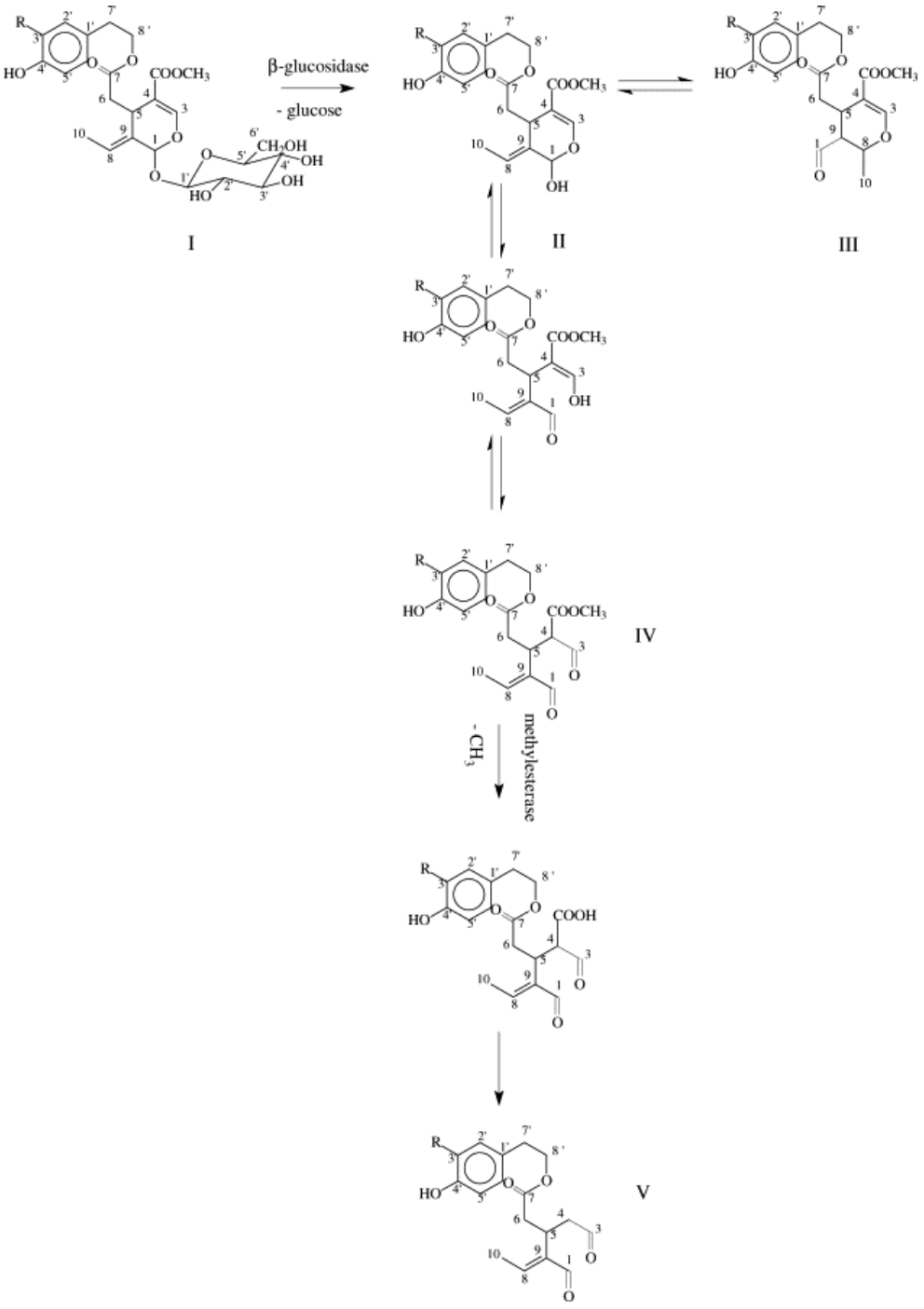


**Figure 6.** Biochemical mechanism of secoiridoids derivatives formation: (I) R = H: ligstroside; R = OH: oleuropein; (II) R = H: ligstroside aglycon; (III) R = OH: 3,4-DHPEA-EA; (IV) R = H: dialdehydic form of ligstroside aglycon; R = OH: dialdehydic form of oleuropein aglycon; (V) R = H: p-HPEA-EDA; R = OH: 3,4-DHPEA-EDA.

## 1.3. Health benefits of phenolic compounds

In Figure 7, the biological activities of phenolic compounds on health are summarized.

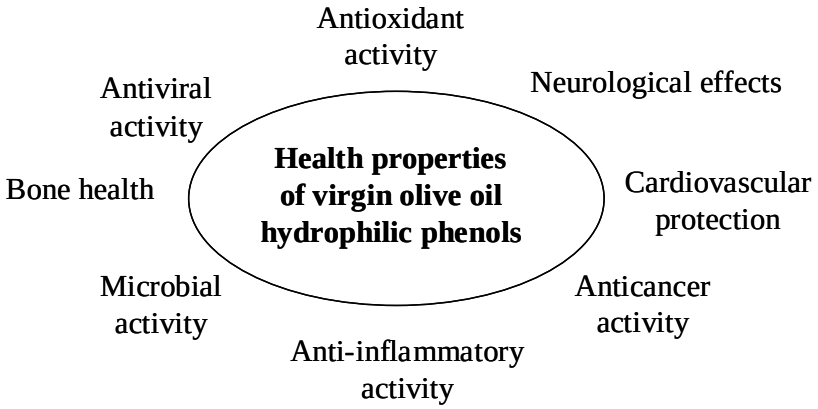


**Figure 7**. Biological activities of phenolic compounds.

Assessing the olive oil phenols bioavailability was an important step in demonstrating *in vivo* effects on humans. Up to now the research on the bioavailability of olive oil phenolic compounds, was mainly focused on hydroxytyrosol, tyrosol and oleuropein (Cicerale *et al.*, 2010). In particular it was found that hydroxytyrosol and tyrosol, absorbed dose-dependently from olive oil after oral ingestion in humans, rise quickly in plasma, reaching a peak at around 1h (Weinbrenner *et al*., 2004; Miró-Casas *et al.*, 2003) and 0-2 h in urine (Miró-Casas *et al*., 2001). The excretion of hydroxytyrosol and tyrosol in humans is between 30-60% and 20-22% of the total amount ingested, respectively (Visioli *et al*., 2000b). The different polarities of these compounds were supposed to play an important role in the absorption process; hydroxytyrosol and tyrosol are polar and permeate cell membranes of human intestinal cells via a passive diffusion mechanism (Manna *et al*., 2000). Hydroxytyrosol and tyrosol are present in plasma and urine in their glucoronide conjugated forms, (Caruso *et al*., 2001) after

virgin olive oil ingestion; an extensive first pass intestinal/hepatic metabolism of the ingested primary forms has been proposed (Miró-Casas *et al.*, 2003). Bonanome and co-authors (2000) showed that the olive phenolic compounds are incorporated in human lipoproteins after ingestion and it has been proven that hydroxytyrosol preserves its antioxidant properties following ingestion (Visioli *et al.*, 2005). Miró-Casas *et al.*, (2003) developed a method to quantify absorption of hydroxytyrosol and its metabolites, and also found that absorption of hydroxytyrosol is nearly complete and its plasma half-life is 2.43 hours. The high bioavailability represents a starting point to confirm its beneficial effects on health.

### *1.3.1. Antioxidant activity*

Elevated levels of high density lipoprotein cholesterol (HDL-C) have protective and anti-inflammatory properties (Chrysohoou *et al*., 2006). However, on the other hand elevated levels of total cholesterol and low density lipoprotein cholesterol (LDL-C) have been established as a critical factor in the development of atherosclerosis, which is the primary cause of cardiovascular disease (Gonzalez-Santiago *et al*., 2010; Vázquez-Velasco *et al*., 2011). Coronary events are reduced by 2-3% for every 1% decrease in LDL-C, as reported in the Helsinki Heart Study (Manninen *et al*., 1988) and are reduced by 3% for every 1% increase in HDL-C (Feldman, 2002).

There is evidence that oxidative modification of low density lipoprotein (LDL) plays a key role in the development of atherosclerosis (Witztum *et al*., 1994a). The formation of oxidized LDL depends on its antioxidant content, such as vitamin E and phenolic compounds, present in LDL (Reaven *et al*., 1994). *In vitro* the antioxidant effect is evaluated through the assessment of various markers of LDL oxidation such as reduced formation of short-chain aldehydes and lipid peroxides, high vitamin E content in the residual LDL, protection of the apoprotein layer (Visioli *et al.*, 1995a). Hydroxytyrosol reduces oxidation of the low-density lipoproteins carrying cholesterol (LDL-C); it also has a potential protective effect against oxidative stress induced by tertbutyl hydroperoxide (Goya *et al*., 2007). Both hydoxythyrosol and oleuropein inhibit the

copper sulfate-induced oxidation of LDL (Visioli and Galli, 1994) which indicate that these molecules are potent free radical scavengers.

Up to now in humans it has been proven that phenolic compounds linked to human LDL increases in a dose dependent manner with the phenolic content of the olive oil administered (Covas *et al*., 2006) (Figure 8), hence, the phenolic content of the olive oil protects the LDL phenolic content from degradation (Fitò *et al.*, 2000).

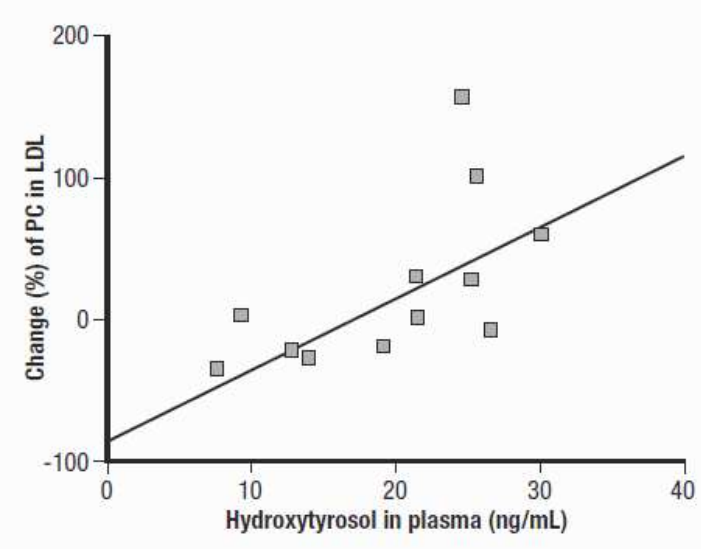


**Figure 8.** Relationship between the change in the total phenolic content (PC) of the LDL and plasma hydroxytyrosol concentrations 30 minutes after ingestion of 40 ml of a high phenolic content (366mg/kg) of olive oil. R=0.780, P=0.009, Spearman's correlation coefficient. Adapted from Covas *et al.*, (2006), by Fitò *et al.*, (2007).

Oxidative stress produced by reactive oxygen species (ROS) has been linked to several diseases such as atherosclerosis, certain cancers and neurodegenerative diseases (Witztum *et al*., 1994b). Manna and co-workers (2002) found that the phenolic fraction of extra virgin olive oils plays a protective role against ROS-mediated (reactive oxygen species) oxidative injuries in erythrocytes and human intestinal cells (Caco-2). It has

also been reported that total plasma antioxidant activity increases in humans after the ingestion of olive oil phenolic compounds (Visioli *et al*., 2005).

The abundance of polyunsaturated fatty acids makes the kidney an organ particularly vulnerable to the lipid peroxidation process (Kubo *et al.*, 1997): lipid peroxidation is considered to be crucial in the mechanisms involved in a wide range of renal diseases, such as tubulointerstitial alterations (Martin-Mateo *et al*., 1999). A recent study has shown that a pre-treatment with hydroxytyrosol inhibits $H_2O_2$ induced oxidative damage in a porcine kidney epithelial cell line (LLCPK1), preserving the level of membrane lipids with no significant detection of oxidation products (Deiana *et al*., 2008).

The olive oil polyphenols contribute to the protection of human blood lipids from oxidative stress; the new European regulation about the "health claims in foods" fixed for the phenolic compounds of EVOO a minimum level of 5 mg of hydroxytirosol and its derivatives (e.g. oleuropein complex and tyrosol) in 20 g of oil (Reg. EU 432/2012).

Oxidative stress is involved in age-related degeneration of retinal pigment epithelium and the photoreceptors in the macular area of the retina; Liu *et al*., (2007), have shown that hydroxytyrosol protects human retinal pigment epithelial cells (ARPE-19) from oxidative damage induced by an environmental toxin (acrolein), endogenous end product of lipid oxidation, that occurs at increased levels in age-related macular degeneration lesions. A further study on the same disease showed that hydroxytyrosol is an important inducer of phase II detoxifying enzymes and an enhancer of mitochondrial biogenesis (Zhu *et al.*, 2010). Several studies reported the protective rule on DNA damage of the phenolic compounds of virgin olive oil, *in vitro* (Fabiani *et al*., 2008) and in animals.

A study conducted, by Salvini and co-workers (2006) reports that in humans the ingestion of phenol rich virgin olive oil decreases oxidative DNA damage by up to 30%, compared to a low phenolic content virgin olive oil. A further study demonstrated that after consumption of phenol rich virgin olive oil there was decreased urinary excretion of 8-oxo-deoxygyuanosine a systemic marker of DNA oxidation (Cooke *et al*., 2003).

### *1.3.2. Neuroprotective effects*

Aging is the result of the oxidative injury, mainly to mitochondria. Some of the oxidative damage leads to cellular dysfunctions during an entire lifetime. Mitochondrial membranes are very sensitive to free radical attacks because of the presence of double bond carbon-carbon in the lipid tails of its phospholipids, which can lead to cognitive and neurodegenerative diseases (Omar, 2010). In a study Bazoti *et al.,* (2006) reported that oleuropein decreases or even prevents *β*-amyloid peptide aggregation, this peptide is the major proteinaceous component of senile plaques formed in Alzheimer's disease brain. An animal study has shown the protective role of virgin olive oil phenols on lipid peroxidation: mice fed from middle age to senescence with extra-virgin olive oil (10% wt/wt dry diet) rich in phenols (total polyphenol dose/day, 6 mg/kg) improved contextual memory to levels similar to young animals and prevented age-related impairment in motor coordination. This effect appeared to be correlated with reduced lipid peroxidation in the cerebellum (Pitozzi *et al*., 2012).

Another study showed that administration of virgin olive oil in mice improves learning and memory, increasing brain glutathione levels, suggesting reduced oxidative stress as a possible mechanism (Farr *et al.,* 2012).

### *1.3.3. Cardiovascular protection*

It is recognized that platelets play a key role in the development of thrombosis, (Osler, 1886) and it is widely accepted that platelets play a central role in the development of cardiovascular diseases (Bhatt and Topol, 2003).

Hydroxytyrosol has been proven to inhibit platelet aggregation completely in human blood (*in vitro*) in the range of 100-400 μM (Petroni *et al*., 1995). A more recent study shows that a number of olive oil phenolic compounds such as oleuropein aglycone and luteolin are also potent inhibitors of platelet aggregation (Dell'Agli *et al*., 2008). Leukocyte adhesion to vascular endothelial represents a key step in the formation of

atherosclerotic, plaque; the ability of phenols to inhibit these kinds of cells was also proven (Carluccio *et al*., 2003).

### *1.3.4. Anticancer activity*

Several epidemiologic studies have demonstrated the association between olive oil consumption and a reduced risk of cancer in different sites such as the breast (La Vecchia *et al*., 1995; Martin-Moreno *et al*., 1994; Trichopoulou *et al.,* 1995), the prostate (Hodge *et al.,* 2004), the lung (Fortes *et al.,* 2003), the larynx (Bosetti *et al.,* 2002a), the ovary (Bosetti *et al*., 2002b) and the colon (Stoneham *et al*., 2000). Cell proliferation is the factor chiefly responsible for tumour formation and progression. It has been proven that hydroxytyrosol shows a pro-apoptotic effect by modulating the expression of genes involved in tumour cell proliferation of promyelocytes (HL60 cells) (Fabiani *et al*., 2006, 2008, 2009, 2011). Moreover, it has been proven that hydroxytyrosol inhibits proliferation of human MCF-7 breast cancer cells (Bulotta *et al.* 2011; Sirianni *et al*., 2010), human HT29 colon carcinoma cells (Guichard *et al*., 2006), human M14 melanoma cells (D'Angelo *et al*., 2005) and human PC3 prostate cells (Quiles *et al*., 2002). Hashim and co-workers (2008) demonstrated a dose-related inhibition of colon cancer cell invasion exerted by olive oil phenolic compounds.

### *1.3.5. Anti-inflammatory activity*

Recent studies on the mechanisms involved in atherosclerosis disease has focused on inflammatory cytokines that are responsible for vascular inflammation, stimulating the generation of endothelial adhesion molecules which may enter the circulation in a soluble form. Elevated concentrations of inflammation markers in serum are associated with increased cardiovascular risk (Packard and Libby, 2008). Plasma thromboxane B2 has the ability to increase blood platelet aggregation and leukotriene B4 has a chemostactic effect on neutrophils, directing the cells to damaged tissue, and both are known proinflammatory agents (Bogani *et al*., 2007). Polyphenols have been shown to

decrease the production of inflammatory markers, such as leukotriene B4, in several systems (Biesalski, 2007).

Recently, it has been established that HT-20, an olive oil extract containing about 20% of hydroxytyrosol, inhibits inflammatory swelling and hyperalgesia, and suppresses proinflammatory cytokine in a rat inflammation model (Gong *et al*., 2009).

### *1.3.6. Microbial activity*

Antimicrobial activity of oleuropein, tyrosol and hydroxytyrosol has been studied *in vitro* against bacteria, viruses and protozoa (Bisignano *et al*., 1999). Hydroxytyrosol and oleuropein have been shown to have antibacterial properties in particular cytotoxic against many bacterial strains, mainly against bacteria responsible for intestinal and respiratory infections (Medina *et al*., 2006). Also, the dialdehydic form of decarboxymethyl ligstroside is not hydrolyzed in the stomach and plays an important role in the inhibition of the growth of the *Helicobacter pylori* bacteria (Romero *et al*., 2007), chiefly responsible for the development of gastric cancer and peptic ulcers.

### *1.3.7. Bone health*

Tyrosol and hydroxytyrosol seem to be involved in increased bone strengthening in rats (Puel *et al.*, 2008). In particular these compounds could represent effective remedies in the treatment of osteoporosis symptoms; Hagiwara and co-authors (2011) evaluated the effects of oleuropein, hydroxytyrosol and tyrosol on bone formation using cultured osteoblasts and osteoclasts. The results showed that oleuropein and hydroxytyrosol inhibited the loss of bone density, stimulating the deposit of calcium in a dose-dependent manner. Both compounds, also, suppressed the loss of bone density of trabecular bone in femurs of ovariectomized mice.

### *1.3.8. Antiviral activity*

Hydroxytyrosol and oleuropein have been identified as a unique class of HIV-1 inhibitors that prevent HIV from entering into the host cell and binding the catalytic site of the HIV-1 integrase (Fernández-Bolaños *et al.*, 2012). Oleuropein and hydroxytyrosol were identified as HIV inhibitors at both the fusion and integration stages (Lee-Huang *et al.*, 2007a, 2007b) in a dose-dependent manner. Hydroxytyrosol was also found to be useful as a microbicide for preventing HIV infection, in fact, thanks to this property, it has recently been patented as a product for topical use (Gómez-Acebo *et al.*, 2011). Furthermore, it has been reported in another study that hydroxytyrosol inactivated influenza A viruses, including H1N1, H3N2, H5N1, and H9N2 subtypes. Electron microscopic analysis revealed morphological abnormalities in the hydroxytyrosol-treated H9N2 virus, this suggested that the structure of the H9N2 virus could be disrupted by hydroxytyrosol (Yamada *et al*., 2009).

## 1.4. Beneficial effect of virgin olive oil monounsaturated fatty acids

Monounsaturated fatty acids intake leads to enhanced resistance of LDL oxidation (Bonanome *et al*., 1992), it also increases the levels of the protective high-density lipoprotein more than polyunsaturated fatty acids when these two classes of fatty acids replace carbohydrates in the diet (Mensink *et al*., 2003). Another effect of dietary MUFA, is lower endothelial activation (Massaro *et al.*, 2002; Massaro and De Caterina, 2002). The intake of monounsaturated fatty acid appeared to be associated with a reduced risk of age-related cognitive decline, mainly due to the role these fats play in maintaining the structural integrity of neuronal membranes (Panza *et al*., 2004a; Solfrizzi *et al*., 1999). Moreover, high ingestion of monounsaturated fatty acids appears to exert a contrasting action on Alzheimer's disease (Panza *et al*., 2004b).

## 1.5. Olive by-products

The virgin olive oil (VOO) extraction system and related processing parameters can strongly affect the qualitative and the quantitative composition in phenols of VOO and their main by-products. In particular, the concentration in the final product and by-products of those compounds, is largely affected by agronomic and technological conditions of VOO production. Cultivar, ripening stage, geographic origin of olives and olive trees irrigation are the main agronomic aspects that can modify the VOO secoiridoid composition as well as some operative conditions applied during crushing, malaxation and VOO separation (Servili *et al.*, 2004, 2009). So far, different extraction technologies, such as pressure and centrifugation and selective filtration (i.e. "surface tension" or "percolation"), are used to enable the separation of oily must from the olive paste (Boskou, 1996; Di Giovacchino *et al.*, 1994), however, the majority of VOO in the Mediterranean area is currently extracted by centrifugation; in Italy this technique represents more than 80% of total production (Roig *et al.*, 2006).

In particular two main different systems have been used, up to now, using centrifugation: the three-phases and the two-phases extraction systems (Sequi *et al.*, 2001). However innovative technologies in recent years have improved, from both environmental and qualitative point of view, the existing extraction systems; for example the new three-phases decanters, and a new two-phases decanter which produces a new kind of pomace, more easily reusable (i.e. paté).

