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TITLE

**STUDY AND PREVENTION OF
LIPID OXIDATION IN MEAT**

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Anyone who has never made a mistake has never tried anything new

Albert Einstein (1879-1955)

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INTRODUCTION

Nowadays in Western Countries major causes of morbidity and mortality are related to poor diet and a sedentary lifestyle, for this reason good nutrition has become absolutely essential for good health, moreover for healthy growth and development of children and adolescents.

Meat is considered a very nutritive food and has been evaluated and associated with a good health, by contributing quality protein, B vitamins, iron (a nutrient most often lacking in the diets of women and children) and zinc (a mineral that is essential for growth and metabolism). Meat fat is important in human nutrition with n-3 PUFA and CLAs playing a beneficial role. In addition to its nutritive value, meat has other important attributes, including its attractive sensory properties.

In 1997 meat consumption in the fifteen countries member of the EU has been of 101 million tons for beef/veal and 215 million tons for pork meat, while the European production of sausages (not of liver) was 2,647,000 tons (1).

Nowadays under a point of view, some kind of meat, can be considered more healthy of the past. In fact, since dietary recommendations favoring the consumption of less saturated, by a target diet it has been possible increase ratio unsaturated/saturated fatty acid in meat.

Meat industry has worked for reduction of fat in meat, reaching important results, but the problem of lipid peroxidation remains still open.

The fatty acids composition of meat will affect the profile of compounds produced in lipid oxidation and the abundance of unsaturated fatty acids will favour the abstraction of a hydrogen atom and the start of the oxidation process. Moreover part of meat, in according to modern trends toward convenience foods, have consumed as precooked and restructured products and such foods are highly susceptible to lipid oxidation, showing all factors favouring oxidation process.

Lipid peroxidation is a main problem that reduces meat quality and this is a ubiquitous phenomenon that can lead to rancid odour and loss of product taste. Moreover, lipoperoxides and some cholesterol epoxides are considered in literature as atherogenic agents and are mutagenic, carcinogenic and cytotoxic (2).

This problem to meat scientist new challenges in develop new strategies to preserve meat quality.

Muscle lipids and meat quality

Lipids are present in muscles as structural components of the muscle membranes, as storage droplets of triacylglycerol between muscle fibres and as adipose tissue (marbling fat). These lipids, or more precisely their fatty acids, contribute to a wide range of quality attributes. For fresh meat these are colour stability, drip loss and the development of oxidative rancidity. Finally, nutritional quality depends upon the fat content of the meat and its fatty acid composition.

The attractiveness of meat to the purchaser is mainly related to colour, after perceived economic value. As meat ages, it turns brown as the myoglobin is converted to oxidized metmyoglobin and is rejected by the consumer. Lipid peroxidation increases the rate of metmyoglobin formation and conversely metmyoglobin acts as a catalyst of lipid peroxidation so that in beef muscle displayed under oxygen permeable film, lipid oxidation and metmyoglobin levels were closely correlated. Lipid peroxidation depends upon the degree of unsaturation of the fatty acids and the levels of the antioxidant vitamin E (α -tocopherol) and prooxidants such as free iron. Increasing the degree of unsaturation of the fatty acids results in a decrease in colour and oxidative shelf-life.

In a study to increase the content of n-3 PUFA in beef, Richardson *et al.*, (1997) (3) observed that minced muscle from steers fed 3% fish oil had more rapid colour deterioration (saturation) and higher levels of lipid oxidation (thiobarbituric acid reacting substances) than muscle from steers fed bruised, whole linseed or a mixture of linseed and fish oil. This difference in shelf-life resulted from relatively small changes in muscle fatty acids. Total PUFA in the phospholipids were similar for meat from the fish oil or control fed steers but a doubling of the amounts of the main fish oil PUFA, 20:5 n-3 (EPA) and 22:6 n-3 (DHA) decreased shelf-life even when accompanied by a halving of the content of 20:4 n-6 (arachidonic acid).

The recommendations for the amount and type of fat in the human diet in relation to coronary heart disease (CHD) and stroke were set out in the LARN (Livelli di Assunzione Raccomandati di Energia e Nutrienti, Italy). Similar recommendations were made in other countries. In addition to cardiovascular disease, these recommendations are also relevant in terms of obesity and diabetes, both of which are risk factors for CHD, and also for cancer.

However, in considering the possible role of meat lipids in disease it must be remembered that most diseases are of complex aetiology and that fat is only one risk factor.