The three-phase system, introduced in the 1970s to improve extraction yield, produces olive oil, vegetation water (VWs), which consists of the water plus organic matter incorporated into drupes and the water added during the process, pomace made up of pulp bulk and pit; the dilution of the malaxed pastes produces 50-90 l of VWs/100 kg of olive pastes (Servili *et al.*, 2011). The traditional three-phases decanters, featuring water addition ranging from 0.5 to 1 m3/ton, while the “new three-phases decanters”, works at low water consumption (ranging from 0.2 to 0.3 m3/ton) (Servili *et al.*, 2012). The two-phase centrifugation system was introduced in the 1990s in Spain as an ecological approach for olive oil production and, thanks to this, there was a significant decrease in water consumption during the process producing two fractions: a semisolid

sludge called by various names (“two-phases olive mill waste”, “alperujo”, “olive wet husk”, or “wet pomace”) and olive oil (Roig *et al*., 2006). From an environmental point of view, VWs from the three-phase system is considered the worst waste in both quantity and quality terms; the two-phases decanters, which can operate without the addition of water do not produce vegetation water as a by-product of the extraction oil process (Servili *et al*., 2012). Figure 9 reports the comparison of the two main different olive oil extraction systems.

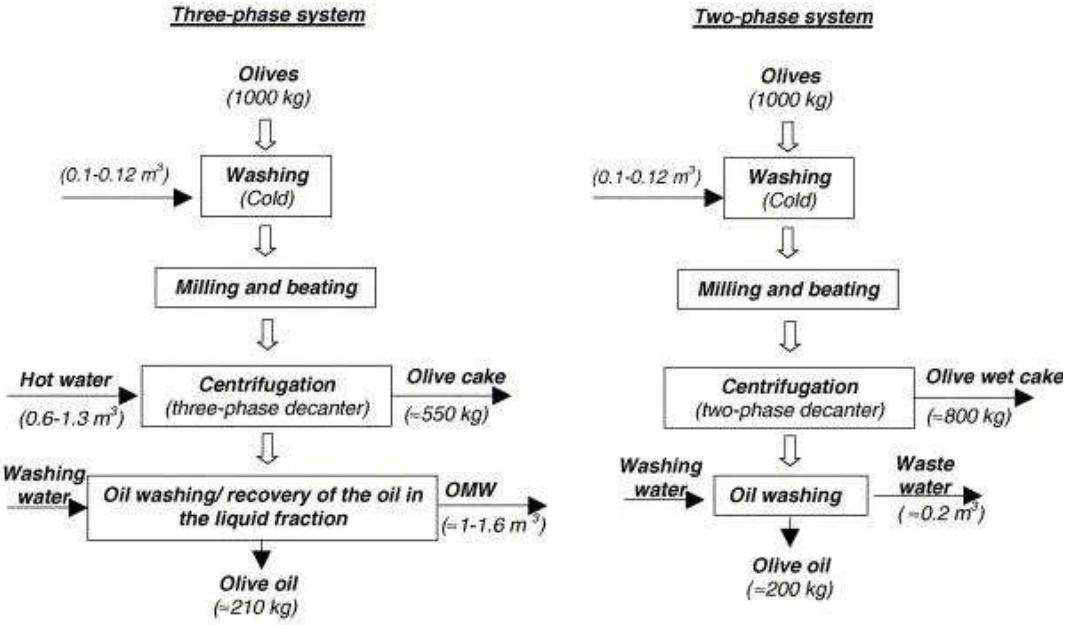


**Figure 9.** Comparison of the three-phases and two-phases centrifugation systems for olive oil extraction (Alburquerque *et al.*, 2004).

Lesage-Messen and co-workers (2001) studied the phenolic composition of by-products obtained from three-phase and two-phase extraction systems. The study reported that the phenolic composition, identified by HPLC, after acid extraction with ethyl acetate, was similar in VWs and pomace from the two different extraction systems, with hydroxytyrosol, approximately 1% dry residue, and tyrosol being the main compounds

detected. Nevertheless, the contents of individual compounds (hydroxytyrosol, tyrosol, caffeic acid, ferulic acid and *p*-coumaric acid), with the exception of vanillic acid, were higher for the two-phase system.

The phenolic composition of virgin olive oil and by-products is strongly affected by the enzymatic reactions such as *β*-glucosidase, polyphenoloxidase (PPO), peroxidase (POD), occurring during the different phases of the mechanical extraction process of the oil (Di Giovacchino *et al.*, 1994; Servili *et al.*, 2004).

The first ones cause the release of phenols in the oil and in the water, while the latter promote their oxidation. In recent years the understanding of those phenomena, acknowledged by the industry in some innovations in the mechanical extraction process, has led to the production of a VOO significantly richer in phenolic compounds and at the same time an increase in those compounds in the VWs.

### *1.5.1. Vegetation water*

The vegetation waters (VWs) is characterized by the following special features and components (Lòpez, 1993; Vásquez-Roncero *et al*., 1974):

- intense violet-dark brown up to black colour;
- strong specific olive oil smell;
- high degree of organic pollution (COD values up to 220 g/l);
- pH between 3 and 6 (slightly acid);
- high electrical conductivity;
- high content of polyphenols (0.5-24 g/l);
- high content of solid matter.

The composition of the VWs varies according to cultivar, degree of maturity, cultivation soil, harvesting time, use of pesticides and fertilizers, olive's water content and climatic conditions. The VWs pH value ranges from 4.5 to 6 and the VWs contain an average of 3-16% of organic compounds, in which 1-8% of sugars, 1.2-2.4% of nitrogen containing compounds and 0.34-1.13% of phenols (Niaounakis and Halvadakis, 2004). VWs contain various amounts of sugars depending on olive cultivar,

climatic conditions during growth and the extraction system. The sugars constitute up to 60% of the dry substance, and are, in order of concentration, fructose, mannose, glucose, saccharose, and traces of sucrose, and some pentose. The olive oil phenolic compounds are amphiphilic in nature and are more soluble in the water than in the oil phase, consequently, a large quantity of antioxidants is lost with the wastewater during processing. These compounds in the VWs reach concentrations ranging from 0.5 to 24 g/L and are strictly dependent on the processing system used for olive oil production (Niaounakis *et al.*, 2006).

The hydrophilic phenols identified and quantified in VWs include phenolic alcohols, phenolic acids, phenyl alcohols, secoiridoids, flavonoids, and lignans. Up to now, more than 30 phenolic compounds have been identified in VWs. The main phenolic compounds detected in VWs are:

- Derivatives from cinnamic acid: (cinnamic acid), *o*-and *p*-coumaric acid (4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), and ferulic acid (4-hydroxy-3-methoxycinnamic acid).
- Derivatives from benzoic acid: (acid benzoic), protocatechuic acid, and b-3,4-dihydroxyphenyl ethanol derivatives. A large amount of phenolic compounds detected in vegetation water are represented by secoiridoids, in particular p-HPEA (p-hydroxyphenylethanol or tyrosol), 3,4-DHPEA (3,4-dihydroxyphenylethanol or hydroxytyrosol), and *p*-HPEA-EDA and the 3,4 DHPEA-EDA (dialdehydic form of decarboxymethil elenolic acid linked to tyrosol and hydroxytyrosol respectively) and also verbascoside (Servili *et al*., 1999).
- catechol, 4-methylcatechol, p-cresol, and resorcinol (Capasso *et al*., 1992a, 1992b; Vinciguerra *et al*., 1993).
- VWs can contain, under certain conditions, small amounts of oleuropein, demethyloleuropein and verbascoside (Servili *et al*., 1999).
- VWs also contains relatively high concentrations of flavonoids: the main flavonoids detected in VWs are anthocyanins (cyanidin-3-glucoside, cyanidin-3-rutinoside), flavones (luteolin, luteolin-7-glucoside, apigenin, apigenin-7-

glucoside), and flavonols (quercetin, rutin) (Duràn Barrantes, 1990; Servili *et al*., 1999).

A specific complex that has an active biological and specific activity, consisting of hydroxytyrosol (3,4-DHPEA), tyrosol (*p*-HPEA) and the dialdehydic form of decarboxymethyl elenolic acid, linked with (3,4-dihydroxyphenyl) ethanol (3,4-DHPEA-EDA) and accounting for 6% of the total phenolic content of the VWs, has recently been identified in VWs (Angelino *et al*., 2011).

The chemical composition of VWs as reported by Morillo *et al*., 2009, is shown in Table 4.

**Table 4**. Chemical composition of VWs from two-phase olive mill extraction system.

| Parameter | Mean | Range |
|---|---|---|
| Dry matter (%) | 6.72 | 6.33–7.19 |
| pH | 4.84 | 4.2–5.17 |
| EC (dS/m) | 8.36 | 5.5–12 |
| Organic Matter (g/l) | 55.80 | 46.5–62.1 |
| TOC (g/l) | 37.00 | 34.2–39.8 |
| TN (g/l) | 0.96 | 0.62–2.1 |
| C/N | 53.32 | 52.3–54.3 |
| $P_2O_5$ (g/l) | 0.57 | 0.31–0.7 |
| $K_2O$ (g/l) | 4.81 | 2.37–10.8 |
| Na (g/l) | 0.26 | 0.11–0.42 |
| Ca (g/l) | 0.35 | 0.2–0.64 |
| Mg (mg/l) | 121.25 | 44–220 |
| Fe (mg/l) | 81.70 | 18.3–120 |
| Cu (mg/l) | 3.15 | 1.5–6 |
| Mn (mg/l) | 5.15 | 1.1–12 |
| Zn (mg/l) | 6.13 | 2.4–12 |
| Density ($g/cm^3$) | 1.04 | 1.02–1.048 |
| Lipids (g/l) | 6.39 | 1.64–12.2 |
| Phenols (g/l) | 4.98 | 0.98–10.7 |
| Carbohydrates (g/l) | 7.16 | 1.4–16.1 |
| COD (g/l) | 124.67 | 67–178 |
| BOD5 (g/l) | 65.00 | 46–94 |

Data were calculated from eight independent studies reported in Roig *et al.*, (2006) and adapted by Morillo *et al.*, 2009.

### *1.5.2. Pomace*

Olive pomace, the other olive mill waste, is the solid phase resulting from the virgin olive oil mechanical separation process. The chemical composition and nutritive value of the olive pomaces are very different from those of pomaces coming from different extraction systems, and depends on the proportion of the different physical components such as skin, stone, pulp, water, year, geographic origin, contamination with soil and the residual oil (Molina Alcaide and Yáñez-Ruiz, 2008). The pomace is made up by residual oil (5-8%), vegetation waters (25-55%) while the remaining fraction is made up by solid components. The pomace produced by the two-phase extraction system has a moisture content in the range of 55-70%, while traditional pomaces have a moisture content of around 20-25% in press systems and 40-45% in three-phase decanters (Alba-Mendoza *et al*., 1990; Alburquerque *et al*., 2004). It also contains some residual olive oil (2-3%) and 2% ash with 30% of potassium content. Tyrosol and hydroxytyrosol are the most abundant phenolic compounds in pomaces (Fernández-Bolaños *et al*., 2002) together with *p*-coumaric, caffeic (Lesage-Meessen *et al*., 2001), while vanillic acid is found in lower amount. These phenolic compounds together with the lipid fraction have been connected with the phytotoxic and antimicrobial effects attributed to olive-mill wastes. The chemical composition is reported in Table 5. The recovery of the pomace residual oil by means of solvent and its subsequent refining, will increase problems for the placement of the product, considered as a low quality oil.

**Table 5**. Chemical composition of pomace from two-phase olive mill extraction system.

| Parameter | Mean | Range |
|---|---|---|
| Humidity (%) | 62.16 | 49.6–71.4 |
| pH ($H_2O$) | 5.48 | 4.9–6.8 |
| EC (dS/m) | 2.99 | 1.2–5.24 |
| Organic matter (%) | 90.66 | 60.3–98.5 |
| C/N | 44.99 | 29.3–59.7 |
| TN (g/kg) | 11.99 | 9.7–18.5 |
| P (g/kg) | 0.97 | 0.3–1.5 |
| K (g/kg) | 18.73 | 6.3–29 |
| Ca (g/kg) | 5.08 | 2.3–12 |
| Mg (g/kg) | 1.03 | 0.5–1.7 |
| Na (g/kg) | 0.67 | 0.2–1 |
| Fe (mg/kg) | 1,107.80 | 526–2,600 |
| Cu (mg/kg) | 41.20 | 13–138 |
| Mn (mg/kg) | 25.80 | 13–67 |
| Zn (mg/kg) | 19.60 | 10.01–27 |
| Lignin (%)[a] | 38.82 | 19.8–47.5 |
| Hemicellulose (%)[a] | 29.70 | 15.3–38.7 |
| Cellulose (%)[a] | 23.47 | 17.3–33.7 |
| Lipids (%)[a] | 11.01 | 3.76–18 |
| Protein (%)[a] | 6.95 | 6.7–7.2 |
| Carbohydrates (%)[a] | 12.32 | 9.6–19.3 |
| Phenols (%)[a] | 1.36 | 0.5–2.4 |

Data were calculated from eight independent studies reported in Roig *et al.* (2006) and adapted by Morillo *et al.*, 2009.
[a] (% w/w) of total organic matter.

The fibrous components vary depending largely on the proportion of stones in pomaces. In the case of de-stoned pomaces the crude fibre value is lower (Sansoucy, 1985). Analysis of fibres by the Van Soest (1975) method shows that pomace has high cell wall constituents (NDF), ligno-cellulose (ADF) and lignin (ADL) contents (Table 6). The crude fat (CF) and neutral detergent fiber (NDF) are the most variable components.

**Table 6**. Average chemical composition (g/kg D.M.) of olive pomace.

| Parameters | Pomace | S.D. |
|---|---|---|
| Dry matter (g/kg fresh matter) | 805 | 178 |
| Organic matter | 901 | 62 |
| Ether extract | 54,5 | 42.1 |
| Gross energy (MJ/kg DM) | 19,7 | 2 |
| Crude protein | 72,6 | 23,4 |
| Amino acid N (g/kg N) | 846 | |
| Acid detergent insoluble | 10,7 | 3,9 |
| Neutral detergent fibre | 676 | 119 |
| Acid detergent fibre | 544 | 83 |
| Acid detergent lignin | 289 | 30 |
| Total extractable polyphenols | 13,9 | 4,8 |
| Total extractable tannins | 9,78 | |
| Total extractable condensed tannins | 0,81 | |
| Total condensed tannins | 12,4 | 0,7 |
| Free condensed tannins | 1,64 | 0,08 |
| Fibre bound condensed tannins | 4 | 0,3 |
| Protein bound condensed tannins | 5,87 | 0,65 |

Lignin content is high and Crude protein content (CP) is generally low, and a substantial part is linked to cell wall components, in fact a large proportion of the proteins (80 to 90%) is linked to the ligno-cellulose fraction (ADF-N) (Nefzaoui, 1983). Pomace fat is high in unsaturated C:16 and C:18 fatty acids which constitute 96% of total fatty acids (Chiofalo *et al*., 2002). Pomace is highly vulnerable to air oxygen which is the main cause of spoilage of its organoleptic properties (Sansoucy, 1985).

### *1.5.3. Olive mill waste: regulations and management*

In Italy land spreading of waste, wastewater and/or wet husk, is regulated by Law n° 574 dated 11/11/1996 regarding olive mill waste waters and olive pomace. "*Nuove norme in materia di utilizzazione agronomica delle acque di vegetazione e di scarichi*

*dei frantoi oleari*" which in Art. 1, envisages that: "*Le acque di vegetazione residuate dalla lavorazione meccanica delle olive che non hanno subito alcun trattamento né ricevuto alcun additivo ad eccezione delle acque per la diluizione delle paste ovvero per la lavatura degli impianti possono essere oggetto di utilizzazione agronomica attraverso lo spandimento controllato su terreni adibiti ad usi agricoli*". The provisions of Italian Law n° 574 on land spreading of VWs and olive pomace, contain many points and the main ones are summarized below.

Art.1. Agronomic use

1. Olive-mill wastewater: olive-mill wastewater without pre-treatment.

2. Olive pomace "*…ai fini dell'applicazione della presente legge le sanse umide provenienti dalla lavorazione delle olive e costituite dalle acque e dalla parte fibrosa di frutto e dai frammenti di nocciolo possono essere utilizzate come ammendanti in deroga alle caratteristiche stabilite dalla legge 19 ottobre 1984, n. 748, e successive modificazioni."* (Replaced by Legislative Decree 217 dated 29 April 2006 "*Revisione della disciplina in materia di fertilizzanti"*).

Art. 2. Limits of acceptability

1. Olive-mill wastewater: from traditional press at 50$m^3$/ha/year or from centrifugation at 80$m^3$/ha/year.

2. The mayor can stop spreading operations if there is a chance of damage to the environment or reduce the limits of acceptability.

Art. 3. Authorization

Spreading operations must be notified to the mayor 30 days before. Communication must include: type of soil, spreading system, spreading time, hydrological condition.

Art. 4. Spreading systems

- Distribution must be uniform and by-products must be ploughed in.
- During spreading operation run off must be avoided.

Art 5. Prohibition

Spreading is forbidden, where:

- Distance is less than 300m to the groundwater draining areas.
- Distance is less than 200m to the built up areas.
- Soil is used for growing vegetables.
- Soil with a water table depth of less than 10 m.
- Soil where percolation water could reach the water table.

Art. 6 Storage

As far as the storage is concerning:

- Storage period max 30 days.
- Storage must be in a water-proof container.
- The mayor must be notified of storage location.

The following articles (Art 8. and Art. 9) refer to the sanctions and inspections, respectively.

What is meant by agronomic utilization is defined in Legislativa Decree n. 152 dated 3 April 2006, entitled "Norme in materia ambientale" article 74 paragraph 1 letter p envisages: "*utilizzazione agronomica: la gestione di effluenti di allevamento, acque di vegetazione residuate dalla lavorazione delle olive, acque reflue provenienti da aziende agricole e piccole aziende agro-alimentari, dalla loro produzione fino all'applicazione al terreno ovvero al loro utilizzo irriguo o fertirriguo, finalizzati all'utilizzo delle sostanze nutritive e ammendanti nei medesimi contenute"*

The norms on the olive mill waste, were later completed with the provisions enviseged in the Ministerial Decree dated 06/07/2005 "*Criteri e sulle norme tecniche generali per la disciplina regionale dell'utilizzazione agronomica delle acque di vegetazione e degli scarichi dei frantoi oleari*" and with the relative regional resolution.

When the use of waste is not defined as "agronomic" use, the Cassation Court defined the sphere of application "*Al di fuori dell'applicazione agronomica per i residui oleari non possono comunque trovare applicazione le disposizioni contenute nella L. n. 574*

*del 1996 ma vanno invece applicate le disposizioni generali in tema di inquinamento o di rifiuti*". Cass., Sez. III, 27 Marzo 2007, N° 21773 In: Ambiente e sviluppo, 2007, 11 p.1024. e recentemente Cass., Sez. III, Sentenza del 24 Luglio 2012, N°30124.

*1.5.3.1. Waste or by-product: regulations*

Norms regarding waste, are established by the Legs. Decree dated 3 April 2006, n° 152 updated on 9 May 2012 with the "*Modifiche al decreto legislativo 3 aprile 2006, n. 152, e altre disposizioni in materia ambientale*" establish by the Senate of the Repubblic.

The Legs. Decree n. 4 dated 2008 entitled "*Ulteriori disposizioni correttive ed integrative del decreto legislativo 3 aprile 2006, n. 152, recante norme in materia ambientale*" introduces important innovations in the definition of the by-product (art. 183, paragraph 1, lett. p), howevere in point 2 of lett. P it establishes that " *il loro impiego sia certo, sin dalla fase della produzione, integrale e avvenga direttamente nel corso del processo di produzione o di utilizzazione preventivamente individuato e definito"*. This definition has created doubts concerning the context in which the by-product may be used, in particular if the production process or the use could be different from the original one.

The "*Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives*" proposes the necessity to avoid "*confusion between the various aspects of the waste definition, and appropriate procedures should be applied, where necessary, to byproducts that are not waste, on the one hand, or to waste that ceases to be waste, on the other hand*". In fact in Article 5 the by-product definition is given.

In Italy with Legs. Decree dated 3 December 2010, n. 205 the "*Disposizioni di attuazione della direttiva 2008/98/CE del Parlamento europeo e del Consiglio del 19 novembre 2008 relativa ai rifiuti e che abroga alcune direttive*" have been defined.

In particular in Art. 12 *"Sottoprodotto e cessazione della qualifica di rifiuto"* after Art. 184 of Legs. Decree dated 3 April 2006, n. 152, Art. 184-bis is inserted; it defines the "by-product". In particular in point b of point 1 the context of use of the by-product is

defined; now it is separate from the initial production process. This article is cited below:

*“E' un sottoprodotto e non un rifiuto ai sensi dell'articolo 183, comma 1, lettera a), qualsiasi sostanza od oggetto che soddisfa tutte le seguenti condizioni…*

*a) la sostanza o l'oggetto e' originato da un processo di produzione, di cui costituisce parte integrante, e il cui scopo primario non e' la produzione di tale sostanza od oggetto;*

*b) e' certo che la sostanza o l'oggetto sara' utilizzato, nel corso dello stesso o di un successivo processo di produzione o di utilizzazione, da parte del produttore o di terzi;*

*c) la sostanza o l'oggetto puo' essere utilizzato direttamente senza alcun ulteriore trattamento diverso dalla normale pratica industriale.*

*d) l'ulteriore utilizzo e' legale, ossia la sostanza o l'oggetto soddisfa, per l'utilizzo specifico, tutti i requisiti pertinenti riguardanti i prodotti e la protezione della salute e dell'ambiente e non portera' a impatti complessivi negativi sull'ambiente o la salute umana.*

*2. Sulla base delle condizioni previste al comma 1, possono essere adottate misure per stabilire criteri qualitativi o quantitativi da soddisfare affinche' specifiche tipologie di sostanze o oggetti siano considerati sottoprodotti e non rifiuti. All'adozione di tali criteri si provvede con uno o piu' decreti del Ministro dell'ambiente e della tutela del territorio e del mare, ai sensi dell'articolo 17, comma 3, della legge 23 agosto 1988, n. 400, in conformita' a quanto previsto dalla disciplina comunitaria.”*

In point 1 of Art. 184-ter the requisites necessary for the“*Cessazione della qualifica di rifiuto”* is defined:

*“Un rifiuto cessa di essere tale, quando e' stato sottoposto a un'operazione di recupero, incluso il riciclaggio e la preparazione per il riutilizzo, e soddisfi i criteri specifici, da adottare nel rispetto delle seguenti condizioni:*

*a) la sostanza o l'oggetto e' comunemente utilizzato per scopi specifici;*

*b) esiste un mercato o una domanda per tale sostanza od oggetto;*

*c) la sostanza o l'oggetto soddisfa i requisiti tecnici per gli scopi specifici e rispetta la normativa e gli standard esistenti applicabili ai prodotti;*

*d) l'utilizzo della sostanza o dell'oggetto non porterà a impatti complessivi negativi sull'ambiente o sulla salute umana.*

At the end of point 5 of the above mentioned article it is established that: *"La disciplina in materia di gestione dei rifiuti si applica fino alla cessazione della qualifica di rifiuto."*

As far as the use of pomace is concerned, until few years ago, not all types of olive residues could be used for heating purposes. In fact the Italian decree dated 8 March 2002, mentioned that only olive pomace without physical or chemical treatment processing could be used. However since 2006 with the D.Lgs n. 152 del 3/04/06 ed al d.p.c.m. 8/10/04) all pomaces resulting from mechanical processing of agricultural products, without chemical additives can be used and are considered a biomass fuel.

In Part 1, in the attachments of the fifth part, section 4 the "nuovo testo unico dell'ambiente" defines the "Caratteristiche delle biomasse combustibili e relative condizioni di utilizzo". Point f of point 1, states that: *"Sansa di olive disoleata…ottenuta dal trattamento delle sanse vergini con n-esano per l'estrazione con l'olio di sansa destinato all'alimentazione umana, e da successivo trattamento termico, purchè i predetti trattamenti siano effettuati all'interno del medesimo impianto; tali requisiti nel caso di impiego del prodotto al di fuori dell'impianto stesso di produzione, devono risultare da un sistema di identificazione conforme a quanto stabilito al punto 3"*. The point 3 is related to the *"Norme per l'identificazione delle biomasse"*. The normative recognition of potential uses of these wastes represents a fundamental point for their valorisation.

## 1.6. Environmental problems posed by olive-mill waste

Around 30 million $m^3$ of by-products are produced annually in the Mediterranean area, generating many environmental problems (Meksi *et al.,* 2012) because of their polluting effects on soil fertility and water (Piotrowska *et al*., 2011; Sierra *et al*., 2001) and its potentially pathogenic consequences. Problems arise also from the fact that olive oil production is seasonal; a large amount of waste is applied over a short period and storage of such large amounts of liquid waste is difficult. Because of their

amphiphilicity a high fraction of phenols (>98%) is lost with the waste stream during the mechanical extraction of olive oil (Rodis *et al*. 2002). In fact, the olive pulp is very rich in phenolic compounds (Cardoso *et al*., 2005), but only 2.7% of the total phenolic content of the olive fruit is released in the oil phase (Rodis *et al.*, 2002) and the remaining amount is lost in the VWs and in the pomace (Figure 10); the values shown in Figure 10 refer to the pomace obtained from three-phases decanters, while the percentage of phenolic compounds contained in the pomace obtained from two-phases system are higher, because it consists also of the phenolic fraction generally present in the vegetation waters.

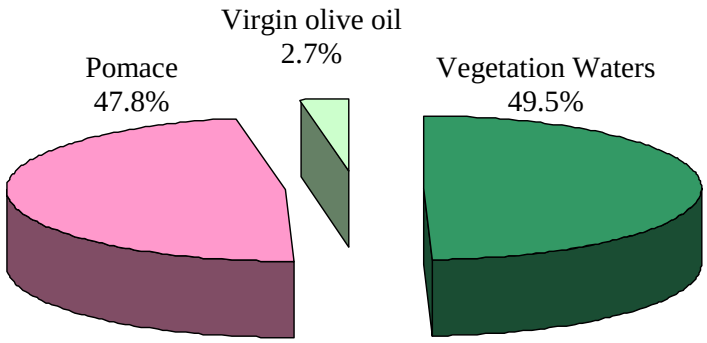


**Figure 10.** Distribution of phenols in VOO and in by-products.

The phenolic components are the various phenolic acids such as caffeic, protocatechuic, α-hydroxycinnamic, vanillic, then flavonoids, anthocyanes, and seleoprotein. The high concentration of phenols provides a batteriostatic property to the VWs due to their antimicrobial activity, so they become less biodegradable. Phenols are unstable and tend to polymerise during storage into condensed high-molecular-weight polymers that are very difficult to degrade (Crognale *et al*., 2006). The pollutant load of VWs is elevated because of the maximum biochemical oxygen demand ($BOD_5$) ranges between 15.000 and 120.000 mg/L and the chemical oxygen demand (COD) concentration of VWs ranges between 40.000 and 220.000 mg/l. This high polluting power makes wastewaters

resistant to degradation and represents a severe environmental problem related to its high organic content made up largely of simple phenolic compounds. The toxic load of VWs in terms of phenolic compounds is estimated to be up to a thousand times greater than that of domestic sewage (Niaounakis and Halvadakis 2004).

The antimicrobial and phytotoxic properties of olive mill wastes have been widely investigated (Bonari *et al*., 1993; Capasso *et al*., 1992; Casa *et al*. 2003; Obied *et al*., 2005). These phytotoxic effects are particularly evident during germination and seedling development (Aliotta *et al*., 2000). Casa *et al*., (2003) and Asfi *et al*., (2006) showed that phenolic compounds have the main phytotoxic properties using the durum wheat and spinach (*Spinacea oleracea* L.), respectively, germinability as a biotest. El Hadrami *et al*. (2004), reported that crude and undiluted VWs was lethal when applied to the crops studied, i.e., maize (*Zea mays* L.), chickpea (*Cicer arietinum* L.), tomato and wheat. Boz *et al*. (2003) studied the herbicidal effect of solid and liquid olive mill waste on common weeds in wheat and maize crops, finding a high inhibition of the germination of *Portulaca oleracea* species. However, Cayuela and co-workers (2008) reports that inhibition of seed germination is species-specific and therefore the toxic effect of these compounds should be investigated for each crop-weed system. Waste spreading can be carried out without significant crop yield losses, at rates lower than 40 $m^3$ $ha^{-1}$, during the advanced growth stage of winter cereals (Bonari *et al*., 2001), on the contrary spreading of higher rates before sowing caused phytotoxic effects and decreases in yield, unless at least 60 days were allowed to elapse between spreading and sowing (Bonari and Ceccarini, 1993).

The uncontrolled disposal of VWs can lead to an alteration in the balance of soil (Moreno *et al*., 1987; Paredes *et al*., 1986) that results in anomalous fermentation of the organic compounds, changing the environmental conditions for the microorganisms and the reduction of soil fertility, also the sugar content of VWs leads to an alteration of the invertebrate community (Cicolani *et al*., 1992). Moreover, the discharge of olive oil mill waste into soils leads to the release of heavy metals retained in the waste, such as Pb, Fe, Cu, Zn, Mn. The solubilisation tends to be a pH-dependent value for some of these metals. The discharge of VWs can also affect the movement of pesticides in soil and in streams. Furthermore, the olive mill waste is sometimes disposed in the latter and

nearby rivers with a considerable impact on the waters; as a result of these malpractices many rivers in Spain and Italy become anoxic (Cabrera *et al*., 1984; Di Giovacchino *et al*., 1976). The subsequent change in colour of these rivers, due to the oxidation and polymerization of tannins giving darkly colored polyphenols, is difficult to be removed (Hamdi, 1992).

Numerous studies have been carried out in order to solve the VWs disposal problem and at the same time valorise it, through physico-chemical or biological pre-treatments to reduce the pollutant load. Concerning this topic, treatments with fungi have been successfully used to mitigate its phytotoxicity (D'Annibale *et al*., 1998; Kissi *et al*., 2001; Sayadi and Ellouz, 1993) such as *Pleurotus* (Tsioulpas *et al*., 2002) and *Lentinula edodes* (D'Annibale *et al.*, 2004) whose cultures in VWs are able to reduce the total phenol content by about 88% in 240 hours. This effect was preferentially exerted on ortho-diphenolic structures, such as catechol, 4-methyl-cathechol and caffeic acid (D'Annibale *et al.*, 2004). Furthermore, some researchers have found that toxicity is also found despite of the total removal of phenols, suggesting that other chemical products contribute to the total amount of toxicity (Capasso *et al*., 1992; Greco *et al*., 2006); it seems to be attributed to long-chain fatty acids and volatile acids (Linares *et al*., 2003; Ouzounidou *et al*., 2008; Paixao *et al*., 1999). Several research works are directed towards the exploitation of microorganisms isolated from VWs or from substrates treated with it over long periods; an example is provided by *Azotobacter vinelandii* strains originating from soil treated with VWs and used as an inoculum for the aerobic biodegradation of the VWs; it was found to reduce the phytotoxicity significantly by reducing COD (70% after three days) and phenolic compounds, up to 100% within seven days, with consequent enhancement of soil fertility (Ehaliotis *et al*., 1999; Piperidou *et al*., 2000).

## 1.7. Possible uses of olive-mill waste

The growing interest in environmental protection has promoted studies on the evaluation of the possible uses of the olive industry by-products.

### *1.7.1. Vegetation water*

Numerous studies have suggested the use of vegetation waters as a substrate for the production of methane (Fiestas Ros de Ursinos *et al*., 1982)**.** Another use is the production of pectic enzymes (Federici *et al.,* 1988) through the use of the microorganism *Cryptococcus albidus* var. albidus, strain IMAT-4735 grown on suitably treated VWs. Vegetation water and in general all the olive industry by-products is also used as a fertilizer (Garcia-Ortiz *et al*., 1999; Tejada and Gonzalez, 2004a), mixed with irrigation water (Briccoli-Bati and Lombardo, 1990) and in animal rations (Martilotti, 1983). VWs is an organic ammendant, according to Italian law, so it is a soil fertilizer and has been considered as an inexpensive method of disposal and recovery of the mineral and organic components present and a valid method of improving soil fertility (Di Giovacchino *et al*., 1990, 2000). The greatest benefits of the effective use of the plant nutrients of the waste, mainly K, N, P, and Mg the fact that they are a low cost source, an important resource for irrigation and supply of organic matter for the soil, which enhances microbial activity and improves the physical and chemical properties of soil. However, this use as a fertilizer is controversial, mainly due to the presence of phytotoxic compounds and their toxic effects, especially phenols, high mineral salt content and low pH.

Nevertheless, high concentration of antioxidants provides nutraceutic properties to VWs and the interest of the pharmaceutical industries in natural antioxidants is growing constantly. VWs could represent an important source of polyphenolic compounds; in fact as the phenolic compounds in virgin olive oil, the waste water extracts have shown powerful antioxidant activity *in vitro* (Visioli *et al*., 1999). Visioli and co-workers (2000a) have shown that in rats the intake of a hydroxytyrosol-rich VWs extract (10 mg/kg) was associated with an increase in the antioxidant capacity in plasma. Another human study tested the antioxidant effect of a hdroxytyrosol-rich phenolic extract from VWs, administered during breakfast to patients with uncomplicated type I diabetes; the main result was the plasma enhanced antioxidant capacity, hence, an antiaggregating platelet action (Leger *et al*., 2005). Concerning the molecules responsible for the protective effect, Angelino and co-workers 2011, found in VWs a bioactive a

phytocomplex made up of hydroxytyrosol (3,4 DHPEA), tyrosol (*p*-HPEA) and the dialdehydic form of decarboxymethyl elenolic acid, linked with (3,4-dihydroxyphenyl) ethanol (3,4-DHPEA-EDA); this is able to permeate the cell membrane, exhibiting antioxidant activity inside the red blood cells.

One of the methods used for recovering hydrophilic phenols and reduce polluting power from VWs is a process consisting of three consecutive membrane-filtration steps with decreasing cut-off values: microfiltration, ultra filtration and reverse osmosis, facilitated by previous enzymatic treatment (Ghanbari *et al.*, 2012; Paraskeva *et al.*, 2007; Servili *et al.*, 2011). The products obtained is a crude phenolic concentrated that has been proven to improve VOO phenolic content when is re-utilised in a virgin olive oil extraction process (Servili *et al.*, 2011) and a permeate without any phenolic compounds and organic residue. Another extraction method for phenolic compounds is super critical fluid extraction with carbon dioxide.

### *1.7.2. Pomace*

Traditionally this by-product is sent for further treatment to obtain an olive pomace oil. The recovery is carried out by extraction with solvent, and by subsequent deacidification and bleaching, in order to make the oil edible. After refining, olive pomace oil is mixed with virgin olive oil and then it is eligible for the commercial class of olive pomace oil as defined by European Community (EC) Regulation No. 356/92. The refining process generates a final solid waste, whose main use is as a fuel resource, in drying ovens or steam boilers because of its thermal capacity, even though, environmental problems associated with smoke emission from burning have led to restrictions on this practice. The use of olive pit for the production of bio-energy is very important, mainly from an environmental point of view; to confirm this in a recent study Russo *et al*., 2008 applied Life Cycle Assessment (LCA) methodology to compare the environmental performance of the recovery of by-products from the olive-oil sector with that of wood-pellet production, and the results showed that the recovery of olive pit offers environmental advantages with respect to other alternative fuels, mainly due to

the higher net calorific value and also the simple recovery method. Therefore, with the continued production of these by-products the need for utilization strategies represent an important step. Further possible uses of pomace are gasification (Ollero *et al*. 2003) and recycling of pomace as a soil amendment (Brunetti *et al*., 2005; López-Piñeiro *et al*., 2006).

Another possible use of olive pomace is in the production of "compost" that is an aerobic exothermic process implemented by micro-organisms that make a partial biodegradation and bioconversion of organic matter that provides a final product fertilizer for agricultural use. This shows significant improvements of the physical-chemical characteristics of soils due to the nitrogen, phosphorus and organic carbon deposited. Many studies looked into the utilization of olive cake for composting purposes (Alburquerque *et al*., 2004; Giannoutsou *et al*., 2004). Pomace contains a large amount of organic matter (90%), and valuable nutrients, especially potassium, and this makes pomace a valuable resource. Pomace has also been successfully tested as a foliar fertilizer (Tejada and Gonzalez, 2004b) and as a soil-less substrate in combination with peat (García-Gómez *et al.*, 2002).

Several studies showed the high potential of olive mill wastes as biobased pesticides against several species of fungi, weeds and nematodes. In particular Cayuela *et al.,* (2008) reports the nematicidal potential of pomace and pomace compost which is able to pass through the nematode egg.

Compared to traditional uses, new uses of pomace, such as the use of virgin pomace in agriculture and in floro-vivaistic productions, and the use of olive stoned pomace as supplements in animal feeding in the livestock sector are being evaluated

## 1.8. Animal feed supplements

The valorization of by-products from various food industries, is nowadays an important area of research. Concerning this topic, in 2007 the FAO conducted a study entitled "Feed supplementation blocks", on the use of solidified blend of agro-industrial by-products as supplement in ruminants feeding, carried out in areas of the world where the

farming conditions is difficult, such as India, Bangladesh, China, Thailand, Sri Lanka, Vietnam, Venezuela, Pakistan, Sudan, Malaysia, and parts of tropical Africa are collected. Ruminants in arid, semi-arid or mountainous areas rely almost exclusively on pasture as a major component of their diet, however a wide range of secondary compounds, particularly tannins, have been implicated as anti-nutritional components of shrubs and trees, and these limit the nutritional potential, reducing the absorption-process. This is mainly due to the ability of tannins to form complexes with proteins, carbohydrates, amino acids and minerals.

However, it is now recognized that these phenolic compounds can be toxic, innocuous or beneficial depending on the type, chemical structure, amount administrated and animal species (Makkar, 2003; Mueller-Harvey, 2006; Toral *et al.,* 2011).

The negative effects of tannins include inhibition of digestive enzymes, hence, lower digestibility, loss of endogenous protein and systemic effects as a result of the absorption of hydrolysable tannin degradation products from the digestive tract. Furthermore tannins limit N availability (Mangan, 1988).

Vermeire and Wester, (2001) highlighted the fact that animal performance is reduced when tannins exceeded 5% of forage weight, and Hegarty *et al*., (1985) assessed that both condensed and hydrolysable tannins can exert detrimental effects, however tannins can also exert a preventive action against gastrointestinal parasitism (Paolini *et al*., 2002). Several methods have been tested to de-activate tannins and reduce the negative effect of these in animal nutrition; the use of Polyethylene glycol (PEG) is the only approach that is proven to reduce the negative effect of tannins by forming complexes with them and seems to be the more effective way to increase the nutritive value of tree foliage and shrubs as found in goats (Ben Salem *et al*., 2003; Decandia *et al*., 2000; Titus *et al*., 2001), and in sheep (Villalba and Provenza, 2002). PEG was usually incorporated into feed blocks that are an efficient supplement for increasing intake, rumen fermentation, digestibility and daily weight gain. The use of solid feed supplementation blocks provides nitrogen, the minerals and vitamins missing in fibrous feeds; other advantages are ease of transport and storage.

Furthermore feed blocks can be used as a carrier for some additives such as minerals, to increase reproductive performance or anthelmintic drugs for the control of

gastrointestinal parasites. Formulation of this supplement includes one or more binders such as lime, cement or bentonite, common preservatives such as salt, urea as a source of non-proteic nitrogen, molasses and other ingredients to ensure an adequate supply of energy, nitrogen and minerals. "Molasses-free" blocks have demonstrated equivalent benefits both in terms of degree of acceptance by ruminants and the nutritional value of low quality roughage. The development of "mini-blocks" without urea, has facilitated integrated feeding for rabbits (Ramchurn and Ragoo, 2000). Most research on feed-blocks has been published over the last three decades (Sansoucy, 1995, 1996) however Cordier (1947) highlighted that the use of these feed supplements was already implemented in Tunisia in 1930. In recent years, about 25 formulations have been used (Ben Salem and Nefzaoui, 2003) and at least 60 countries have adopted this type of dietary supplement for ruminants in difficult conditions, especially cows, sheep and goats. The widespread use of these blocks in the world reveals the importance of this supplement in the livestock sector and the improvement in revenue for farmers and ranchers. Their using in fact, not only provides a continuous and balanced supply of energy and nutrients such as nitrogen, minerals and vitamins a low cost, but it is also represents an effective method for the enhancement of agro-industrial by-products with a high moisture content which are not always eco-compatible and otherwise expensive to dispose of.

## 1.9. Olive pomace in livestock feeding

In the last 50 years olive growing has undergone an expansion due to strong demand; furthermore the increasing interest concerning sources of fats with a healthy fatty acid profile has led to an increase in production, moreover the use of by-products as sources of nutrients for animals can improve the economy and the efficiency of agricultural, industrial and livestock production (Molina-Alcaide, 1996). In Italy from the early decades of the 1900s, several studies were conducted to assess the effect of olive pomace feeding on animals (Gugnoni, 1920; Maymone and Giustozzi, 1935) and the

results showed the positive effect of pomace intake as a feed integrator on the end products.

The main types of pomace that are subjected to the drying process are virgin olive pomaces, pomaces destoned in pre-extraction and pomace destoned in post-extraction, among these, only the last two are the more suitable for a zootechnical use. The use of virgin olive pomace can, however, be evaluated, but only after its enzymatic stabilization. This process is involved in the degradation of the polysaccharide fraction of the pomace, which consists mostly of cellulose, pectins and hemicelluloses, increasing the amount of soluble fiber and consequently its dietary value as animal feed (Servili *et al.*, 2007b).

### *1.9.1. Digestibility*

The low degradable fraction of dry matter, crude protein and NDF of olive by-products are the most important limitation factors in ruminant nutrition. In fact, crude fibre is mainly constituted of lignin, which limits the feed value of olive cake; furthermore a large proportion of protein is linked to the ligno-cellulose fraction decreasing the bio-digestion of pomace. This evidence induced many studies to improve the nutritive value of olive pomace. However, among domestic ruminants, goats seem to be the ones most suitable for utilizing these high lignin-cellulose and low protein forages (Beede *et al*., 1986; Molina-Alcaide *et al*., 1997) while the other ruminants require an adequate supply of protein nitrogen for the ruminal microorganisms in addition to specific de-activating tannin compounds (Molina-Alcaide *et al*., 2003).

Alkali treatments have been the most studied procedures, treatments with NaOH seems to improve *in vitro* digestibility (Abdouli, 1979). Another trial study was carried out by Nefrazoui (1986), ensiling the pomace with different levels of sodium hydroxide and ammonia on a laboratory scale and assessing the digestibility of the pomace as feed for sheep; the main results were that the ammonia treatment increased the organic matter digestibility by 4 % units and the nitrogen retention by 6%.

The partial destoning is considered a valid economic possibility to improve the nutritive value of this by-product and increase its digestible content. Sadeghi *et al.,* (2009) in a recent study evaluated the effect after feeding ewes with four different olive pomaces: crude, exhausted, partly destoned and partly destoned exhausted pomace. These were included in experimental rations of ewes and tested the effect on fattening performance. The results of this trial show that destoned olive pomace has a higher nutritive value than the other pomaces and improves body weight gain and growth rate.

### *1.9.2. Effect of olive pomace supplementation on fatty acid composition of milk and meat*

Several studies have shown positive effects arising from the use of virgin olive pomaces as animal feed supplements such as the increase in the amount of unsaturated fatty acids from the meat and milk of ruminants (Molina-Alcaide *et al*., 2008), increased oxidation stability of both milk and cheese products, and an increase in vitamin E (α-tocopherol) in milk fat (Pauselli *et al*., 2007; Servili *et al*., 2007). The intake of SFAs is strongly related to an atherogenic and thrombogenic risk (Givens, 2005). At this regard, it has been estimated that for every 1% reduction in dietary SFA intake, there is an associated 3% decrease in cardiovascular risk (Minihane, 2006). Therefore olive pomace with its high amount of oleic acid has been considered in dairy ewe nutrition to modify the fatty acid composition of milk (Chiofalo *et al*., 2004) increasing the unsaturated-saturated fatty acid ratio, improving oleic acid content and the oxidative stability of milk due to its high content of antioxidants (Chiofalo *et al*., 2004; Pauselli *et al*., 2007).

Another study conducted by Vera and co-workers (2009) evaluated lamb carcass quality and fat composition providing a destoned dry pomace-based ration, instead of a conventional ration or pasture feeding. The results showed a decrease of the amount of saturated fatty acid and an increase in monounsaturated fatty acid with a high increase in oleic acid. Pork fed with olive pomace (10% inclusion rate) was significantly lower in saturated fatty acids, polyunsaturated fat and ω-6 fatty acids ($p<.05$) compared to the control diet, evaluated on the *Longissimus* muscle (Doyle *et al.,* 2006).