In fact muscle, if consumed without any adhering adipose tissue, can easily contribute to this aim since, as discussed above for texture, most muscles have less than 50g/kg lipid and hence can be classed as a low fat product. However, even a lamb or pork chop or sirloin steak can be included in meals that meet dietary guidelines. It is the consumption of “hidden” fat in burgers, pates, sausages and the like that contribute to the high fat intake from meat since a high proportion of people discard adipose tissue on the plate. The saturated fatty acids, lauric (12:0), myristic (14:0) and palmitic (16:0) acid contribute to heart disease by raising plasma low density lipoprotein cholesterol whereas linoleic acid and α -linolenic acid lower it thereby decreasing the risk of heart disease. Stearic acid (18:0) does not affect plasma cholesterol concentrations. However, it may contribute to thrombosis, the final event in CHD that produces the heart attack. It is therefore included in the ratio of PUFA to saturated fatty acids (P:S) used to assess fat quality in terms of human nutrition and which has an acceptable value of 0.4 or above for the diet as a whole. The P:S ratio for pig muscle is generally above this value but for muscle from cattle and sheep is around 0.1 or less.

Another consideration in addition to increasing the intake of total PUFA is the relative levels of those derived from linoleic acid, the n-6 or ω 6 series, and those derived from α -linolenic acid, the n-3 or ω 3 series. The longer-chain PUFA act as precursors for oxidized derivatives called eicosanoids and these function as regulators of many physiological processes. In haemostasis, thromboxane A₂ produced from arachidonic acid (20:4 n-6) is a powerful 245 clotting agent whereas thromboxane A₃ from eicosapentaenoic acid (20:5 n-3) is much less active. The levels of the two eicosanoids depend on the quantities of their precursor fatty acids in the phospholipids of blood platelets. These amounts, in turn, depend upon the relative amounts of linoleic acid and α -linolenic acid in the diet since these are the precursors of the longer-chain PUFA. It is believed that primitive man evolved with a ratio of 18:2/18:3 of 1 in his diet but because we now consume large quantities of linoleic acid in vegetable oils the ratio ranges from 7-20. This contributes to heart disease by raising 20:4 n-6 and increasing the thrombotic tendency and contributes to autoimmune disease like arthritis because the leukotrienes produced from arachidonic acid stimulate the immune responses more than those from 20:5 n-3.

Because of these competitive metabolic effects between n-6 PUFA and n-3 PUFA the recommended level for the ratio of linoleic to α -linolenic acid is 4 or less. Muscle from forage finished cattle and sheep usually has an n-6:n-3 ratio below 2 whereas for pigs it is nearer 20. Fatty acids of considerable interest at present are the conjugated linoleic acids (CLA). One isomer, 9-cis, 11-trans is present in ruminant meats and milk. It is formed either in the rumen as the first step in biohydrogenation of linoleic acid or by Δ^9 desaturation in body tissues of transvaccenic acid, itself produced in the rumen. CLA isomers inhibit carcinogenesis, decrease atherosclerosis, modify the immune response and partition energy toward the growth of muscle rather than adipose tissue (4).

Amounts in ruminant meat and milk can be altered by dietary means (5, 6).

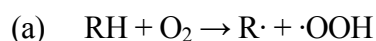
However, the value of CLA in human nutrition and the amounts required for therapeutic effects remain to be established.

Lipid oxidation process in meat

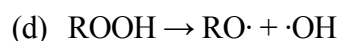
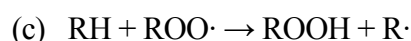
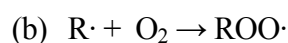
The oxidation of lipids is commonly described as an oxidative, oxygen dependent, deterioration of fats, notably the unsaturated fatty acids.

This modification of fatty acids is principally carried out by an autocatalytic mechanism of 'free radicals', called auto oxidation, consisting of three phases (7)

1. Initiation:



2. Propagation:



3. Termination:



Lipids hydroperoxides (ROOH) are the primary lipid oxidation products and once formed, they are relatively stable at moderate reaction conditions, such as low temperature and absence of metal ions. However, these conditions are never found in muscle foods and hydroperoxides become susceptible to further free radical chain reaction such as isomerization and decomposition.

Their breakage causes secondary products such as pentanal, hexanal, 4-hydro-xynonenal and malondialdehyde (MDA).