### *1.9.3. Effects of olive pomace supplementation on animal's health*

Olive stone removal before the crushing can reduce the phenolic oxidative degradation, during processing providing by-products having an increased content of phenols. In fact as previously shown, about 48% of the total phenolic content of the olive fruit, is transferred into the olive pomaces (Figure 10), consequently the virgin pomaces destoned in pre-extraction are more richer in natural antioxidants, with a residual oil content ranging from 8% to 15% (Dal Bosco *et al*., 2007; Pauselli *et al*., 2007; Servili *et al*., 2007a, 2007b). Stoned pomaces have a fatty acid composition similar to that of virgin olive oil and also show a high concentration of bio-active phenolic compounds such as lignans and secoiridoids. For this reason, the virgin destoned pomace is considered as a good supplement animal feed in the livestock sector and an important source of monounsaturated fatty acids and antioxidants.

The use of dried destoned pomace could be considered interesting in dairy production to increase the oxidative stability of milk and cheeses. Several studies have shown that the phenolic compounds of pomace are able to prevent lipid oxidation with a potent free radical scavenging activity (Amro *et al*., 2002).

The effect of pomace antioxidant and radical scavenging activity, such as hydroxytyrosol (3,4-DHPEA), tyrosol (p-HPEA) and their secoiridoid derivatives (dialdheydic form of decarboxymethyl elenolic acid, 3,4-DHPEA-EDA or p-HPEA-EDA) as well as verbascoside, is proven by several studies.

Dal Bosco *et al*., (2012) shows the effects on a rabbit diet of including (5%) pomace from different olive cultivars characterized by different phenolic concentrations; this feed integration leads to an increase in oxidative stability and nutritional value, as revealed by the low concentration of lipid peroxidation and the high nutritional indexes, the greater the increase, the greater the phenol content. This shows how pomaces with high polyphenols content are able to prevent the oxidation of unsaturated lipids, contributing to the preservation of the dietetic-nutritional value of the meat, moreover, also stone removal contributed to reducing the oxidative degradation of phenols, considering that seed has the highest peroxidative activity (Dal Bosco *et al.*, 2007).

### *1.9.4. Storage*

The use of pomace as a supplement animal feed in the livestock sector has been taken into consideration even if the production is seasonal and therefore requires adequate techniques for storage (Molina-Alcaide and Yáñez-Ruiz, 2008). Concerning this topic the main problem is related to the high water content and the large amount of oil retained, which quickly becomes rancid when exposed to air. It has been estimated that pomace obtained by centrifugation deteriorates after 4-5 days, whereas pomace obtained by pressure deteriorates after about 15 days (Sansoucy, 1985).

Silage has been reported to be an efficient procedure to preserve pomace, alone (Hadjipanayiotou, 1999), or mixed with other feed (Hadjipanayiotou, 1994) or with urea (Al-Jassim *et al*., 1997) or with alkali. In particular the ensiling technique can be used for long term storage of pomaces, and can partially replace conventional roughage in diets of mature, growing and lactating ruminants (Hadjipanayiotou, 2000). Often molasses is added in order to supply water soluble carbohydrates necessary for the ensiling fermentation. In a recent study it was concluded that molasses added at 3% could improve the ensiling fermentation (Weinberg *et al*., 2008) of pomaces without substantial losses, while with a higher application of molasses more yeasts developed and ensiling losses increased. However results from a recent study conducted by Rowghani and Zamiri (2007), indicated that treating pomaces before ensiling with 8% molasses, 0.4% formic acid and 0.5% urea could provide a good and economical source of a non-conventional feed and help to improve the diet formulation for ruminants.

Vera *et al*., (2009) evaluate the stability of destoned, unexhausted olive cake, originating from the two-phase process dry pomace over time, the results showed that dehydrated pomace can be preserved for several months with no detrimental effects on its lipid composition and quality.

## 1.10. Unsaturated fatty acid sources as supplements in animal feeding

Dairy products are the main source of 12:0 and 14:0 in the human diet and make a significant contribution to 16:0 and trans fatty acids intake (Shingfield, 2008a). To improve the quality of milk from a nutritional point of view is one of the most important purposes of the milk industry.

The reduction of saturated fatty acids, in favour of polyunsaturated fatty acids, increasing the content of n-3 series, including α-linolenic acid (ALA), has been proven to have beneficial effects. In particular ALA is recognized as minimizing the risk of cardiovascular disease, modulation of the inflammatory response, and shows a positive impact on both central nervous system function and behaviour (Stark *et al.*, 2008). Also the increase in concentration of conjugated linoleic acid (CLA) has been reported to decrease tumorigenesis in animals both *in vitro* and *in vivo* (Deker, 1995; Ip *et al.*, 1994; Parodi, 1997). CLA refers to a group of polyunsaturated fatty acids that exist as positional and stereo-isomers of conjugated dienoic octadecadienoate (18:2) and the predominant geometric isomer in foods is the c9, t11-18:2, also called “rumenic acid” (Kramer *et al.*, 1998). The diet influences milk composition and fatty acid profile, hence, the use of vegetable lipid sources aimed at increasing the level of MUFA, PUFA and CLA in milk has been tested in numerous studies both in cattle and in sheep (Castro *et al.*, 2009; Gómez-Cortés *et al.*, 2011; Mele *et al.*, 2007; Zhang *et al.*, 2006a, 2006b). However, is well known the importance of ingestion of grass grazing directly, on the level CLA in milk and its derivatives both in cattle (Bargo *et al.*, 2006) and in the sheep (Addis *et al.*, 2005); in fact the highest concentrations of CLA in milk fat and unsaturated fatty acids (UFA) are mostly found in milk from animal diet based on long periods of pasture feeding. Studies on grazing cows shows high proportions of UFA and more CLA content than cows fed just on silage, which on the contrary shows an increased proportion of saturated fatty acids (Elgersma *et al.*, 2004; Gonzalez-Rodríguez *et al.*, 2010).

The two main sources of linolenic acid are grass, both fresh and ensiled (Boufaied *et al.*, 2003), and linseed (Doreau *et al.*, 2009). Linseed is particularly rich in ALA and it has been proven that its intake can lead a significantly increase in linolenic acid content in

both milk (Loor *et al.,* 2005) and meat (Normand *et al*., 2005). These benefits are independent of the form of administration; whole seed (Ward *et al*., 2002), ground seed (Collomb *et al*., 2004), extruded seed (Schori *et al*., 2006), or oil (Dhiman *et al*., 2000). Oilseeds can be either provided as whole seeds or processed by different techniques, the most common is the extrusion. The animal products, after linseed intake are enriched in rumenic acid, which has been proven to have positive effect on human health, decreasing the incidence of cancer (Shingfield *et al.,* 2008b). The moderate amount of linseed added to the diet has no detrimental effects on organic matter or fiber digestion (Ueda *et al.,* 2003). Concerning this topic, Doreau *et al.,* (2009), investigates the consequences of a linseed supply on the changes in organic matter and fiber digestion comparing three linseed forms; rolled, extruded and oil. They found that linseed supplementation increases the duodenal flow of unsaturated intermediates of biohydrogenation, and this effect is more evident using extruded seeds. The ability of extruded linseed in modifying fatty acids composition and conjugated linoleic acid concentration, by dietary supplementation, were assess in several studies, in ewes (Mele *et al*., 2007) in goats (Nudda *et al*., 2006) and cows (Pezzi *et al*., 2007). Hurtaud *et al*., (2010) found that increasing the amount of extruded linseed dietary ratio in dairy cow feeding leads a linearly decrease of milk fat content and globule size and at the same time a linearly increase the percentage of milk unsaturated fatty acid, specifically α-linolenic acid and trans fatty acid. A similar result was obtained by Oeffner *et al.,* (2013) whose have been shown that the increase of supplementation rates of extruded linseed improved the fatty acid profile of milk, butter, and cheese gradually with a simultaneous decrease in saturated fatty acids in serum in Holstein cows.

Mele and co-workers (2010) studied the milk fatty acid composition in Sarda ewes, particularly evaluating the change in rumenic acid content, in dietary supplementation with whole extruded linseed over a long period (70 days). The results showed that concentrations of cis-9, trans-11, and vaccenic acid (VA), reached the highest levels of enrichment after 7-8 weeks (3.06, 7.31 g/100 g milk fat for RA and VA, respectively), furthermore, the milk from the ewes fed with linseed showed a significant reduction:

(-17%) in saturated fatty acid concentration in the milk, improving its quality and providing it of important transferable properties.

# 2. PURPOSE OF THE THESIS

This research project is aimed at the valorisation of two types of pomace obtained from the extra virgin olive oil mechanical extraction process such as olive pomace and a new by-product named 'paté'. For this purpose, these by-products have been used in the livestock sector as important sources of antioxidants and unsaturated fatty acids. This work project includes two parallel researches:

***Research 1.*** The suitability of dried stoned olive pomace as a dietary supplement for dairy buffaloes was evaluated. The effectiveness of this utilization in modifying fatty acid composition and improving the oxidative stability of buffalo milk and mozzarella cheese was assessed by means of the analysis of qualitative and quantitative parameters;

***Research 2.*** The use of paté as a new by-product in dietary feed supplementation for dairy ewes, already fed with a source of unsaturated fatty acids such as extruded linseed, was studied in order to assess the effect of this combination on the dairy products obtained. The characterization of paté as a new by-product was also carried out, studying the optimal conditions of its stabilization and preservation at the same time.

The transfer of bioactive compounds and the improvement of the quality of milk fat could positively interact in the prevention of some human cardiovascular diseases and some tumours.

# 3. MATERIALS AND METHODS

## 3.1. Research 1: olive pomace supplementation in buffalo feeding

This research project was carried out in 2008; however in the following year (2009) a partial repetition of the trial was carried out, in order to evaluate how the processing system to obtain mozzarella cheese could affect the difference in the fatty acid composition of the lipid fraction, due to dietary treatment. The phenolic and acidic composition of the pomace, the fatty acid composition of the milk obtained, mozzarella fatty acid composition and its oxidative stability were evaluated for the year 2009. The experimental plan implemented in the second year and the feed used were the same as those of the previous year, except for the amount of the daily pomace ration that was slightly higher: 1.05 kg DM/d of pomace per head in 2008 vs. 1.2 kg DM/d of pomace per head in 2009.

### *3.1.1. Production of stoned olive pomace*

The fresh pomaces were obtained, in 2008 and 2009, from virgin olive oil mechanical extraction using the following operative conditions: the olives were stoned and malaxed for 40 min at 25°C, the oil extraction was performed using an RCM Rapanelli three phase decanter mod. 400 eco. The stoned pomaces were stored at room temperature for a maximum period of 36 hours before drying, then were dried using a fluid-bed dryer, with a capacity of 20 kg/hour of evaporated water, following the process defined by Servili *et al*., (2007a); the initial temperature of the flow of drying air was 120°C and the maximum temperature of the pomaces during the drying process was 45°C. After drying, the dried stoned olive pomaces (DSOP) were stored at room temperature.

### *3.1.2. Animals and diets*

Sixteen pluriparous Mediterranean buffaloes were used, divided at the beginning of the trial into two uniform groups: control and experimental. The two groups were not statistically different for the following parameters: milk production (2192 and 2102 kg) and duration of the lactation (254 and 252 d) of the previous year; distance from calving (51 and 43 d), milk production (9.71 and 10.18 kg/d), body condition score (BCS) (6.44 and 6.31) and weight (617 and 653 kg) at the beginning of the trial. The trial lasted 40 days. The animals were weighed at the beginning and the end of the trial and the nutritional status was determined using the BCS, utilizing the scale of Wagner *et al.* (1988) modified, for the buffalo species, by Campanile *et al.* (1998); this method provides for the use of a score from 0 to 9. The feedstuffs used were second cut alfalfa hay, corn silage and two concentrates especially formulated using the same feed materials (Table 7), the experimental concentrate contained DSOP (15.50% as fed). The control and experimental diets were isoenergetic and isoproteic. Both the diets had the following formulation dry matter basis: second cut alfalfa hay 20%, maize silage 42%, and concentrate 38%. The feeding was by group and the feed was given as a mixed ration once a day; 17 kg DM/d was administered to each animal and each buffalo in the experimental group received 1.05 kg DM/d (2008) and 1.2 kg DM/d (2009) of DSOP which represented respectively 6.17% and 7.05% of the diet. The ingestion of 17 kg DM/d per head for each group was constantly maintained for the duration of the trial; in this phase of lactation, this quantity represents the maximum ingestion capacity (Bartocci *et al.*, 2006).

**Table 7.** Formulation of the two concentrates (% as fed).

| Items | Control | Experimental Concentrates |
|---|---|---|
| Bran flour | 27.00 | 20.00 |
| Maize flour | 21.00 | 25.00 |
| Soya extract flour | 12.00 | 15.00 |
| Flour from distillery residues | 10.00 | 5.00 |
| Flour of dehydrated alfalfa | 10.00 | - |
| Flour from sunflower extract | 9.00 | 12.00 |
| Flour from beet pulp | 3.50 | - |
| Flour from stoned olive pomace | - | 15.50 |
| Molasses | 3.00 | 3.00 |
| Vitamin-mineral supplementation | 4.50 | 4.50 |

### *3.1.3. Chemical analyses of animal feeds*

The following analytical determinations were frequently performed on samples of the feedstuffs used: dry matter (DM), crude protein (CP), crude fibre (CF), ether extract (EE) and ash (AOAC, 1995); neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) according to the method reported by Goering and Van Soest (1970), the non-structural carbohydrates were calculated according to the method reported by Van Soest *et al.* (1991).

The phenolic compounds of DSOP were extracted following the procedure described by Servili *et al.* (2011), using 10 g of the sample and reported below:

*Preparation of the aqueous extract*: 10 gr of the sample was homogenized with 100 ml of a mixture of methanol and water (80:20, v/v) containing 20 mg/L of sodium diethyldithiocarbamate (DIECA); the extraction was performed in triplicate. After methanol removal, using rotavapor, the aqueous extract was used for SPE phenol separation.

*Solid phase extraction (SPE):* the cartridge was activated with 10 ml methanol + 10 ml of distilled water and then dried with N2. Than the SPE procedure was applied by loading 1 ml of the aqueous extract into an Extraclean High-load C18 cartridge (Alltech

Italia S.r.l.) and eluting with methanol (50 ml). After solvent removal under vacuum at 30°C, the phenolic extract was resolubilized with 5 ml of methanol and after sovent removal, re-dissolved in methanol (1 ml).

*Sample preparation for HPLC analysis*: the phenolic extract was recovered with 1 ml of methanol, filtered with 0.2 m PVDF filter and recovered in a vial.

The HPLC-DAD/FLD analyses of the phenolic extracts were conducted according to Servili *et al.* (2011) with an Agilent Technologies system, model 1100, composed of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, a DAD and a fluorescence detector (FLD). The C18 column used was a Spherisorb ODS-1 measuring 250 mm X 4.6 mm with a particle size of 5 µm (Phase Separation Ltd., Deeside, United Kingdom); the injected sample volume was 20 µl.

To extract the residual oil from the DSOP, 250 ml of hexane was added to 100 g of DSOP. The mixture was homogenized with the Ultra-Turrax T50 (IKA-Labortechnik, Staufen, Germany) at 6000 rpm for 10 minutes at 20°C and then filtered with filter paper. The extraction was performed twice. The filtered homogenate was evaporated under vacuum in a nitrogen flow at 35°C. In the residual oil, the acidic composition was determined according to the 1989/03 Commission Regulation (2003).

Feed fatty acid composition was ascertained by gas chromatography, as methyl ester derivatives after transesterification with sulphuric acid. A MEGAL FISONS gas chromatographic apparatus was equipped with a detector and a capillary column (D-B Wax 30 m in length, 0.25 mm in thickness and 0.25 µm in thickness of the internal film) with helium as the carrier gas. The temperature was programmed at 130°C for 5 min followed by an increase to 230°C at a rate of 20°C/min. The temperatures of the injector and detector were 270°C and 260°C, respectively. The peak areas of the individual fatty acids were identified by means of previous fatty acid standard injection (Sigma-Aldrich) and quantified as a percent of total fatty acids.

The extraction of tocopherols, tocotrienols and retinol from the feeds was carried out according to the method of Havemose *et al.* (2004), by means of chromatography.

### *3.1.4. Buffalo milk and mozzarella cheese analyses*

Milk production, representative of two milkings at intervals of 12 h, was determined at the beginning of the trial and after 15, 30 and 40 days. The individual milk samples, representative of the two milkings, taken at each control, underwent the following analytical determinations: fat, protein (Nx6.38), lactose, urea and pH (ASPA, 1995). The parameters for milk coagulation were determined: rennet clotting time (r), curd firming time ($k_{20}$), curd firmness ($a_{30}$) by means of the thromboelastograph Formagraph as reported by Zannoni and Annibaldi (1981).

During the last two weeks of the trial, the bulk milk of the two experimental groups was processed according to the following artisanal method: a pilot plant consisting of thermoregulated (37°C) vats and the natural whey culture ("cizza") were used. The natural starter was added to the vat milk to bring the acidity of the mixture to 10°SH. Eight cubic centimeters (1:20,000) of liquid rennet was added to obtain milk clotting within 20 min, as required by the traditional method of cheese making. After one hour, the curd was cut and left to rest for approximately five hours under serum. Then, the curd was stretched in hot tap water (95ºC), and afterward cheese pieces of about 70 g were docked. The cheese pieces were then cooled in cold water for 1 h and then kept at room temperature in skim water resulting from the stretching, diluted with skim whey from previous manufacturing up to 5-6 °SH (pH 3.842) and 1% NaCl (referred to in this study as "governing liquid").

After each processing, the mozzarella and governing liquid samples were collected according to the following procedure; Mozzarella was divided into two halves and was subsequently homogenized with an Omnimixer homogenizer at low speed without other solutions. The governing liquid filtered with Whatman N°1 filters.

The milk and mozzarella fatty acid composition were evaluated by gas chromatographic analyses as were the methyl ester derivatives after transesterification with sulphuric acid following the procedure reported above for the animal feeds samples. To evaluate the risk of coronary disease, atherogenic and thrombogenic indexes were calculated as suggested by Ulbricht and Southgate (1991). Tocopherol, tocotrienol and retinol extraction was conducted according to the method proposed by Havemose *et al.* (2004).

The evaluation of the oxidative stability was carried out by the determination of thiobarbituric acid reactive substances, expressed as mg/L of malondialdehyde according to the method proposed by Fenaille *et al.* (2001).

### *3.1.5. Analysis of the phenolic compounds of milk*

A specific method has been developed for the extraction and purification of the phenolic fraction from milk, and it is reported at the end of "Results and Discussion" section.
For LC-MS/MS analysis, 20 μl aliquots of purified phenolic fraction, were injected into an HPLC system consisting of two 218 pumps and a Varian 1200L triple-quadrupole mass spectrometer equipped with an electrospray ionization interface (Varian, Palo Alto, CA). A Varian C18 Intertsil ODS-3 column (250 x 4.6 mm, 5 μm) was used as the HPLC column. Elution was performed at a flow rate of 1.0 ml/min using a mixture of water/acetic acid (99.8:0.2 v/v) (solvent A) and methanol (solvent B) as mobile phases. The gradient was changed as follows: to 95% solvent A for 2 min, to 75% solvent A in 8 min, to 60% solvent A in 10 min, maintained for 2 min, to 100% solvent B in 4 min, maintained for 14 min, and returned to initial conditions in 7 min. The total run time was 47 min. Data were acquired using the Varian MS Workstation data system. A splitting valve allowed a solvent flow of 0.2 ml/min from the HPLC to the electrospray source. LC MS/MS analysis was performed in negative multiple reaction monitoring (MRM) mode. The mass spectra were obtained under the following conditions: a spray voltage of 4500 V, shield voltage of 600 V, capillary voltage of 60 V, nebulizing gas pressure of 50 psi, drying gas pressure of 18 psi, temperature of 230°C and electron multiplier voltage of 1500 V. The formation of product ions in the MS/MS experiments was induced by collision (CID) of the selected precursor (3,4-DHPEA m/z 153) with argon.

### *3.1.6. Statistical analysis*

The differences between the two groups were tested by means of the GLM procedure (SAS, 2001) using the monofactorial model:

$Yij = \mu + \alpha i + \varepsilon ij$

where $\mu$ = general mean; $\alpha i$ = diet (i = 1, 2); $\varepsilon ij$ = error of model.

## 3.2. Research 2: olive paté supplementation in sheep feeding

### *3.2.1. Production of olive paté*

The paté is a new by-product obtained by virgin olive oil mechanical extraction, by separation from the innovative two-phases Pieralisi decanter, named DMF.

This decanter, even though is a two-phases, and does not require the use of added water, produces three types of products: oil, a fairly dry pomace (like that of a three-phase extraction system, but contains all the hardest parts of the olive, such as epicarp, stone, seed as well as a small amount of pulp) and the paté (consisting of pulp and olives water). The estimated amounts of this products for 100 kg of processed olives are: 15-20% oil, 42-43% of the remaining percentage is pomace and 42-43% is paté.

It represents, as well as the virgin olive pomace, the solid fraction separated from the horizontal centrifuge, but without the woody fraction and epicarp of the drupe. The paté used for the tests of storage in silos was obtained from olives harvested in three successive batches of product, during the month of December 2008, in advanced state of maturation. The third batch was also used for testing storage in silos; samples of this batch were analyzed in the following months (January, February, March and April 2009), in order to assess the stability of the ensiling by-product over time. The paté used for the supplementation in ewes' feed was obtained from olives harvested during the month of November 2009. The paté, both in year 2008 and in year 2009, was dried using a fluid-bed dryer, following the procedure mentioned above for pomace.

### *3.2.2. Preparation of feedstuff*

Concerning the three batches of fresh paté a part of the first two, and a part of the third batch related to the trial on freshly ensiled paté, was analyzed fresh, and the following samples were dried before analysis:

-pate fresh as it is;

-pate fresh + 20% alfalfa;

-pate fresh + 20% soybean meal;

-pate fresh + mixture of soybean meal 10% and alfalfa meal 10 %.

Only the mixing of the paté with vegetable flours at a rate of 20% was evaluated for the second batch.

### *3.2.3. Animals and diets*

Fifteen multiparous Comisana ewes an average body weight of 63 ± 4.5 kg and 161 ± 14 days of lactation were kept at the Experimental Section of the Department of Applied Biology, University of Perugia. The animals were allotted into 3 experimental groups, corresponding to three different dietary treatments. The experiment was conducted in 2010 and lasted 28 days after 14 days of acclimatization. The animals were grazed each day from 6:00 p.m to 7:00 a.m, on a natural pasture characterized by the botanical composition reported in Table 8, following the strip-grazing technique. Grazing periods lasted 3 days each by the displacement of the electrified fence, yielding approximately 40 $m^2$/ewe/day, water was always available.

Treatments were: 1) 150 g/head/d rolled linseed (L), 2) 162 g/head/d dried paté (mixed with 20% of dehydrated alfalfa hay) (OP) and 24 g/head/d olive oil and 3) 75 g/head/d rolled linseed, 81 g/head/d dried paté (mixed with 20% of dehydrated alfalfa hay) and 12 g/head/day olive oil (LOP).

The percentage composition of the three test concentrations is shown in Table 9. The experimental concentrates (600 g/head/d) were offered after the morning and the afternoon milking, while a 100 g/head of rolled barley were offered during milking. The ewes were milked daily at 07:30 AM and 17:30 PM, and daily milk yield recorded.

**Table 8.** Pasture grasses (%).

| | |
|---|---|
| *Bromus sterilis* L. | 40 |
| *Medicago arabica* (L.) Hudson | 30 |
| *Bellis perennis* L. | 5 |
| *Bromus hordeaceus* L. | 5 |
| *Hordeum murinum* L. | 5 |
| *Avena barbata* Potter | 5 |
| *Calamintha nepeta* (L.) Savi | + |
| *Dactylis glomerata* L. | + |
| *Geranium dissectum* L. | + |
| *Orchis purpurea* Hudson | + |
| *Poa pratensis* L. | + |
| *Trifolium incarnatum* L. | + |
| *Vicia sativa* L. | + |

**Table 9.** Dietary treatments composition (%).

| Feeds | Experimental concentrates | | |
|---|---|---|---|
| | L | P | PL |
| Wheat middlings | 13.9 | 4.5 | 9.0 |
| Soybean meal | 1.0 | 6.0 | 3.4 |
| Corn | 21.0 | 22.0 | 22.0 |
| Dried Beet Pulp | 20.0 | 1.9 | 11.0 |
| Corn Gluten Meal | 2.0 | 2.0 | 2.0 |
| Barley | 10.0 | 10.0 | 10.0 |
| Rolled Linseed | 25.0 | - | 12.5 |
| olive paté mixed with alfalfa meal | - | 44.5 | 22.0 |
| Olive oil | - | 4.0 | 2.0 |
| Molasses | 4.0 | 2.0 | 3.0 |
| Calcium Carbonate | 1.0 | 1.0 | 1.0 |
| Sodium Chloride | 0.5 | 0.5 | 0.4 |
| Sodium di-carbonate | 0.5 | 0.5 | 0.5 |
| Di-calcium Phosphate | 0.5 | 0.5 | 0.5 |
| Mineral-Vitamins Premix | 0.6 | 0.6 | 0.6 |

### *3.2.4. Chemical analyses of animal feeds*

The analytical determinations were frequently performed on samples of the feedstuffs used as above reported with pomace. The phenolic compounds of feed were extracted and analysed by HPLC-DAD/FLD following the procedure previously described by Servili *et al.* (2011), using 10 g of product (20 g for the phenolic extraction from fresh paté). The feed fatty acid composition was evaluated by gas chromatographic analyses as reported above for the buffalo feeds and milk samples. The extraction of residual oil from paté was carried out following the method above reported for residual oil from pomace. In the residual oil, the acidic composition was determined according to the 1989/03 Commission Regulation (2003). The extraction of tocopherols, tocotrienols and retinol from the feeds was carried out according to the method of Havemose *et al.* (2004), by means of chromatography.

### *3.2.5. Ewes' milk and cheese analyses*

Production was recorded daily and milk samples were collected weekly to assess chemical, physical parameters (following the method reported above for buffalo milk).
The collected milk was promptly place to freezing and stored at -80° C until the time of analysis while another part was processed into cheese.
The milk of each group was processed into cheese using a polyvalent tank, built in stainless steel with a maximum capacity of about 50 l.
The starter used was obtained by inoculating ewes' milk treated at 90 ° C x 7 minutes, allowed to cool and maintained at 42° C in an oven, with a lyophilized culture of various selected strains of *Lactobacillus delbrurckii* (ssp. Bulgaricus and Streptococcus thermophilus). Then the inoculum was fractionated and frozen. The amount of rennet used was 30g/100L of milk, diluted in a ratio of 1:10 with water at 25° C. After the the frozen inoculum was added, the milk, stirred continuously, was brought, to a temperature of 35° C in the polyvalent. After about 6-7 minutes, with a milk temperature of 37.5° C the diluted rennet paste was added; after 15-17 minutes from the uniform distribution of the rennet, a first break was carried out with the help of the

cutter and a knife to obtain a checkerboard with squares approximately 2.5 cm from the side. After 5 minutes, the breaking of the curd was carried out with a simultaneous heating of the mass until a temperature of 38-40° C was reached. The gradual increase of the speed of agitation allowed the curd to refine in about 15 minutes. Subsequently, with the aid of perforated molds, the curd was extracted from polyvalent and the serum recovered for the subsequent production of ricotta. The shapes, slightly pressed initially, were then turned over every 30 minutes for the first two hours in order to facilitate the elimination of the serum still retained by the cheese. The cheese was then maintained at a temperature of about 40-45° C for about 3h to ensure the multiplication of the inoculum. After about 15-20 hours from the production, the shapes were placed in brine (saturated NaCl solution) for 6-7 hours. The process ended with the aging at a temperature of 10-13 °C with RH 80%.

After each processing, the cheese samples were were grated at low temperature to to make it more homogeneous and then homogenized. The milk fatty acid composition was evaluated by gas chromatographic analyses as were the methyl ester derivatives after transesterification with sulphuric acid following the procedure reported above for buffalo milk. To evaluate the risk of coronary disease, atherogenic and thrombogenic indexes were calculated as suggested by Ulbricht and Southgate (1991). Tocopherol, tocotrienol and retinol extraction was conducted according to the method proposed by Havemose *et al.* (2004). The reactive substances with tiobarbituric acid (TBARs), expressed as µg MDA/g of fat, (Fenaille *et al*., 2001) per ml of milk measured at 0, 24 and 72 hours of exposure to light at 4°C, were determined.

The same method was used to asses the oxidative stability of cheese at the time of sampling, after 3 and 7 days of storage in the dark and at a temperature of 4 ° C in a cell. On cheese (40 d ripening) stored at 4°C and dark TBARs after 0, 3 and 7 d was evaluate. Also, yellow index was evaluated on cheese following the method proposed by Giangiacomo and Messina (1988).

### *3.2.6. Analysis of the phenolic compounds of milk*

A specific method has been developed for the extraction and purification of the phenolic fraction from milk, and it is reported at the end of "Results and Discussion" section. The analysis of these compounds was carried out following the procedure reported above for buffalo milk

### *3.2.7. Statistical analysis*

Data were performed according to the following model:

$Y_{ijkl} = \mu + \alpha_i + \beta(\alpha)_{ij} + dim_{ijk} + \varepsilon_{ijkl}$

where:

Yijk = experimental parameters

$\mu$ = general mean

$\alpha_i$ = fixed effect due to treatment (Linseed, Olive Paté, Linseed+Olive Paté);

$\beta_{ij}$ = random effect due to ewe within tratment;

$dim_{ijk}$ = covariate for days in milking;

$\varepsilon_{ijk}$ = residual errror.

# 4. RESULTS AND DISCUSSION

## Research 1

The results reported below refer to the first year of research (2008).
In addition the ripetition of the analysis of the phenolic and acid composition of the pomace and the fatty acid composition of milk carried out in the following year (2009), are reported below. During this second year mozzarella was made and then the fatty acid composition and the oxidative stability were evaluated.

## 4.1. Characterization of dried pomace and feedstuff

The chemical characteristics of the stoned olive pomace (DSOP) are reported in Table 10, and the protein content is similar to that reported by Malossini *et al.* (1983). The fibrous component was lower than the values reported by Molina-Alcaide and Yañez-Ruiz (2008) for olive cakes. The destoning process determines a reduction in ADL content (207.8 g/Kg), this value represents a further reduction of the values obtained by Chiofalo *et al*. (2004) in partially stoned virgin pomace (308.0 g/kg DM). The DSOP shows a high level of ether extract (206.6 g/kg DM).
The fatty acids profile is characterized by a high amount of C18:1cis9 (75.5%) (Table 11), this value is consistent with that reported by Chiofalo *et al.* (2002); the reduction in lignin associated with the high content of ether extract determine an improvement in the dry matter digestibility (Sadeghi *et al.*, 2009).

**Table 10.** Dried Stoned Olive Pomace (DSOP) chemical characteristics (% D.M.).

| | |
|---|---|
| Dry Matter | 95.67 |
| Crude Protein | 10.08 |
| Ether Extract | 20.66 |
| Neutral Detergent Fiber | 42.16 |
| Acid Detergent Fiber | 32.92 |
| Acid Detergent Lignin | 20.78 |
| Non Structural Carbohydrates | 19.53 |
| Ash | 7.56 |

**Table 11**. DSOP fatty acids profile (% FAME).

| Fatty acids | 2008 | 2009 |
|---|---|---|
| C14:0 | 0.1 | 0.1 |
| C16:0 | 12.3 | 12.7 |
| C16:1 | 0.9 | 0.7 |
| C17:0 | 0.1 | 0.1 |
| C18:0 | 1.9 | 2.0 |
| C18:1n9 | 75.5 | 76.5 |
| C18:2n6 | 8.3 | 6.9 |
| C18:3n3 | 0.6 | 0.7 |
| C20:0 | 0.2 | 0.1 |
| C20:1n9 | 0.3 | 0.2 |

The phenolic composition of the DSOP (2008 and 2009) shows high amounts of secoiridoids, such as 3,4-DHPEA (1.2 g/kg DM), 3,4-DHPEA-EDA (12.6 g/kg DM), *p*-

HPEA-EDA (5.6 g/kg DM) and lignans, including 1-acetoxypinoresinol (Table 12). For the year 2009 also the phenolic composition of fresh pomace is shown (Table 22), in comparison with paté.

**Table 12**. Phenolic compounds of the dried stoned olive pomace (g/Kg D.M.) (± Rmse).

| Phenolic compounds | 2008 | | | 2009 | | |
|---|---|---|---|---|---|---|
| 3,4-DHPEA | 1.2 | ± | 0.8 | 3.5 | ± | 0.1 |
| *p*-HPEA | 0.9 | ± | 0.07 | 2.8 | ± | 0.1 |
| Verbascoside | 10 | ± | 0.5 | 8.0 | ± | 0.9 |
| 3,4-DHPEA-EDA | 12.6 | ± | 0.7 | 9.4 | ± | 1.1 |
| *p*-HPEA-EDA | 5.6 | ± | 0.4 | 3.8 | ± | 0.5 |
| (+)-1-Acetoxypinoresinol | 0.2 | ± | 0.002 | 0.5 | ± | 0.1 |
| Sum of phenols | 30.4 | ± | 1.2 | 27.9 | ± | 1.5 |

Several phenolic compounds occurring in the DSOP, such as 3,4-DHPEA, 3,4-DHPEA-EDA, p-HPEA-EDA, are now considered to be the main bio-active phenols of extra-virgin olive involved in the prevention of cardiovascular disease and cancer in humans (Servili *et al.*, 2009; Covas, 2008; EFSA, 2011).

The chemical characteristics of the feedstuff are shown in Table 13.

Table 14 shows the tocopherols and tocotrienol content and the acidic composition of the lipid fraction of the feedstuffs. The experimental concentrate shows a higher amount of α-T ($P<0.05$), of γ e δ-T ($P<0.01$) and consequently of the total tocopherols (62.88 vs 55.87 μg/g, ($P<0.05$) compared to the control group, however a good level of α-T is present in the maize silage.

The DSOP supplementation improved acidic composition of the lipid fraction of the experimental concentrate with a significant decrease, with the exception of C18:0, of all the saturated fatty acids reported.

There is significant increase ($P<0.01$) both in C18:1ω9 and for C22:6ω3, on the contrary there is a decrease ($P<0.01$) both of C18:2ω6 and of C18:3ω3 and also C20:5ω3 was lower ($P<0.05$) compared to the value of the control group.

**Table 13.** Dry matter (g/kg as fed) and chemical composition (g/kg D.M.) of the feedstuffs.

| Items | DM | CP | CF | EE | NSC | Ash | NDF | ADF | ADL |
|---|---|---|---|---|---|---|---|---|---|
| Alfalfa hay | 879.6 | 179.9 | 339.2 | 27.1 | 207.2 | 85.3 | 500.5 | 385.1 | 87.6 |
| Maize silage | 334.2 | 83.9 | 216.7 | 24.8 | 327.9 | 46.9 | 516.5 | 259.0 | 44.9 |
| Control concentrate | 907.3 | 189.6 | 124.4 | 29.7 | 366.0 | 99.8 | 314.9 | 175.3 | 65.5 |
| Experimental concentrate | 912.5 | 198.3 | 124.7 | 47.5 | 348.2 | 84.8 | 321.2 | 168.6 | 78.2 |
| Control diet | 661.1 | 143.3 | 206.1 | 27.1 | 318.2 | 74.7 | 436.7 | 252.4 | 61.3 |
| Experimental diet | 663.1 | 146.6 | 206.2 | 33.9 | 311.4 | 69.0 | 439.1 | 249.9 | 66.1 |

**Table 14.** Tocopherols, tocotrienol (μg/g) and fatty acids (%) of the feedstuffs.

| Items | Alfalfa hay | Maize silage | Control concentrate | Experimental concentrate | Rmse |
|---|---|---|---|---|---|
| α-tocopherol | 2.36 | 43.32 | $45.64^{b}$ | $50.26^{a}$ | 1.33 |
| γ-tocopherol | 0.16 | 2.79 | $1.59^{B}$ | $2.72^{A}$ | 0.19 |
| δ-tocopherol | - | 1.10 | $1.43^{B}$ | $1.73^{A}$ | 0.14 |
| γ-tocotrienol | - | 7.94 | 7.21* | 8.18* | 0.47 |
| Toc. totals | 2.52 | 55.15 | $55.87^{b}$ | $62.88^{a}$ | 2.17 |
| Fatty acid profile | | | | | |
| C10:0 | 0.24 | 0.06 | $0.20^{A}$ | $0.07^{B}$ | 0.01 |
| C12:0 | 1.04 | 0.39 | $1.34^{A}$ | $0.56^{B}$ | 0.03 |
| C14:0 | 1.76 | 0.57 | $0.71^{A}$ | $0.49^{B}$ | 0.02 |
| C16:0 | 29.01 | 17.20 | $17.13^{a}$ | $15.01^{b}$ | 0.40 |
| C18:0 | 4.83 | 2.02 | 3.00 | 2.86 | 0.07 |
| C18:1ω9 | 5.51 | 20.67 | $27.53^{B}$ | $42.05^{A}$ | 0.89 |
| C18:2ω6 | 16.85 | 45.92 | $42.18^{A}$ | $32.76^{B}$ | 0.94 |
| C18:3ω3 | 22.30 | 7.03 | $4.02^{A}$ | $2.95^{B}$ | 0.09 |
| C20:4ω6 | 1.03 | 0.23 | 0.05 | 0.06 | 0.01 |
| C20:5ω3 | 1.40 | 0.27 | $0.11^{a}$ | $0.07^{b}$ | 0.01 |
| C22:6ω3 | 1.37 | 0.17 | $0.10^{B}$ | $0.24^{A}$ | 0.01 |

A, B: $P < 0.01$; a, b: $P < 0.05$; *: P = 6.28%

## 4.2. Effects of pomace supplemetation in buffalo

### *4.2.1. Production and quality of buffalo milk*

As can be seen from the comparison with the control group, shown in Table 15, the use of DSOP as supplemented feed does not affect the BCS (body condition score), ADG (average daily gain) the fat, protein, lactose and urea content of the milk. These results demonstrate, as found by Chiofalo *et al.* (2004) and Pauselli *et al.* (2007), that the experimental diet did not alter the level of the parameters considered and did not lead to any detrimental effect on the activity of the ruminal bacteria due to the long-chain unsaturated fatty acids, as has already been proved by Nefzaoui and Vanbelle (1986) and by Chiofalo *et al.* (2004). Moreover it is important to note that water buffalo has a better fibre utilization than the dairy cow (Batista *et al.*, 1982; Bartocci *et al.*, 2002). The use of the dried stoned olive pomace does not affect the milk production, this confirms what was reported by Hadjipanyiotou (1999) on dairy cows and Chiofalo *et al.* (2004) and Pauselli *et al.* (2007) on milk of ewes. No difference was found between the milk produced by the control group and the experimental group.

**Table 15.** Live weight, body condition score (BCS ), milk yield and quality.

| Items | Control group | DSOP group | Rmse |
|---|---|---|---|
| Ingestion of DM (kg/d) | 17.00 | 17.00 | - |
| Live weight (kg) | 625.93 | 662.50 | 90.16 |
| ADG (g/d) | 421.88 | 437.50 | 391.47 |
| BCS (1÷9) | 6.41 | 6.53 | 0.45 |
| Milk production (kg/d) | 9.69 | 10.08 | 2.53 |
| Fat (%) | 7.16 | 7.36 | 1.07 |
| Protein (%) | 4.51 | 4.45 | 0.33 |
| Lactose (%) | 4.88 | 4.90 | 0.19 |
| Urea (mg/100ml) | 32.58 | 33.13 | 3.22 |

The quantity

of crude protein administered per head in the DSOP animal group was 110.60 g/d (4.42% of the total protein). The pH, the milk coagulation parameters of the two groups are shown in Table 16. No significant differences were found for the pH values, for the rennet clotting time (20.34 and 22.35 min), for the curd firming time (2.30 and 2.66 min) and for the curd firmness (44.40 and 35.25 mm). These data are similar to those obtained by Bartocci *et al.* (2006) and Tripaldi *et al.* (2010).

**Table 16.** Acidity, thromboelastographic parameters, of the milk of the two groups.

| Items | Control group | DSOP group | Rmse |
|---|---|---|---|
| pH | 6.78 | 6.80 | 0.08 |
| r (min) | 20.34 | 22.35 | 4.15 |
| $k_{20}$ (min) | 2.30 | 2.66 | 1.28 |
| $a_{30}$ (min) | 44.40 | 35.25 | 14.22 |

### *4.2.2. Buffalo milk fatty acid composition*

Table 17, shows the fatty acid composition of the lipid fraction of buffalo milk, refer to the first year of research (2008).

A significant difference was found in the DSOP group in C18:0, C18:3ω6 and C18:1ω7 milk content and also a large amount of C18:1ω9, although not significant.

The use of non-rumen-protected, vegetable C:18 unsaturated fatty-acid sources resulted in an increase in rumen of the C18:0 synthesis due to bacterial biohydrogenation activity (Mosley *et al.*, 2002), which could be partially converted to C18:1ω9 in the mammary gland; in fact the increase of C18:1ω9, could in part be attributed to the activity of desaturation of the mammary gland both for C18:1ω7 and C18:3ω6 (Chilliard and Ferlay, 2004). In dairy cows, 40% of the C18:1ω9 is formed in the mammary gland by $\Delta^9$-desaturase activity (Lock and Gainsworth, 2003). The fatty acid composition of the milk of the experimental group has been proven to be similar to that observed by Selner and Schultz (1980) in cattle fed with rumen unprotected oleic acid

sources. The DSOP intake led to an increase in the MUFA represented principally by C18:1ω9 and an increase in the PUFA represented principally by C18:2ω6. The consequent reduction of SFA led to a reduction of the saturated/unsaturated ratio (2.75 and 3.04), of the atherogenic (3.60 and 3.84) and thrombogenic indices (3.75 and 3.87) of the milk produced by the animal of the experimental group. In the milk of the experimental group there was a slight reduction in content of the short-chain (C6:0-C10:0) and medium-chain fatty acids (C11:0-C16:3ω4) and a slight increase in the long-chain fatty acids (C17:0-C24:1).

**Table 17.** Fatty acid composition (%) of the milk fat of the two groups (1st year 2008).

| Fatty acid composition | Control group | DSOP group | Rmse |
|---|---|---|---|
| C6:0 | 5.20 | 4.72 | 1.00 |
| C8:0 | 3.17 | 2.74 | 0.79 |
| C10:0 | 4.08 | 3.72 | 1.01 |
| C12:0 | 4.31 | 3.95 | 0.56 |
| C14:0 | 14.43 | 14.30 | 1.02 |
| C16:0 | 34.10 | 33.53 | 2.91 |
| C18:0 | 7.34[b] | 8.60[a] | 0.93 |
| C18:1ω9 | 16.83 | 17.81 | 2.58 |
| C18:1ω7 | 0.96* | 1.27* | 0.33 |
| C18:2ω6 | 2.15 | 2.29 | 0.39 |
| C18:3ω3 | 0.43 | 0.46 | 0.08 |
| C18:3ω6 | 0.06[b] | 0.09[a] | 0.02 |
| C18:4ω3 | 0.45 | 0.52 | 0.11 |
| C20:4ω6 | 0.18 | 0.20 | 0.08 |
| C20:5ω3 | 0.07 | 0.06 | 0.03 |
| C22:6ω3 | 0.04 | 0.03 | 0.03 |
| SFA | 74.52 | 73.30 | 2.97 |
| MUFA | 21.08 | 22.15 | 2.69 |
| PUFA | 4.40 | 4.55 | 0.74 |
| SFA/UFA | 3.04 | 2.75 | 0.41 |
| ω3 | 1.47 | 1.45 | 0.42 |
| ω6 | 3.47 | 3.64 | 0.46 |
| ω6/ω3 | 2.40 | 2.51 | 0.26 |
| Atherogenic index | 3.84 | 3.60 | 0.57 |
| Thrombogenic index | 3.87 | 3.75 | 0.51 |
| SCFA | 12.35 | 10.93 | 2.53 |
| MCFA | 57.56 | 56.17 | 3.11 |
| LCFA | 30.09 | 32.90 | 3.69 |

a, b: $P < 0.05$; *: $P = 8.67\%$

The fatty acid composition of milk from buffalo cows supplemented with DSOP relative to the year 2009, is reported in Table 18. The milk of the DSOP group shows a significant higher proportion in C18:1 n9 content (P<0,001), with respect to the content of the control group, that consequently leads to an important increase in LCFA, UFA and MUFA milk content. The simultaneous reduction in the SAT amount in the milk from the pomace-feeding group resulted also in a reduction of the atherogenic and thrombogenic index; similar results were obtained on the fatty acid composition of the milk fat of sheep and on the indices by Chiofalo *et al.* (2004). These results are similar to those found by Molina Alcaide *et al.* (2008) in milk from ewes fed with olive cake and by Pauselli *et al.* (2007) in ewes fed with a concentrate of stoned olive pomace. Also a significant increase in of the long-chain fatty acids (LCFA) emerged; this could be determined by the protective effect from oxidation exerted by the vitamin E on the long-chain fatty acids (Chiofalo *et al.*, 2004).