Meat is rich in heme proteins such as oxymyoglobin and oxyhemoglobin, that are particularly susceptible to oxidation and are affected by transition metals, lipoxy radicals and active oxygen species such as H_2O_2 , OH^\bullet , $HOCl$ and NO_2^\bullet . Several investigations have shown that the free iron redox cycle, provided by ascorbic acid, is the main initiator of lipid peroxidation in fresh muscle foods and that such a system could greatly affect the oxidation of oxymyoglobin. Haemoglobin and myoglobin as inhibitors of hydroxyl radical generation in a model system of "Iron Redox" cycle. (8). It was postulated that there is interrelation between lipid peroxidation in muscle tissue and oxymyoglobin (9, 10) and recent works have shown that there are two pathways that affect oxymyoglobin: a) oxygen active species (H_2O_2 , OH^\bullet , $O_2^{\bullet-}$ and perferryl), generated during autoxidation of myoglobin and oxidation of ferrous ions; b) lipid radicals (ROO^\bullet , RO^\bullet) and hydroperoxides generated during lipid peroxidation (11).

During oxidation processes it is necessary to consider also the effects of enzymatic component (autocatalyzed oxidation) that operate after slaughtering. In the *post mortem* step, endogenous antioxidant systems (for example superoxide dismutase and glutathione peroxidase) available *in vivo* in the cell are not active and this doesn't permit to balance free radical production.

Factors influencing lipid peroxidation

Many factors can influence fat composition of meat, i.e. diet, race, weight, age, tissue of deposit, sex, hormones etc. (12-15).

Meat industry has worked for reduction of fat in meat, reaching important results, but the problem of lipid peroxidation remains still open.

The fatty acids composition of meat will affect the profile of compounds produced in lipid oxidation and the abundance of unsaturated fatty acids will favour the abstraction of a hydrogen atom and the start of the oxidation process.

Dietary supplementation to animal foods and the tendency of the species to accumulate certain fatty acids in the membrane phospholipids, affect the lipid composition of the membrane and, consequently, its susceptibility to peroxidation. Polyunsaturated fatty acids (PUFA) of muscle membrane cells are particularly susceptible during storage to peroxidation when the degree of unsaturation of membrane lipids is increased with reduction of oxidative stability of muscle (16).

Furthermore, other factors that will affect the lipid oxidation of muscle foods are exposure to light, oxygen availability, temperature conditions and microbial growth (17).

Cooking process can affect lipid compounds in meat, especially the fatty acids component, changing the nutritional value of cooked products respect to raw sample (18-20).

These factors can make the quality of the foodstuff not acceptable for human consumption, but before these conditions take place, lipid oxidation could generate toxic molecules with possible hazards for human health.

Effects of oxidized lipids in nutrition

The toxicity of dietary oxidized lipids was studied in various animal species and involved highly oxidized fats, particularly thermoxidized frying fats. Among oxidation products contained in such fats, polymers appear to be harmless because they are minimally absorbed and are removed with feces. Nonpolar dimer FA showed digestibility below 5% (21). Noncyclic dimeric FA were also poorly absorbed (10%). On the other hand, as much as 50% of the total oxidized monomeric acids and 95% of the total cyclic monomeric acids from thermoxidized fats and TAG were recovered in the lymphatic lipids (22). Rats fed mackerel fried in coconut oil over the course of 4 days showed initial stages of cell damage in the liver and kidney as well as an increase in total lipids and cholesterol in heart and serum, compared with the control group fed steamed mackerel (23). Eder (1999) (24) showed that the consumption of oxidized lipids (thermally, at 150°C, 6 days) caused a reduction in the desaturation rate of linoleic acid and α -linolenic acid by microsomal $\Delta 4$, $\Delta 5$, and $\Delta 6$ desaturases and also a reduction in $\Delta 9$ desaturation. On the other hand, recent research on

humans demonstrated the conversion of *trans*- α -linolenic acid isomers into the following long-chain PUFA: 19-*trans*-C22:5n-3, 19-*trans*-C22:6 n-3, and *trans*-C24:5. These *trans*-LC-PUFA can interfere with platelet and endothelium metabolism.

The *trans*- α -linolenic acid isomer-rich diet raised the LDL- to HDL-cholesterol and total cholesterol ratio, which would increase the risk of cardiovascular disease by 8% (25).