The differences between the pomace group and the control group appear to be more pronounced in the second year of experimentation; this is due to a higher amount of pomace used in the diet, 1.2 kg DM/d in year 2009 compared to 1.05 kg DM/d in year 2008.

**Table 18.** Fatty acid composition (%) of the milk fat of the two groups (2nd year 2009).

| Fatty acid composition | Control group | DSOP group | Rmse |
|---|---|---|---|
| C6:0 | 3.55 | 3.28 | 1.37 |
| C8:0 | 2.52 | 2.19 | 0.58 |
| C10:0 | 5.15 | 4.26 | 0.73 |
| C12:0 | 0.10 | 0.09 | 0.02 |
| C14:0 | 17.37 | 17.39 | 2.94 |
| C14:1n6 | 1.23 | 1.34 | 0.24 |
| C16:0 | 41.49 | 39.77 | 1.81 |
| C16:1n7 | 1.84 | 3.00 | 0.42 |
| C18:0 | 5.90 | 6.36 | 0.59 |
| C18:1n9 | 13.49$^{B}$ | 16.67$^{A}$ | 1.54 |
| C18:2n6 | 1.50 | 1.52 | 0.14 |
| C18:3n3 | 0.35 | 0.36 | 0.05 |
| C20:4n6 | 0.08$^{a}$ | 0.07$^{b}$ | 0.01 |
| C20:5n3 | 0.06 | 0.08 | 0.03 |
| C22:6n3 | 0.0009 | 0.0004 | 0.00 |
| SCFA | 11.46 | 9.98 | 2.51 |
| MCFA | 61.21 | 59.87 | 2.34 |
| LCFA | 26.82$^{b}$ | 30.25$^{a}$ | 2.08 |
| SAT | 78.39$^{a}$ | 75.33$^{b}$ | 1.89 |
| INS | 21.61$^{b}$ | 24.67$^{a}$ | 1.90 |
| MUFA | 18.37$^{b}$ | 21.44$^{a}$ | 1.73 |
| PUFA | 3.23 | 3.22 | 0.30 |
| n6 | 3.09 | 3.20 | 0.30 |
| n3 | 0.74 | 0.81 | 0.10 |
| n6/n3 | 4.26 | 4.00 | 0.48 |
| Atherogenic Index | 5.06$^{a}$ | 4.37$^{b}$ | 0.74 |
| Thrombogenic index | 3.78 | 3.41 | 0.29 |

a-b: P< 0.05; A-B: P < 0.01

### *4.2.3. Antioxidants and oxidative status of buffalo milk*

The occurrence of tocopherols, retinol and TBARs values of the milk of DSOP-group and control group was evaluated for the first year (2008) and is reported in Table 19. Among tocopherols only α-T, γ-T, δ-T and γ-T3 were present in appreciable quantities in the milk of both groups. Alpha-T was present in a high concentration (79.42%), followed by y-T (15.93%) and δ-T (1.98%) in the milk of the control group. The DSOP dietary supplementation resulted in a significant increase in all the aforementioned tocopherols, except for δ-T, in the milk of the treated group, without altering their distribution. As a consequence, the total amount of vitamin E in the milk reached 8.6 and 10.45 μg/g fat (P<0.01) for the control and the treated group respectively.

**Table 19.** Tocopherols, tocotrienol, retinol (μg/g fat ) and oxidative stability (μg MDA/g fat) of the lipid fraction of the milk of the two groups (1st year 2008).

| Items | Control group | Experimental group | Rmse |
|---|---|---|---|
| α-tocopherol | $6.83^{b}$ | $8.19^{a}$ | 1.00 |
| γ-tocopherol | $1.37^{B}$ | $1.70^{A}$ | 0.21 |
| δ-tocopherol | 0.17 | 0.19 | 0.03 |
| γ-tocotrienol | $0.23^{B}$ | $0.37^{A}$ | 0.05 |
| Total (Vitamin E) | $8.60^{B}$ | $10.45^{A}$ | 1.12 |
| Retinol | $2.54^{B}$ | $3.17^{A}$ | 0.28 |
| TBARs | $15.05^{A}$ | $12.09^{B}$ | 1.79 |

A, B: P < 0.01; a, b: P < 0.05

These results may be ascribed, in part, to the occurrence of a higher amount of vitamin E in the experimental concentrate (62.88 vs. 55.87 μg/g, P<0.05). These findings are consistent with those reported by Weiss *et al.* (2003), who showed evidence of the relationship between dietary α-T content and its concentration in milk independently from the tested fat sources. The high amount of phenols characterized by strong antioxidant activity, such as 3, 4-DHPEA, 3, 4-DHPEA-EDA, found in the DSOP,

might also be implicated in the increase in the amount of α-T in the milk. In fact, the DSOP phenolic antioxidants can protect tocopherols from oxidation thereby increasing the total amount absorbed through digestion and thus their concentration in the milk.

In this regard, one of the main results of this research work was the detection and partial characterization of hydrophilic phenols in the milk. Figure 11 shows the LC-MS/MS chromatogram and the mass spectra of the occurrence of 3,4-DHPEA in the milk of water buffaloes fed with DSOP. The other phenols, such as 3,4-DHPEA-EDA, *p*-HPEA-EDA and lignans, which are the most concentrated compounds of DSOP, were not found in the milk of DSOP-fed animals.

These results suggest that 3,4-DHPEA-EDA, very abundant in DSOP, could be probably hydrolyzed during digestion, releasing the 3,4-DHPEA that is transferred and accumulated in the milk. In terms of the absolute concentration of 3,4-DHPEA found in the milk of water buffaloes treated with DSOP, the average value was 36.0 μg/l, but the disparity due to subject variability was very large, ranging between a minimum of 8.9 μg/l and a maximum of 56.2 μg/l.

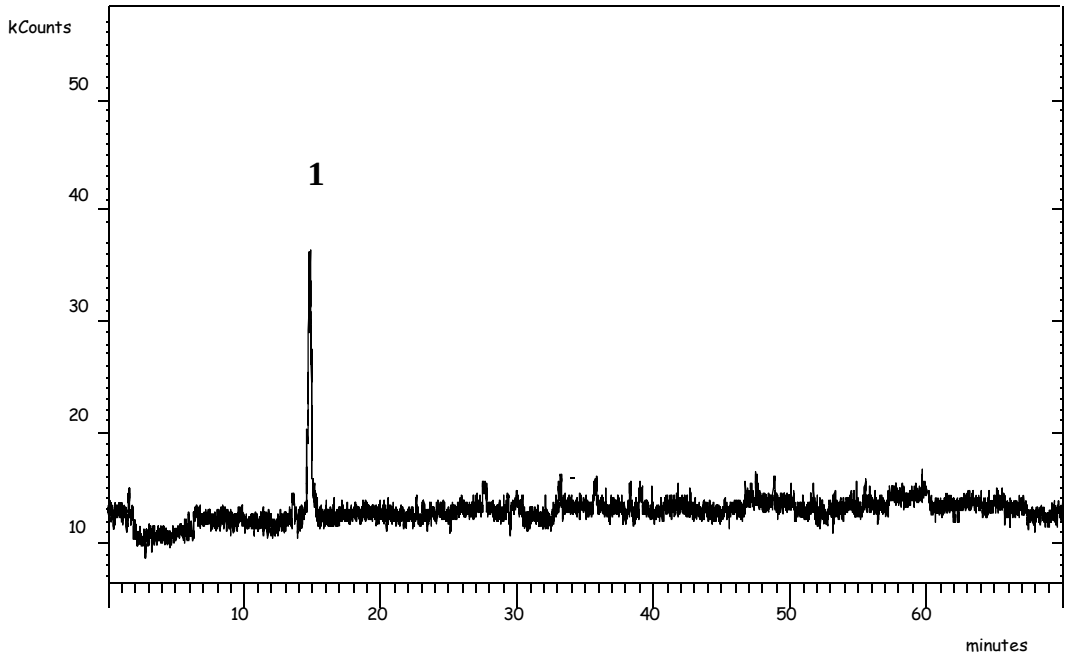


**Figure 11.** HPLC-ESI-MS/MS chromatogram obtained from a milk sample produced by a DSOP-fed buffalo cow. [1] 3,4-DHPEA (hydroxytyrosol).

The milk from DSOP-fed animals also revealed a higher retinol level (3.17 vs. 2.54 μg/g fat, $P<0.01$) in comparison to the milk from the control group (Table 19). The higher amount of tocopherols and retinol might have contributed to reducing the level of TBARs (12.09 vs. 15.05 μg MDA/g fat, $P<0.01$) in the milk of the treated group and consequently to a better oxidative status of this milk with respect to the milk derived from the untreated animals. These results are similar to those reported by Pauselli *et al.* (2007) in the milk of ewes fed with a concentrate enriched with stoned olive pomace. The presence of a powerful natural antioxidant such as 3,4-DHPEA, in the milk of DSOP-fed animals, an antioxidant that is normally present in virgin olive oil and its by-products, might also contribute to the better oxidative status of milk fat from the DSOP-treated group, acting either directly or indirectly (synergism with vitamin E) against free radicals (Owen *et al.*, 2000; Mirò Casas *et al.*, 2001; Servili *et al.*, 2004). Among DSOP phenols, ortho-diphenols, such as 3,4-DHPEA and 3,4-DHPEA-EDA, are known to

possess the highest antioxidant activity and are also effective radical scavengers (Baldioli *et al.*, 1996). In particular, 3,4-DHPEA scavenges aqueous peroxyl radicals near the membrane surface, while oleuropein scavenges chain-propagating lipid peroxyl radicals within membranes (Saija *et al.*, 1998). Moreover, phenolic compounds have been shown to play a role in vitamin E recycling and might have also accounted for the higher milk tocopherol levels in the DSOP-treated group. A similar hypothesis has been formulated for flavonoids on α-T recycling (Zhu *et al.*, 1999; Pedrielli and Skibsted 2002; Pazos *et al.*, 2002). However, further studies are required to better clarify the role of each DSOP compound in the total milk antioxidant capacity.

### *4.2.4. Fatty acid composition of buffalo mozzarella cheese*

Milk (year 2009), processing to obtain mozzarella cheese, led to an increase in the difference in the fatty acid composition of the lipid fraction, due to dietary treatment (Table 20). The percentages of C6:0, C8:0 and C10:0 were higher in the mozzarella from the control group milk, while in the mozzarella from the milk of DSPO group, the presence of C14:0 was lower ($P<0.05$) and the C18:1n9 content was higher ($P<0.01$).
Mozzarella of the DSPO group was also characterized by a higher MUFA content ($P<0.01$), a reduced n6/n3 ratio ($P<0.05$) and lower atherogenic and thrombogenic indexes.

**Table 20.** Effects of dietary treatment on mozzarella cheese lipid fraction (% FAME) (2nd year 2009).

| Fatty acid composition | C | DSOP | Rmse |
|---|---|---|---|
| C6:0 | 3.89$^{A}$ | 2.44$^{B}$ | 0.38 |
| C8:0 | 3.01$^{A}$ | 2.24$^{B}$ | 0.21 |
| C10:0 | 4.12$^{a}$ | 3.60$^{b}$ | 0.23 |
| C12:0 | 0.09 | 0.08 | 0.02 |
| C14:0 | 18.66$^{a}$ | 16.92$^{b}$ | 1.00 |
| C16:0 | 39.00 | 36.07 | 2.00 |
| C18:0 | 6.82 | 8.05 | 0.92 |
| C18:1n9 | 14.06$^{B}$ | 20.90$^{A}$ | 1.57 |
| C18:2n6 | 1.52 | 1.69 | 0.32 |
| C18:3n3 | 0.52 | 0.47 | 0.53 |
| C20:4n6 | 0.07 | 0.08 | 0.01 |
| C20:5n3 | 0.06 | 0.08 | 0.03 |
| C20:6n3 | 0.0009 | 0.0005 | 0.00 |
| SCFA | 11.00$^{A}$ | 8.39$^{B}$ | 0.59 |
| MCFA | 60.97$^{a}$ | 55.29$^{b}$ | 2.31 |
| LCFA | 27.92$^{B}$ | 36.31$^{A}$ | 2.31 |
| SAT | 77.69$^{A}$ | 71.63$^{B}$ | 1.79 |
| INS | 22.31$^{B}$ | 28.37$^{A}$ | 1.79 |
| MUFA | 18.77$^{B}$ | 24.65$^{A}$ | 1.61 |
| PUFA | 3.53 | 3.71 | 0.75 |
| n6 | 3.33 | 2.76 | 0.30 |
| n3 | 0.74 | 0.81 | 0.10 |
| n6/n3 | 3.76$^{a}$ | 2.73$^{b}$ | 0.54 |
| Atherogenic Index | 4.95$^{A}$ | 3.68$^{B}$ | 0.44 |
| Thrombogenic Index | 3.68$^{a}$ | 3.14$^{b}$ | 0.30 |

a,b: P<0,0 5; A, B: P<0,01

### *4.2.5. Oxidative status of buffalo mozzarella cheese*

No statistical differences due to dietary treatment were found in the concentration of tocopherols, whose contents were similar to those found by Balestrieri *et al.* (2002) in commercial mozzarella cheeses produced from water buffalo milk (Table 21).

**Table 21**. Effects of diet and storage on mozzarella tocopherol content and oxidative status.

| Items | Dietary treatment | | Rmse |
|---|---|---|---|
| | C | DSOP | |
| α-tocopherol (µg /100 g) | 159.39 | 148.25 | 23.31 |
| γ-tocopherol (µg /100 g) | 1.88 | 1.65 | 0.36 |
| α-tocopherol (µg /g fat) | 6.13 | 5.68 | 0.88 |
| γ-tocopherol (µg /g fat) | 0.07 | 0.06 | 0.01 |
| Total tocopherols (µg /g fat) | 6.20 | 5.75 | 0.88 |
| TBARs (µg MDA/g fat) | 2.690 | 2.569 | 0.6009 |
| Governing liquid TBARs (µg MDA/ml) | 0.375 | 0.259 | 0.21 |

The storage period from 0 to 4 days resulted in a significant reduction in the α-tocopherol content, which was slight higher in mozzarella from the control group (Figure 12).

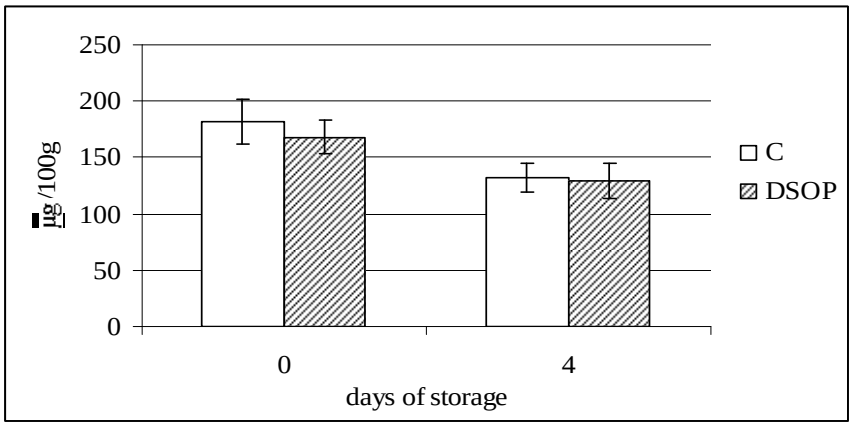


**Figure 12.** Effect of storage on α-tocopherol content in mozzarella cheese obtained with the milk from the two groups of buffaloes with different dietary treatments (C and DSOP).

In dairy products, tocopherols are scavengers of free radicals that catalyze the beginning and propagation of chain reactions in lipid peroxidation (Burton and Ingold, 1986; Madhavi *et al.* 1996). The reduction in the tocopherol content from milk to mozzarella cheese is due to the strong oxidative conditions that occur during mozzarella manufacturing, such as high temperature and oxygen exposure (Balestrieri *et al.*, 2002), taking in account also that buffalo milk has a lower content in ß-carotene than cow milk (Addeo *et al.*, 1995). Thiobarbituric acid-reactive substances in mozzarella cheese put in evidence the consumption of antioxidants such as tocopherols during storage (Figure 13).

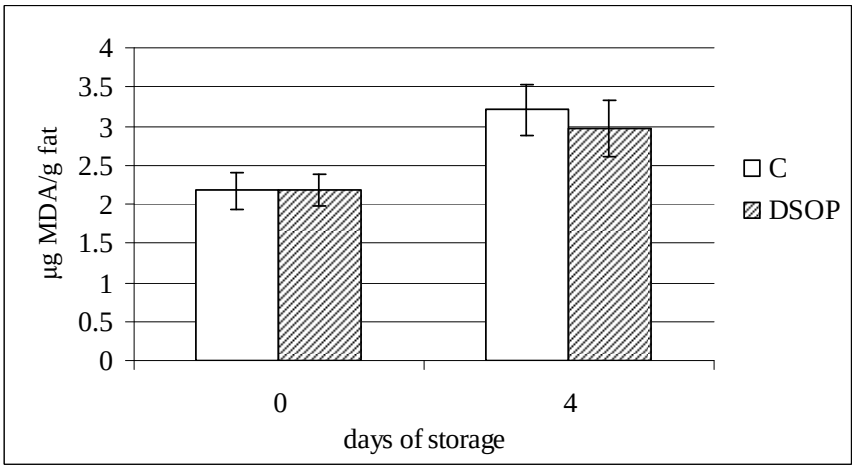


**Figure 13.** Effect of storage on the oxidative status (TBARs) of mozzarella cheese obtained with the milk from the two groups of buffaloes with different dietary treatments (C and DSOP).

The slightly lower level of MDA content in the governing liquid from mozzarella of the DSOP group (Table 21) could be due to the tranfer of hydrophilic polyphenols in such liquid, and their consequent antioxidant action (Figure 14).

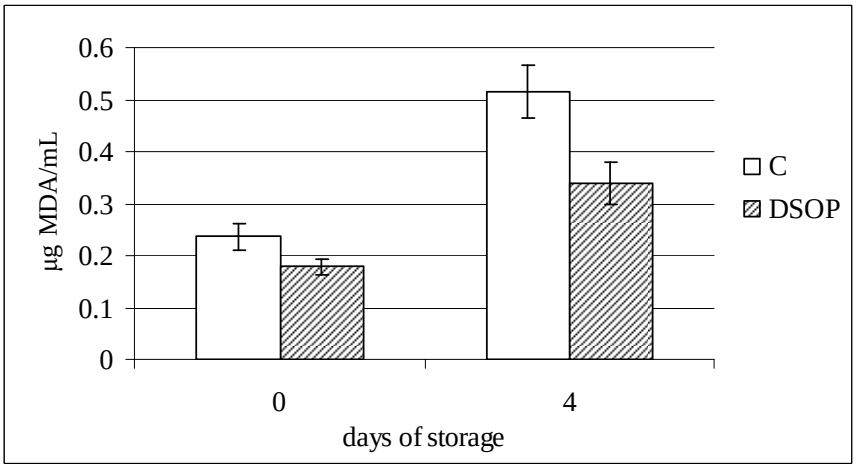


**Figure 14.** Effect of storage on the oxidative status (TBARs) of the governing liquid of mozzarella cheese obtained with the milk from the two groups of buffaloes with different dietary treatments (C and DSOP).

## Research 2

### 4.3. Phenolic characterization of fresh paté

From a comparison of the phenol content of the fresh olive paté with that of a fresh pomace (Table 22), the patè shows a considerably high content in phenolic compounds (76-96g/ kg), such as secoiridoids and verbascoside despite the late harvest of the olives (December).

**Table 22.** Phenolic composition (g/kg D.M.) of fresh paté and fresh pomace.

| Items | Fresh Paté | | | Fresh Pomace |
|---|---|---|---|---|
| | I batch | II batch | III batch | |
| 3,4-DHPEA | 24.3 ± 0.2 | 20.9 ± 0.2 | 11.4 ± 1.0 | 4.1 ± 0.1 |
| *p*-HPEA | 11.3 ± 0.1 | 12.7 ± 0.1 | 6.4 ± 0.5 | 4.9 ± 0.2 |
| 3,4-DHPEA-EDA | 31.5 ± 1.7 | 24.9 ± 0.6 | 28.1 ± 0.8 | 18.5 ± 2.4 |
| Verbascoside | 29.1 ± 1.3 | 18.0 ± 0.3 | 29.9 ± 1.3 | 23.8 ± 2.2 |
| Sum of phenols | 96.1 ± 2.1 | 76.5 ± 0.7 | 75.8 ± 5.7 | 51.3 ± 3.3 |

### 4.4. Preservation of fresh paté over time

The pre-drying storage conditions over time were evaluated on the third batch.

The silos were experimentally tested for the preservation of fresh paté. The evaluation of storage stability of the by-product was carried out on paté samples after 30, 60 and 120 days of storage. It emerged from the results that the phenolic fraction shows a good stability until day 30 of conservation, with a loss of just 10% compared to the initial sample; then this percentage tends to increase to 48% after 60 days and to 76% after 120 days of conservation. Is important to consider that this trial was tested on the third batch, which was carried out in late December and it is characterized not only by higher initial moisture content (Table 23) but also by a lower concentration of phenolic

compounds (Table 24) comparing to the others two (Table 22). The results obtained from the analysis of silage paté show that this type of storage could be an interesting prospective.

**Table 23.** Moisture (%) of fresh pate, and paté blended with vegetable flours, stored in silos.

| Items | Initial point (III batch) | 30 days | 60 days | 120 days |
|---|---|---|---|---|
| Paté | 78.8 ± 3.6 | 77.5 ± 4.1 | 71.5 ± 3.8 | 78.2 ± 4.1 |
| Paté + dehydrated alfalfa 20 % | 63.3 ± 3.4 | 62.3 ± 3.1 | 56.3 ± 2.8 | 63.8 ± 3.2 |
| Paté + soybean meal 20 % | 64.1 ± 3.9 | 63.4 ± 3.5 | 58.5 ± 3.3 | 65.2 ± 3.6 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 62.8 ± 4.3 | 62.6 ± 3.7 | 56.8 ± 3.3 | 63.5 ± 3.7 |

**Table 24.** Phenolic composition (g/kg D.M.) of fresh paté during storage in silos.

| Phenols | Initial point (III batch) | 30 days | 60 days | 120 days |
|---|---|---|---|---|
| 3,4-DHPEA | 11.4 ± 1.0 | 11.7 ± 0.5 | 7.3 ± 0.6 | 3.2 ± 0.2 |
| *p*-HPEA | 6.4 ± 0.5 | 6.1 ± 0.6 | 6.3 ± 0.5 | 4.3 ± 0.5 |
| Verbascoside | 28.1 ± 0.8 | 24.0 ± 0.7 | 12.7 ± 0.4 | 6.4 ± 0.1 |
| 3,4-DHPEA-EDA | 29.9 ± 1.3 | 25.8 ± 0.9 | 15.9 ± 0.2 | 8.1 ± 0.2 |
| Total phenols | 75.8 ± 5.7 | 67.6 ± 5.2 | 42.2 ± 2.8 | 22.0 ± 1.7 |

The results on the characterization of dried paté after ensiling and after mixing with vegetable flours have shown that the latter practice has had a stabilizing effect on polyphenols in the drying phase from the thirtieth day. In fact from this moment on the percentage of "recovery" of such substances on mixed paté was higher compared to non-enriched paté (Table 25) despite the fact that the polyphenol content of the mixture pate-vegetable flours was lower than that of the paté alone, due to a dilution effect.

This result could be due to an absorbent effect on the free water, exerted by vegetable flour: during the fresh ensiling period of the paté, the vegetation water tended to separate from the solid phase of the paté and was then absorbed by the vegetable meals

at the time of mixing, making the product more stable during drying, and consequently more resistant to high temperatures.

The data relating to the fat fraction of the fresh paté stored in silos (Table 26), show also a remarkable stability over time, both with respect to hydrolysis and oxidation; in fact the differences in free acidity and peroxide value between the samples taken at different storage times and then dried, are low.

**Tabella 25.** Phenolic composition (g/kg D.M.) and its recovery (%) in dried paté (after storage in silos) alone or mixed with vegetable flours.

| | 3,4-DHPEA | *p*-HPEA | Verbascoside | 3,4-DHPEA-EDA | Total phenols | Recovery |
|---|---|---|---|---|---|---|
| | | | Paté (III batch) | | | |
| Initial point | 5.8 ± 0.5 | 6.0 ± 0.5 | 15.8 ± 0.8 | 14.8 ± 0.7 | 42.4 ± 3.2 | 55.9 |
| 30 days | 5.9 ± 0.3 | 6.0 ± 0.6 | 14.7 ± 0.6 | 11.5 ± 0.5 | 38.0 ± 2.9 | 56.3 |
| 60 days | 4.2 ± 0.3 | 5.5 ± 0.4 | 7.5 ± 0.4 | 5.5 ± 0.1 | 22.7 ± 1.6 | 53.8 |
| 120 days | 1.1 ± 0.1 | 3.3 ± 0.5 | 3.3 ± 0.1 | 2.3 ± 0.1 | 10.0 ± 1.0 | 45.4 |
| | | | Paté + dehydrated alfalfa 20 % | | | |
| Initial point | 3.7 ± 0.4 | 2.7 ± 0.1 | 7.7 ± 0.4 | 10.6 ± 0.5 | 24.8 ± 0.7 | 58.8 |
| 30 days | 3.8 ± 0.3 | 2.6 ± 0.2 | 7.2 ± 0.5 | 9.3 ± 0.6 | 22.9 ± 0.9 | 57.6 |
| 60 days | 3.3 ± 0.3 | 3.4 ± 0.3 | 5.2 ± 0.4 | 5.5 ± 0.1 | 17.4 ± 1.0 | 65.1 |
| 120 days | 1.4 ± 0.2 | 1.2 ± 0.1 | 2.8 ± 0.2 | 1.6 ± 0.3 | 7.1 ± 0.6 | 51.0 |
| | | | Paté + soybean meal 20 % | | | |
| Initial point | 3.5 ± 0.4 | 2.9 ± 0.1 | 6.8 ± 0.3 | 9.8 ± 0.5 | 23.0 ± 1.2 | 54.3 |
| 30 days | 3.5 ± 0.2 | 2.5 ± 0.1 | 6.6 ± 0.4 | 9.2 ± 0.4 | 21.8 ± 1.1 | 61.0 |
| 60 days | 3.4 ± 0.2 | 2.5 ± 0.1 | 5.4 ± 0.4 | 6.0 ± 0.4 | 17.4 ± 1.6 | 63.1 |
| 120 days | 1.7 ± 0.1 | 1.5 ± 0.1 | 2.6 ± 0.2 | 2.6 ± 0.1 | 8.5 ± 0.8 | 58.2 |
| | | | Paté + dehydrated alfalfa 10 % + soybean meal 10% | | | |
| Initial point | 3.7 ± 0.4 | 3.4 ± 0.2 | 7.1 ± 0.4 | 7.8 ± 0.4 | 22.0 ± 1.6 | 55.2 |
| 30 days | 3.7 ± 0.3 | 3.4 ± 0.2 | 6.9 ± 0.4 | 7.6 ± 0.4 | 21.6 ± 1.7 | 57.5 |
| 60 days | 3.6 ± 0.3 | 3.2 ± 0.2 | 5.5 ± 0.4 | 5.1 ± 0.4 | 17.4 ± 1.7 | 61.7 |
| 120 days | 1.3 ± 0.1 | 1.9 ± 0.1 | 2.8 ± 0.1 | 2.4 ± 0.1 | 8.3 ± 0.8 | 57.3 |

*The results represent the average of two independent experiments ± standard deviation

**Tabella 26.** Values of the stability parameters of the residual oil of fresh paté (after storage in silos), alone or mixed with vegetable flours*.

| Items | Acidity (oleic acid g/100g oil) | | | Peroxide (meq $O_2$/Kg oil) | | |
|---|---|---|---|---|---|---|
| | | | Initial point | | | |
| Paté (III batch) | 1.5 | ± | 0.2 | 3.7 | ± | 0.3 |
| Paté + dehydrated alfalfa 20 % | 2.2 | ± | 0.2 | 5.5 | ± | 0.4 |
| Paté + soybean meal 20 % | 1.8 | ± | 0.1 | 5.7 | ± | 0.5 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 2.3 | ± | 0.1 | 5.9 | ± | 0.4 |
| | | | 30 days | | | |
| Paté (III batch) | 1.4 | ± | 0.1 | 3.0 | ± | 0.2 |
| Paté + dehydrated alfalfa 20 % | 2.2 | ± | 0.1 | 5.1 | ± | 0.3 |
| Paté + soybean meal 20 % | 1.8 | ± | 0.1 | 5.8 | ± | 0.3 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 2.3 | ± | 0.1 | 6.1 | ± | 0.4 |
| | | | | | | |
| | | | 60 days | | | |
| Paté (III batch) | 1.8 | ± | 0.1 | 3.3 | ± | 0.2 |
| Paté + dehydrated alfalfa 20 % | 2.5 | ± | 0.1 | 5.5 | ± | 0.3 |
| Paté + soybean meal 20 % | 2.2 | ± | 0.1 | 5.6 | ± | 0.3 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 2.4 | ± | 0.1 | 6.0 | ± | 0.4 |
| | | | 120 days | | | |
| Paté (III batch) | 2.2 | ± | 0.1 | 4.0 | ± | 0.2 |
| Paté + dehydrated alfalfa 20 % | 2.7 | ± | 0.1 | 5.8 | ± | 0.3 |
| Paté + soybean meal 20 % | 2.5 | ± | 0.1 | 5.9 | ± | 0.3 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 2.6 | ± | 0.2 | 6.2 | ± | 0.4 |

*The results represent the average of two independent experiments ± standard deviation

## 4.5. Paté pre-drying treatment

The high level of humidity of the fresh paté made direct drying difficult, using the normal process applied in zoo-technical flour industry, which envisages a maximum level of humidity of the row material at approximately 50%. Therefore the humidity of the paté was reduced by pre-drying mixing with soya flour and alfalfa hay, usually used in zoo-technical feed. It emerged from the tests that the addition of 20% vegetable flours, reduces significantly the level of initial humidity of the fresh paté; in fact, in this way, the humidity went down, as is shown in Table 27, to a value near of 50%, and consequently the drying-time was significantly reduced.

**Table 27.** Moisture (%) and drying times, of fresh pate of the first two samples.

| Items | Moisture % | | Drying time | |
|---|---|---|---|---|
| | I batch | II batch | I batch | II batch |
| Paté | 68.8 | 68.7 | 1 h 46' | 1 h 40' |
| Paté + dehydrated alfalfa 10 % | 58.4 | - | 1 h 15' | - |
| Paté + soybean meal 10 % | 57.7 | - | 1 h 10' | - |
| Paté + dehydrated alfalfa 5 % + soybean meal 5% | 56.8 | - | 1 h 10' | - |
| Paté + dehydrated alfalfa 20 % | 53.6 | 53.1 | 35' | 32' |
| Paté + soybean meal 20 % | 54.64 | 54.7 | 39' | 34' |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 53.84 | 53.7 | 35' | 32' |

## 4.6. Characterization of dried paté and feedstuff

The drying process was carried out following the same method above mentioned for the pomace. This process brought the humidity of paté content down to values ranged between 5 and 8% (Table 28). The loss of polyphenols ranged between 30 to 40%, for the I and II batch, and around 50% for the III, both for the dried paté alone and the ones containing vegetable flours (Table 29). The greater loss in the third sampling is due to the fact that the humidity was 79% while for the other two batches was 68%. These results are in line with

what had already been observed for stoned virgin pomace (Servili *et al.*, 2007b). The reduction in absolute value of the phenolic component is due essentially to the increase in the content of vegetable flours, this led a dilution effect, however the antioxidant content remains significant (between 20 and 40 g/kg), compatible with a concentrate to be used as a feed integrator in the zoo-technical industry.

**Table 28.** Moisture content (%) of dry paté of the first two batches.

| Items | I batch | II batch |
|---|---|---|
| Paté | 7.9 | 7.2 |
| Paté + dehydrated alfalfa 10 % | 6.0 | - |
| Paté + soybean meal 10 % | 7.5 | - |
| Paté + dehydrated alfalfa 5 % + soybean meal 5% | 9.5 | - |
| Paté + dehydrated alfalfa 20 % | 5.9 | 5.5 |
| Paté + soybean meal 20 % | 6.8 | 6.3 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 7.0 | 5.8 |

**Table 29.** Phenolic composition* (g/kg DM) of dried paté as it is and mixed with vegetable flours (I batch).

| Items | 3,4-DHPEA | *p*-HPEA | Verbascoside | 3,4-DHPEA-EDA | Total Phenols |
|---|---|---|---|---|---|
| Paté | 13.9 ± 0.3 | 10.3 ± 1.0 | 18.7 ± 1.0 | 24.8 ± 0.4 | 67.6 ± 1.5 |
| Paté + dehydrated alfalfa 10 % | 7.3 ± 0.2 | 5.7 ± 0.8 | 12.9 ± 0.8 | 12.0 ± 0.3 | 38.0 ± 1.2 |
| Paté + soybean meal 10 % | 8.1 ± 0.2 | 6.4 ± 0.1 | 13.8 ± 0.1 | 12.8 ± 0.1 | 41.1 ± 0.3 |
| Paté + dehydrated alfalfa 5 % + soybean meal 5% | 8.8 ± 0.1 | 7.9 ± 0.1 | 12.6 ± 0.1 | 13.0 ± 0.3 | 42.4 ± 0.3 |
| Paté + dehydrated alfalfa 20 % | 5.2 ± 0.1 | 3.9 ± 0.4 | 9.6 ± 0.4 | 10.4 ± 0.1 | 29.1 ± 0.6 |
| Paté + soybean meal 20 % | 6.2 ± 0.3 | 3.1 ± 0.4 | 8.7 ± 0.4 | 10.3 ± 0.6 | 28.3 ± 0.8 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 5.5 ± 0.3 | 4.0 ± 0.0 | 9.6 ± 0.0 | 10.9 ± 0.2 | 30.0 ± 0.4 |

*The results represent the average of three independent experiments ± standard deviation.

Concerning the residual oil of the paté, the drying process did not cause negative effects on the fat stability; in fact, both free acidity and peroxide number of all dried products were low, corresponding to 1.9 and 2.2 g/100 of oleic acid, and 3.9 and 6.3 meq.$O_2$/kg oil, respectively (Table 30).

**Table 30.** Average values* of the first two samples, referred to the stability parameters of the residual oil dried pate as it is and mixed with vegetable flours.

| Items | Acidity (g of oleic acid/100g of oil) | Peroxide value (meq of $O_2$/kg of oil) |
|---|---|---|
| Paté | 1.9 ± 0.1 | 3.9 ± 0.1 |
| Paté + dehydrated alfalfa 20 % | 2.2 ± 0.1 | 5.7 ± 0.1 |
| Paté + soybean meal 20 % | 2.0 ± 0.0 | 6.0 ± 0.1 |
| Paté + dehydrated alfalfa 10 % + soybean meal 10% | 2.2 ± 0.2 | 6.3 ± 0.2 |

*The results represent the average of two independent experiments ± standard deviation

The analysis of dried crude fiber of paté as it is and mixed with vegetables flours shows a good content in NDF and ADF, levels that as expected increases with the addition of vegetable flours, especially with alfalfa (Table 31). Similar values characterized the experimental concentrates (Table 32).

Further interesting elements are the low lignin amount in the paté (Table 31), comparable to the pomace de-stoned in pre-extraction and the crude protein content which values is very similar to those found in the alfalfa flour. The protein content in paté is also low. These characteristic are transferred into concentrates, which show similar values (Table 32).

**Table 31.** Content of crude fiber in the dried paté as it is and mixed with vegetables flours % (DM).

| Items | Paté | Paté + dehydrated alfalfa 20 % | Paté + soybean meal 20 % | Paté + dehydrated alfalfa 10 % + soybean meal 10% |
|---|---|---|---|---|
| Dry Matter (%) | 93.8 | 94.1 | 93.6 | 94.4 |
| Ether Extract | 29.0 | 18.5 | 18.4 | 19.5 |
| Ash | 6.0 | 8.0 | 6.7 | 7.5 |
| Neutral Detergent Fiber | 20.2 | 36.2 | 28.0 | 29.9 |
| Acid Detergent Fiber | 14.8 | 25.2 | 12.4 | 12.4 |
| Lignin | 8.8 | 7.9 | 5.1 | 5.1 |
| Crude Protein | 8.5 | 8.6 | 22.3 | 16.0 |

**Table 32.** Chemical composition (%) of the pasture and the concentrates used.

| Items | Rolled Barley | Pasture | Experimental Concentrates | | |
|---|---|---|---|---|---|
| | | | L | P | LOP |
| Dry Matter | 88.66 | 23.03 | 87.75 | 89.03 | 88.35 |
| Crude Protein | 9.40 | 15.61 | 14.96 | 15.08 | 15.35 |
| Fat | 2.90 | 2.62 | 11.80 | 10.87 | 11.37 |
| NDF | 21.83 | 48.97 | 24.12 | 23.73 | 24.37 |
| ADF | 5.77 | 32.60 | 12.26 | 15.25 | 13.80 |
| ADL | 1.10 | 4.86 | 2.11 | 4.69 | 3.64 |
| Ash | 2.59 | 4.23 | 5.25 | 7.36 | 6.38 |

The addition of flour did not modify the fatty acid composition of the paté (Table 33). An interesting point is that both the paté as it is and the other combination appear very rich in C18:1n-9, consequently a similar amount characterized also the experimental concentrates (Table 34) while, as expected, the group L was characterized by a high content of C18:2n6 and C18:3n3.

**Table 33.** Fatty acid composition (%) of dried paté as it is and mixed with vegetable flours.

| Fatty acid composition | Paté | Paté + dehydrated alfalfa 20 % | Paté + soybean meal 20 % | Paté + dehydrated alfalfa 10 % + soybean meal 10% |
|---|---|---|---|---|
| C16:0 | 12.5 | 12.7 | 12.8 | 12.3 |
| C16:1 | 1.2 | 1.2 | 1.2 | 1.2 |
| C17:0 | 0.1 | 0.1 | 0.1 | 0.1 |
| C17:1 | 0.1 | 0.1 | 0.1 | 0.1 |
| C18:0 | 2.4 | 2.4 | 2.4 | 2.4 |
| C18:1n9 | 72.7 | 72.5 | 71.6 | 72.6 |
| C18:2n6 | 9.7 | 9.5 | 10.4 | 9.8 |
| C18:n3 | 0.6 | 0.7 | 0.7 | 0.7 |
| C20:0 | 0.4 | 0.4 | 0.3 | 0.4 |
| C20:1n9 | 0.3 | 0.2 | 0.2 | 0.3 |
| C22:0 | 0.2 | 0.1 | 0.1 | 0.1 |

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**Table 34.** Fatty acid composition (%) of the hay and the concentrates used.

| Fatty acid composition | Rolled Barley | Pasture | Experimental Concentrates | | |
|---|---|---|---|---|---|
| | | | L | OP | LOP |
| C12:0 | 0.35 | 0.12 | 0.06 | 0.13 | 0.11 |
| C14:0 | 1.49 | 1.90 | 0.11 | 0.20 | 0.16 |
| C16:0 | 18.17 | 16.32 | 10.08 | 20.07 | 14.53 |
| C16:1 | 2.87 | 0.36 | 0.12 | 1.01 | 0.45 |
| C18:0 | 4.58 | 4.20 | 2.82 | 0.28 | 1.72 |
| C18:1n9 | 21.18 | 11.82 | 16.14 | 53.77 | 32.89 |
| C18:2n6 | 44.97 | 22.18 | 43.14 | 18.04 | 32.56 |
| C18:3n3 | 6.06 | 37.50 | 26.65 | 3.75 | 16.48 |
| SFA | 24.92 | 24.38 | 13.61 | 22.30 | 17.21 |
| MUFA | 24.54 | 12.91 | 16.42 | 55.12 | 33.41 |
| PUFA | 50.64 | 62.71 | 69.97 | 22.57 | 49.37 |

The addition of paté to the feed provided to it an high amount of phenolic compounds (Table 35) equal to 10.3 g/kg (on D.M.). In the LOP group (paté mixed with linseed), which has half paté content the concentration of phenolic compounds is halved (5.1 g/kg of D.M). No trace of phenolic compounds was found in L group.

**Table 35.** Phenolic composition (g/kg DM) and fat-soluble vitamins (µg/g D.M.) of experimental concentrates used.

| Items | Rolled Barley | Pasture | Experimental Concentrates | | |
|---|---|---|---|---|---|
| | | | L | OP | LOP |
| Phenolic Compounds | | | | | |
| 3,4-DHPEA | nd | nd | nd | 4.72 | 2.81 |
| p-HPEA | nd | nd | nd | 1.12 | 0.57 |
| Verbascoside | nd | nd | nd | 2.58 | 1.35 |
| 3,4-DHPEA-EDA | nd | nd | nd | 3.26 | 1.47 |
| Fat-soluble vitamins | | | | | |
| γ-Tocotrienol | 7.21 | Nd | 5.16 | 3.72 | 4.43 |
| α-Tocotrienol | 17.12 | Nd | 7.14 | 5.54 | 5.75 |
| δ-Tocotrienol | 1.97 | 0.61 | 0.51 | 0.64 | 0.38 |
| γ-Tocopherol | 3.24 | 1.65 | 7.97 | 2.68 | 5.45 |
| α-Tocopherol | 22.59 | 76.13 | 113.64 | 154.91 | 134.65 |
| ΣTocotrienol | 24.35 | Nd | 12.30 | 9.26 | 10.18 |
| ΣTocopherol | 27.82 | 78.39 | 122.13 | 158.23 | 140.48 |
| Retinol | | | 251.96 | 246.17 | 241.13 |
| lutein + zeaxanthin | 0.39 | 104.95 | nd | 3.32 | 1.98 |
| β-Carotene | - | 51.22 | - | 13.24 | 6.70 |
| ΣCarotenoids | 0.39 | 156.17 | 251.96 | 262.73 | 249.81 |

## 4.7. Effects of paté dietary supplemetation in Comisana ewes

### *4.7.1. Production and quality of ewes' milk*

The results reported in Table 36 show no significant differences between the values of the parameters considered, with exception of the curd firmness value that resuls higher in L group. Supplementing the diet with extruded linseed did not result in an increase in the fat

content of the milk, as observed Flowers *et al.* (2008) in grazing dairy cows fed with a linseed oil supplementation feed. Milk protein and fat contents were not affected by the supplementation, this is in line with that observed by Gómez-Cortés *et al.* (2009) in ewes fed with different levels of extruded linseed and Nudda *et al.* (2006) in goats.

**Table 36.** Milk yield and quality.

| Parameters | | Dietary Treatments | | | Rmse |
|---|---|---|---|---|---|
| | | L | OP | LOP | |
| Milk yield | g/d | 798.1 | 756.6 | 700.5 | 55.3 |
| Fat | % | 8.08 | 8.78 | 8.35 | 0.58 |
| Lactose | % | 4.56 | 4.52 | 4.62 | 0.08 |
| Protein | % | 5.72 | 5.80 | 5.76 | 0.37 |
| r | min | 16’.23” | 17’47” | 16’18” | 2’56” |
| $k_{20}$ | min | 1’31” | 1’32” | 1’27” | 0’26” |
| $a_{30}$ | mm | 58.78a | 47.80b | 50.96b | 6.93’ |

A. B: P<0.01; a. b: P<0.05.

### *4.7.2. Ewes’ milk fatty acids composition*

The ewes’ milk fatty acids composition is shown in Table 37. The treatment with laminated linseed has led a significantly higher content of ALA in the milk of L group compared to OP group, while the levels of group LOP were slightly lower. The values found in group L are slightly lower than those reported by Mele *et al*. (2007), this may be related to the fact that in this research the amount of linseed used is lower. The concentration of ALA in milk is closely related to the intake-amount derived from linseed. as proven by Mughetti *et al*. (2012), who tested the dietary inclusion of extruded linseed at different supplementation levels in dairy sheep feed.