Moderately thermally oxidized soybean oil (130°C, air flow-through) of peroxide value (PV) = 75 mEq O₂/kg diet (compared with the control of 9.5 Eq O₂/kg diet) was fed to rats for 40 days (26). The study showed no adverse effects on liver, heart, kidney, or adipose tissue FA composition, and even a reduction in the osmotic fragility of erythrocytes and hepatic lipogenesis. However, the moderately oxidized oil slightly reduced the vitamin E status in the tissues. A slightly increased susceptibility of LDL to lipid peroxidation, and an increased concentration of thiobarbituric acid reactive substances (TBARS) in LDL, were also observed.

The harmful effects in animal fats are closely related to the potential activity of cholesterol oxidation products, particularly in the presence of unsaturated FA and thermal treatment at temperatures exceeding 100°C.

UV-oxidized fish lipids, and a threefold increase in PV and TBA compared to controls, resulted in weight loss, lower body weight increments, increased liver weight, reduced hemoglobin and hematocrit in guinea pigs fed a diet with a 14% addition of those lipids for 12 weeks (27). The presence of oxidized lipids in the diet of humans and animals results in an increase in TBARS in plasma and tissues. An increase in the peroxide value could not be detected. There was a dose-dependent increase in conjugated dienes in chylomicrons of rats and humans given oxidized lipids (28).

The research on animals conducted so far, including experiments on mammals, shows aldehydes in free form or conjugated with amino acids to be absorbed from the gastrointestinal tract to plasma, muscles, and liver. The absorbable aldehydes adducts with protein from the diet are less toxic than free aldehydes. With regard to hydroperoxides, they are generally thought to be decomposed in the stomach, from where they are not transported any farther. It is possible that at low doses, FA hydroperoxides are converted to the corresponding hydroxy FA in the mucosal membrane before being transported to the blood. A study on structured lipids confirmed that hydroperoxides were absorbed as monohydroxy

and monoepoxy FA. They can influence endothelial dysfunction, promote thrombosis, and induce atherosclerosis (29).

Apart from the antioxidant system, an additional mechanism of protection is furnished by diarrhea, induced in rats by high doses of lipid hydroperoxide (30). The LD50 of the hydroperoxide of highly unsaturated FA methyl ester was between 285 and 545 mg active hydroperoxide oxygen per kilogram body weight (31). Maximum hydroperoxide content in fatty fish products does not exceed 50 mg/100 g. This hydroperoxide level is accompanied by a 20% decrease in EPA and DHA content. Thus, the effects of oxidized lipids in human diet are rather long-lasting.

Antioxidant addition to prevent lipid oxidation

Antioxidants are added to fresh and further processed meat to prevent oxidative rancidity, retard development of off-flavours, and improve colour stability. In food industry they can be grouped into natural antioxidants and synthetic antioxidants, with the latter including, for example, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ). Both types of antioxidants play a very important role in the food market. However, because of toxicological concerns of synthetic antioxidants, nowadays the new trend in food industry includes an enhanced concern for the quality and safety of food products, increased preference for natural products over synthetic ones, and broadened regulations related to nutritional and toxicity levels of active ingredients. Consequently food market is demanding for natural food ingredients free of chemical additives orientated to promote the use of natural products.

The research for natural additives has notably increased in recent years; compounds obtained from natural sources such as grains, oilseeds, spices, fruit and vegetables have been investigated and the effect of antioxidants on controlling oxidative reactions in meat has been well documented.

Moreover another strategy to prevent lipid oxidation is feeding animals with antioxidants. Addition of vitamin E to the feed increased α -tocopherol concentration in sarcosomes and thus significantly increased the stability of the lipids and Mb-Fe²⁺-O₂ against oxidation.

Vitamin E is the primary lipid-soluble antioxidant in biological systems and breaks the chain of lipid peroxidation in cell membranes and prevents the formation of lipid hydroperoxides

(32, 33) for this reason it has been found to improve the quality of farm animal products. Feeding with vitamin E-supplemented diets reduced lipid peroxidation in turkey muscle (34-36), in chicken meat (37-39), in pork (40), in fish (41, 42), and in beef (43, 44).

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OBJECTIVES

In according to the importance of meat in the diet and of its diffuse use in Western Countries, it's necessary to give attention to a particular nutritional aspect of meat, concerning food safety, which is one of the most important goals in food science nowadays.