The LOP combination appears to be interesting; even though it is based an availability of ALA of about half, group L it still leads to a valid presence of it in the milk The administration of linseed in extruded form compared to integral flax resulted in a significantly higher increase in the ALA content in milk of ewes (Gomez-Cortes *et al*. 2009; Mele *et al.* 2007) and in dairy cows (Akraim *et al.* 2007).

The higher content of total n-3 fatty acids (ALA) in milk caused a linear decrease of the n-6/n-3 fatty acid ratio in L group, with the same trend observed by Mughetti and co-workers (2012).

As far as conjugated linoleic acid (CLA) is concerned, the level of RA is more than double in the milk in groups L and LOP compared to that found in the milk of group OP with values of 1.72 and 1.62 g/100 g of FAME. In the same way the level of VA is higher in the milk of animals treated with linseed compared to those found in group P ($P<0.05$). These results confirm the observation made by other authors (Gomez-Còrtes *et al.* 2009; Luna *et al.* 2008) regarding the direct relationship between VA and RA in animals treated with extruded linseed. Much of the RA in the milk originates from endogenous synthesis at the level of the mammary gland from VA by the way of $\Delta^9$-desaturase. Since the biohydrogenation of ALA does not generate RA as an intermediate, the secretion of high concentrations of it in the milk can be obtained from the production of high levels of VA in the rumen (Bauman *et al.* 2006). As for the ALA, the increase in the milk of RA can be attributed both to the lamination process that the extrusion that can increase the availability of ALA at rumen level, whose process of biohydrogenation generates a high amount of VA that is subjected to the action of $\Delta^9$ desaturase at the level of the mammary gland.

The level of RA and VA were found to be higher in group P than observed by Gómez-Cortés (2009) in ewes fed a diet enriched with olive oil and this is probably due to the high concentration of ALA found in the pasture compared to the test conducted by these authors.

**Table 37.** Fatty acid composition (%) of the ewes' milk.

| Fatty acid composition | Dietary Treatments | | | |
|---|---|---|---|---|
| | L | P | LP | Rmse |
| C14:0 | 6.89b | 8.01a | 7.24ab | 0.52 |
| C16:0 | 18.08b | 20.42a | 19.14ab | 0.81 |
| C18:0 | 15.20 | 13.30 | 13.70 | 1.18 |
| C18:1c9 | 20.09 | 23.85 | 22.48 | 1.45 |
| C18:1t11 | 5.14a | 1.96b | 4.21a | 0.81 |
| C18:2c9t11 | 1.72a | 0.87b | 1.62a | 0.24 |
| C18:2c9c12 | 2.21 | 2.24 | 2.33 | 0.19 |
| C18:3c9c12c15 | 1.65A | 0.62B | 1.25A | 0.16 |
| C20:0 | 0.25 | 0.28 | 0.28 | 0.02 |
| C20:4 | 0.09B | 0.13A | 0.11AB | 0.01 |
| C20:5 | 0.06A | 0.04B | 0.05AB | 0.01 |
| C22:5n3 | 0.101 | 0.091 | 0.093 | 0.014 |
| C22:6n3 | 0.035 | 0.047 | 0.034 | 0.007 |
| SFA | 57.69 | 62.31 | 57.74 | 1.75 |
| MUFA | 32.61 | 32.41 | 34.18 | 1.50 |
| PUFA | 8.74A | 4.94B | 7.50A | 0.49 |
| PUFA:SFA | 0.15a | 0.08b | 0.12a | 0.01 |
| PUFA n-6 | 2.38 | 2.47 | 2.52 | 0.20 |
| PUFA n-3 | 1.86A | 0.81B | 1.43A | 0.17 |
| n-6/n-3 | 1.29C | 3.06A | 1.87B | 0.45 |
| AA/DHA | 2.72 | 3.10 | 3.30 | 0.68 |
| LA/LNA | 1.36C | 3.63A | 1.99B | 0.53 |
| C18:2c9t11/C18:1c9t11 | 25.43 | 30.30 | 27.51 | 2.00 |
| TBARs (MDA mg/l) | 0.857 | 0.706 | 0.587 | 0.156 |

a.b: P<0.0 5; A. B: P<0.01

### *4.7.3. Antioxidants and oxidative stability of ewes' milk*

The most important result of this research is the detection of free-hydroxytyrosol in milk; the chromatograms obtained by means of HPLC-ESI-MS/MS in the negative ion mode on the precursor ions of the hydroxytyrosol and tyrosol with a ratio of m/z of 153 (1) and 137 (2) respectively, have been revealed the presence of these phenolic alcohols in the milk of OL and LOP groups (Figure 15). Figure 16 shows the spectra of MS/MS of hydroxytyrosol and tyrosol found in the extract of the phenolic obtained from ewes' milk; data were

confirmed by retention time and from the spectrum of MS/MS of the respective standard samples.

From a quantitative point of view there no proportional correlation between the amount of phenolic substances in the paté used in feed and the concentration of hydroxytyrosol and tyrosol in the milk. the latter was detected only in traces (Figure 15). However, the detection of these compounds in milk is an important step in a better understanding of the bioavailability of this substances; probably enzymatic hydrolysis of these compounds leads to the liberation of the phenolic component with subsequent transfer into milk.

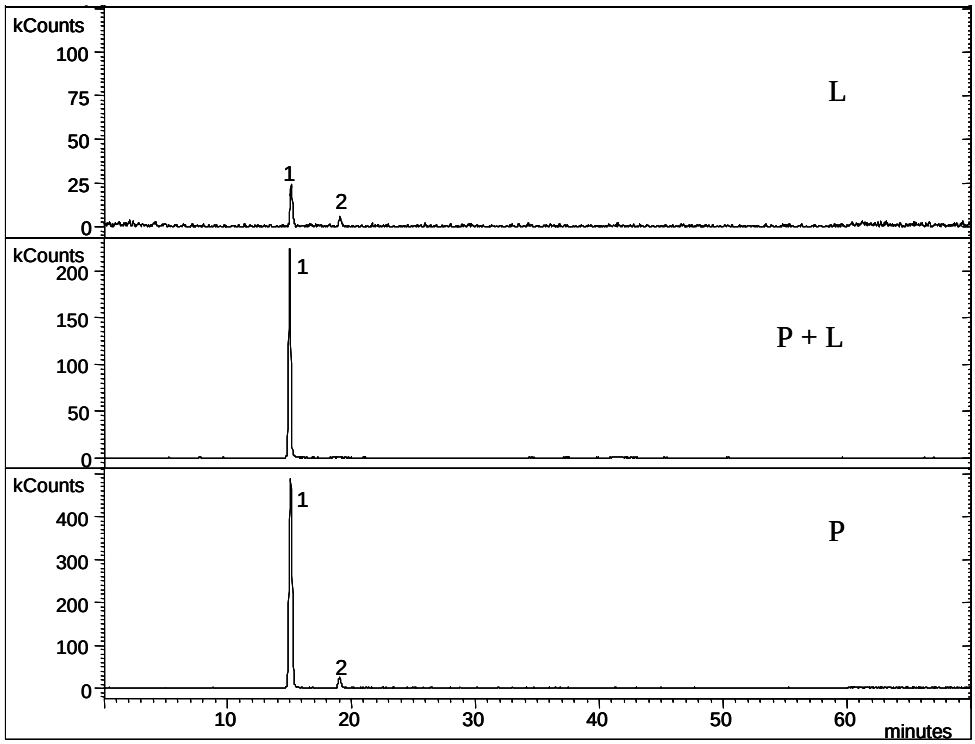


**Figure 15.** Chromatograms HPLC-ESI-MS/MS from milk of ewes fed with linseed (L). paté and linseed (P+L) and paté (P). 1. Hydroxythyrosol; 2. Tyrosol.

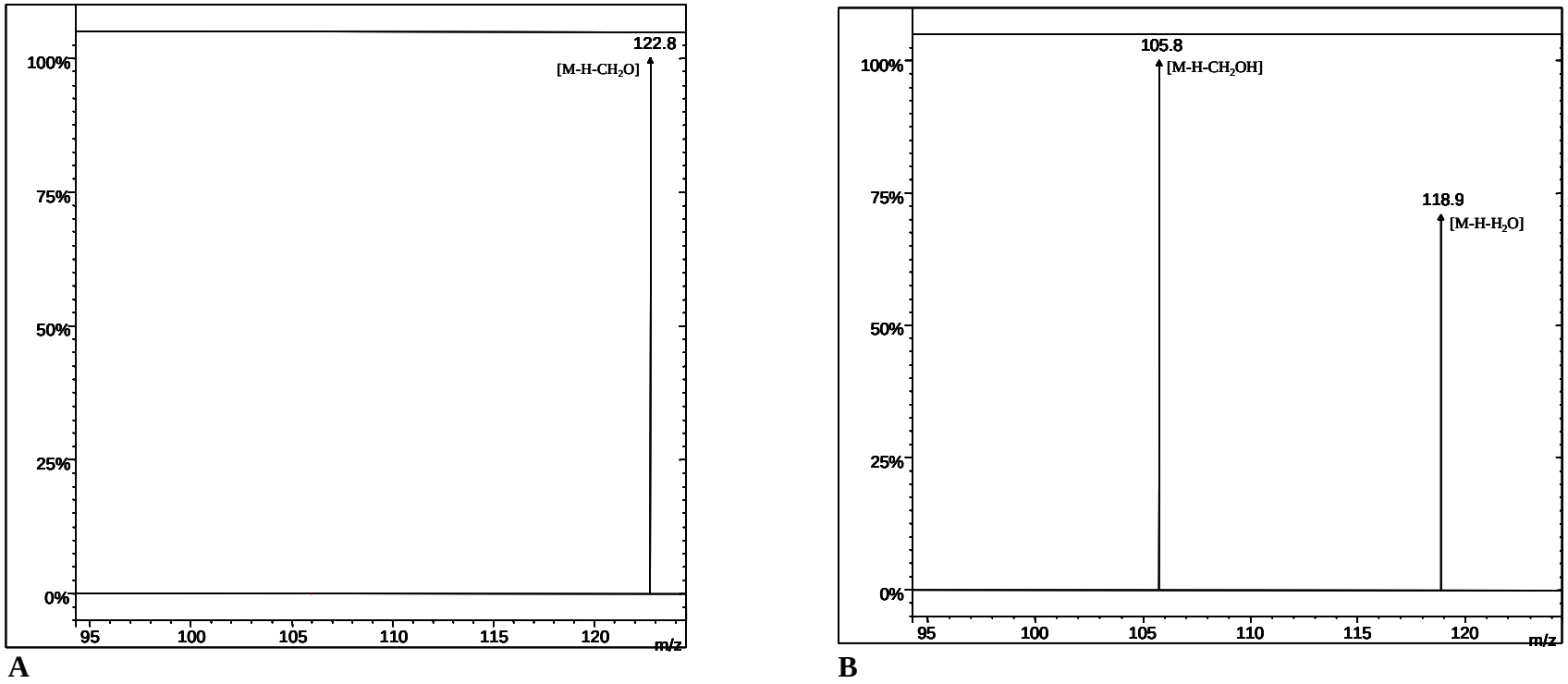


**Figure 16.** ESI-MS/MS spectrum of hydroxytyrosol (A) and tyrosol (B).

As far as the oxidative status is concerned, the milk of L and OP groups is lower compared to the LOP combination (Table 38); the level of TBARs remains higher in milk obtained from animals fed with linseed alone, compared to the others. This differences are not statistically significant. however is important to note that this reseach trial is about a dairy product already partially protected by the large amount of antioxidants present in pasture such as α-Tocopherol and β-Carotene (Table 35).

The concentration of tocopherols and retinol in the milk of the OL and LOP groups (Table 38), reflects the different concentration of hydroxytyrosol, this is probably due to the protective action that the latter has on tocopherols protecting them from oxidation; in fact it has been proven that in olive oil under thermal stress, hydroxythyrosol derivatives are the first compounds to be oxidized, providing oxidative stability to the oil. while tocopherols seems to be oxidized after a significant decrease on hydroxytyrosol derivatives concentration (Nissiotis and Tasioula-Margari, 2001).

Also in this trial a high variability due to the animals emerged; the values of hydroxytyrosol, ranged between a minimum of 2.0 μg/l and a maximum of 37.6 μg/l in the OP group, and between 2.0 μg/l and 13.3 μg/l in the LOP group.

**Table 38.** Effect of dietary treatment on the hydroxytyrosol, tocopherols and retinol content in ewes milk.

| Items | | Dietary Treatments | | | Rmse |
|---|---|---|---|---|---|
| | | L | OP | LOP | |
| 3,4-DPEA-EA | μg/l | $0.02^{B}$ | $10.08^{A}$ | $7.09^{A}$ | 1.57 |
| α-Tocopherol | μg/kg | 215 | 300 | 260 | 160 |
| γ-Tocopherol | μg/kg | 12 | 7 | 10 | 5 |
| Retinol | μg/kg | 167 | 220 | 205 | 97 |
| TBARs | (MDA mg/l) | 0.857 | 0.706 | 0.587 | 0.156 |

A. B: P<0.01.

Further studies have also shown that hydroxytyrosol is a metabolite of dopamine and that small concentrations within human fluids may be due to a combination of exogenous and

endogenous sources (Caruso *et al.* 2001; De la Torre, 2008). This could explain the presence of traces of hydroxytyrosol in milk of L group (Figure 15).

### *4.7.4. Oxidative status of ewes' cheese*

The cheese made from the milk of animals of L group shows a more oxidizable lipid fraction than that obtained from animals of the other two groups (Table 39). The MDA expressed in mg/kg of fat, show good values in OP and LOP cheeses; these values are significantly lower than those of L group cheeses, and about ¼ of those proposed by Hamilton and Rossel (1986) as the value limit. These values are also lower than those observed by Severini *et al.* (1998) in Parmesan cheese stored under vacuum.

The evolution of the oxidative status during storage of cheese, assessed at 3 and 7 days, shows a constant trend in OP and LOP group cheeses (Figure 17), probably due to the protective action that polyphenols exert against tocopherol oxidation protecting them from heat treatment. These data are further confirmed by the "yellow index" of the cheese (Figure 18); the color alteration which is linked to the oxidation process is lower in L group than the others.

**Table 39.** Oxidation of the lipids of cheese as a function of different dietary supplementation.

| Items | Dietary Treatments | | | Rmse |
|---|---|---|---|---|
| | L | OP | LOP | |
| mg MDA /kg cheese | 0.375a | 0.138b | 0.121b | 0.073 |
| mg MDA /kg of fat | 1.291a | 0.622b | 0.493b | 0.235 |

a.b: $P<0.05$.

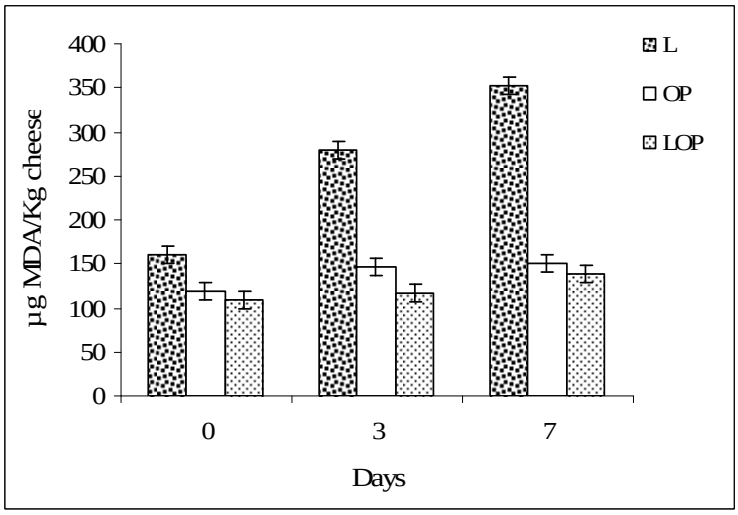


**Figure 17.** Evolution of the oxidative status of cheese during storage, assessed at 3 and 7 days.

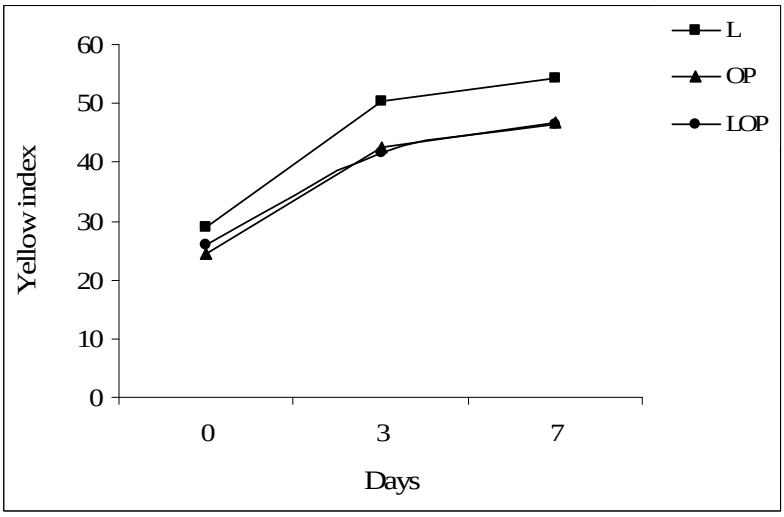


**Figure 18.** Effect of the dietary treatment on cheese yellow index.

## 4.9. Milk phenols: extraction method

A specific method for the extraction and purification of the phenolic compounds contained in buffalo and ewes' milk has been develop during this research work, because up to now, there isn't enough information about a specific procedure in the literature.
The procedure is reported below.

The milk sample (80 ml) was skimmed by centrifuging at 5000 rpm for 1 min at room temperature. A volume of 60 ml of skimmed milk was acidified with a solution of citric acid (1.5 M) to pH 4.6 to precipitate the caseins. Then 120 ml of methanol was added to the sample. and after 1 min of vortex mixing. the solution was centrifuged at 5000 rpm for 2 min. The supernatant was filtered with a fluted filter. The precipitate was washed with 80 ml of a mixture of methanol and water (80:20. v/v). and after 1 min of vortex mixing. the solution was again centrifuged at 5000 rpm for 2 min. The supernatant was filtered and collected with the previous fraction (this operation was repeated twice). The methanol was removed with a rotary vacuum evaporator at 37°C. and the aqueous extract was passed through an Extract-cleanTM SPE C18-HC column (5000 mg/25 ml) that was activated and conditioned with 10 ml of methanol and 10 ml of water. respectively. The column containing the analyte was washed with 10 ml of water. The phenolic compounds were eluted with 100 ml of methanol. and the collected fraction was mixed with 40 ml of hexane in a separatory funnel. After agitation. the methanolic fraction was transferred to the rotary vacuum evaporator to eliminate the solvent. The residue was reconstituted in 5 ml of methanol. filtered with a 0.2 μm PVDF filter (Alltech. Deerfield. IL). dried under a flow of $N_2$ gas and recovered with 1 ml of methanol for LC-MS/MS analysis.

# 5. CONCLUSIONS

This research project provides information on the effectiveness of the use of different types of pomace, such as pomace and patè, obtained from the extra virgin olive oil mechanical extraction process, in dairy animal feeding. The improvement of the quality of dairy product fats could positively lead to beneficial effects in the prevention of some diseases in humans.

The results obtained are summarized in the following points.

**Research 1.**

The results on virgin olive dried pomace supplementation in dairy water buffalo feeding, confirm that its use does not have any detrimental effect on animals: in fact no changes due to the different dietary treatment emerged from the analysis of the qualitative and quantitative parameters of the milk from treated animals, despite the high amount of phenolic compounds in the pomace. The elimination of the stone, also, determines a significant reduction in the ADL content, improving the dry matter digestibility of the olive pomace.

The milk from buffalo fed with pomace shows a higher proportion in C18:1 n9 content and higher amount in LCFA, UFA and MUFA with respect to the control group, a significant reduction in the SAT content and atherogenic index. Notable is the total amount of vitamin E in milk, that increased from 8.605 µg/g of fat to 10.446 µg/g of fat in the milk of treated buffalo with a consequent decrease of the TBARs value. The high amount of phenols, characterized by strong antioxidant activity such as 3,4-DHPEA, 3,4-DHPEA-EDA, found in the pomace may be involved in the increased amount of α- tocopherols in the milk; in fact phenolic antioxidants can protect tocopherols from oxidation improving both their bio-availability during digestion and their concentration in the milk.

A lower level of SFA, a higher MUFA content, a reduced n6/n3 ratio and lower atherogenic and trombogenic indexes were found in mozzarella from buffalo cows fed with stoned pomace.

**Research 2.**

From a comparison of the nutritional-chemical characteristics of the olive paté with those of the pomace, the patè showed better characteristics such as a lower content of lignin and cellulose, and a higher concentration of the lipid fraction, crude fiber and high polyphenol content.

The milk of LOP combination, even though it is based on an availability of ALA of about half of that of the L group, is characterized only by a slightly lower presence of ALA in the milk. The concentration of RA is higher in milk of the L and OP group; a similar trend is followed by VA. The oxidative stability of the milk in diets L and OP is lower compared to the LOP combination, the level of TBARs, even though, not statistically significant, is lower in LOP group. These values, instead, become significant in cheese, where the oxidation of the lipids is lower in the OP and LOP group and lead to a successive constant evolution of the oxidative status during storage of cheese, assessed at 3 and 7 days, with a significant improvement of the cheese shelf-life.

However the main results, common to both researches, have been the detection and the characterization of hydrophilic phenols in the milk through a specific method for the extraction and the purification of the phenolic fraction from milk developed in these years of research. The analytical detection of hydroxytyrosol and tyrosol in the ewes' milk fed with the paté and hydroxytyrosol in buffalo fed with pomace showed for the first time the presence in the milk of hydroxytyrosol, which is one of the most important bioactive compounds of the oil industry products; the transfer of these antioxidants and the proven improvement of the quality of milk fat could positively interact in the prevention of some human cardiovascular diseases and some tumours, increasing in this manner the quality of dairy products and also improving their shelf-life.

These results also provide important information on the bioavailability of these phenolic compounds.

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