It is well know that PUFA fatty acid and iron are among main factors improving lipid oxidation, so their reduction by animal diet may be an efficacy mean to improve oxidative stability in meat. However, this measure may not be acceptable in practice because PUFA and iron are both nutritional elements for human health.

This research work has begun from studying of antioxidant to prevent lipid oxidation until arriving to find a model to induce oxidation to investigate the process in vitro.

Work plan can be schedules in three parts:

CHAPTER I PROTECTIVE EFFECTS OF NATURAL ANTIOXIDANTS AGAINST LIPID OXIDATION DURING MEAT COOKING AND STORAGE

CHAPTER II DETERMINATION OF MDA AND TBARS IN BUFFALO MEAT FROM ANIMALS WITH DIETARY SUPPLEMENTATION OF VITAMIN E

CHAPTER III DEVELOPMENT OF ANALYTICAL PROCEDURES TO STUDY CHANGES IN THE MEAT PHOSPHOLIPIDS COMPOSITION OF CAUSED BY INDUCED OXIDATION

CHAPTER I

PROTECTIVE EFFECTS OF NATURAL ANTIOXIDANTS AGAINST LIPID OXIDATION DURING MEAT COOKING AND STORAGE

Abstract

Lipid peroxidation is a major phenomenon reducing meat quality during cooking and storage and leading to off-flavour formation and rancidity. The use of vegetable extracts to increase shelf life is a promising new frontier in the prevention of lipid oxidation. In this study the efficacy on lipid oxidation during microwave cooking of apple antioxidants at different concentrations have been tested. Moreover the efficacy of natural antioxidants (anise, clove, parsley, sage), extracted with supercritical fluid technology (SFE), and of four oregano and rosemary-based commercial preparations widely used in meat industry have been compared. Total phenols and antioxidant activity of the SFE extracts were used as parameters to characterize the vegetable extracts, while TBARS and MDA were considered to monitor lipid peroxidation. Results show a protective action of apple antioxidants against peroxidation and a higher efficiency of natural antioxidants extracted by SFE respect to the commercial preparation used.

Key words: lipid oxidation, natural antioxidants, supercritical fluid extraction, TBARS, MDA

INTRODUCTION

Oxidation of lipids and muscle pigments is one of the main parameters of meat quality deterioration. Lipid oxidation causes development of off-odours and off-flavours; muscle-pigment oxidation negatively affects colour, appearance and acceptability (Kolakowska, 2003).

Antioxidants are added to fresh and processed meat to prevent oxidative rancidity, to delay development of off-flavours, and to improve colour stability. They can be grouped into natural antioxidants and synthetic antioxidants, with the latter including, for example, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ). Both types of antioxidants play a very important role in the food market, but the trend is to focus on the healthiness of food products, therefore there is an increasing preference for natural products over synthetic ones. Consequently, the search for natural additives, especially of plant origin, has been notably increased in recent years (Meyer, Suhr, Nielsen & Holm, 2002). Compounds obtained from natural sources such as grains, oilseeds, spices, fruit and vegetables have been investigated (Chen, Muramoto, Yamauchi & Huang, 1996).

It has been shown that several plants or their phenolic extracts such as rosemary (Karpinska, Borowski & Danowska-Oziewicz, 2000; McCarthy, Kerry J. P., Kerry J. F., Lynch & Buckley, 2001), potato peel (Kanatt, Chander, Radhakrishna & Sharma, 2005), tea catechins (McCarthy et al., 2001; He & Shahidi, 1997; Tang, Sheehan, Buckley, Morrissey & Kerry, 2001; Rababah et al., 2004), sage (McCarthy et al., 2001; El-Alim, Lugasi, Hovari & Dworschak, 1999), cloudberry, beetroot, willow herb (Rey, Hopia, Kivikari & Kahkonen 2005), rapeseed and pine bark (Vuorela, Salminen, Makela, Kivikari, Karonen & Heinonen, 2005) are efficient meat lipid antioxidants. Rababah et al. (2004) found that tea catechins also inhibited protein oxidation in meat.

The supercritical fluid extraction (SFE) technology is a reliable alternative to the traditional solvent extraction. In fact, it has numerous advantages such as the absence of solvent residues in the food extract and a low environmental impact. In addition, carbon dioxide, which is the most adopted supercritical fluid, do not contain free oxygen, thus the extracts undergo to a limited oxidation damage (Taylor, 1996).

Last, but not least, the parameters of SFE equipments can be modulated to enrich the extract of antioxidant compounds without extracting the essential oil component (Persson, Graziani, Ferracane, Fogliano & Skog, 2003). This is a very important feature as the strong pungent flavour of many plant extracts often represent the bottleneck for their practical use, due to the unpleasant sensorial characteristic of the final product.

Despite SFE technology is more and more common, almost no information is available about the efficacy of plant SFE extracts in the prevention of meat oxidation.

The aims of this work are to evaluate the oxidative stability of meat lipids in presence of apple extracts and using eight extracts obtained by SFE from different vegetable sources, and of 4 commercial antioxidant preparation widely used by the meat industry.

MATERIALS AND METHODS

Reagents

(+)-catechin, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Trolox, CaHPO_4 , Na_2CO_3 , Folin & Ciocalteu's Reagent were from Sigma-Aldrich. Butylated hydroxytoluene (BHT), FeCl_3 , 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (MDA source), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, gallic acid, Celite 545, hydrogen peroxide 30%, dimethylphenylendiamine•2 HCl from Fluka. Trichloroacetic acids (TCA), chloridric acid 37% (HCl) were from Carlo Erba. Other solvents were obtained from Merck.

Experiment with apple antioxidants

Extraction and quantification of apple antioxidant was carried out by HPLC-DAD as described by Napolitano et al., (2004). Apple antioxidants concentration were expressed as catechins equivalents (CEq) as previously described (Peri, Pietraforte, Scorza, Napolitano, Fogliano & Minetti, 2005).

Beef meat was purchased in a local market. Meat was minced and mixed with different concentrations of apple antioxidants: 50 mg Kg^{-1} (CEq); 150 mg Kg^{-1} (CEq); 450 mg Kg^{-1} (CEq). Meat balls of 50g were microwave cooked for 2 min at 650 watt.

After cooking, samples were freeze-dried (Freezer Drier Modulyo Edwards) until analysis.

Sampling of meat treated with SFE extracts and commercial antioxidants

Extraction of antioxidants was performed in a pilot SFE plant (Applied Separation, Italy) equipped with 20 L vessel using the following conditions: CO_2 pressure, 220 bars; T, 40°C ;

flow, 40 Kg/h; time, 9 hours. The starting materials were desiccated leaves of the different vegetables, but seeds for grapefruit and peach (see **Table 3**).

Rosemary and oregano based commercial antioxidant preparations were purchased on the market and indicated with the number: 10, 28, 6 and 31.

Meat used for these experiments had the following compositions (43.3% chicken breast; 43.3% chicken leg; 10% water; 2% binder; 0.8% salt). Meat balls of 100 g were prepared by mixing different concentrations of antioxidant extracts (see **Table 2**)

Meat balls were cooked at T= 100°C in full steam oven, refrigerated at 4°C and packaged in plastic tray. Three samples were prepared for each of the 4 times of shelf life at 4°C: T1 (1 day); T4 (4 days); T9 (9 days); T14 (14 days). At the end of the storage time samples were freeze-dried and stored at -20 °C until analysis.

TBA-test

The method described by Maraschiello, Sárraga and Gargía Regueiro (1999) was used, with some modifications. Briefly 0.5 g of meat were added to 10 mL of deionised water and vigorously mixed (1 min). 2.5 mL of 25 % TCA were added, samples were stored for 15 min at 4 °C and centrifuged (5 min, 4000 rpm, at 4 °C). An aliquot of 3.5 mL of the supernatant was added to 1.5 mL of 0.6 % TBA and incubated in water bath for 30 min at 70 °C. The intensity of the developed color was measured at 532 nm (UV-vis Shimadzu 2100, Japan) against a blank consisting of 2.5 mL of ultrapure H₂O, 1 mL 25% aqueous TCA, and 1.5 mL 0.6 % TBA.

MDA determination

The method proposed by Bergamo, Fedele, Balestrieri, Abrescia & Ferrara (1998) was used with minor modifications. An amount of 1 g of freeze dried meat was dissolved in 4.75 mL of Milli-Q water and 0.25 mL of ethanolic BHT (1000 ppm final concentration). The samples were vigorously mixed and sonicated for 30 min. Samples were centrifuged at 4000 rpm for 5 min and 500 µL of aqueous phase was taken and added to 500 µL of ice-cold 10% TCA. Proteins were removed by centrifugation (5 min, 10000g); 300 µL were taken from the supernatant and added to 700 µL of TBA mix (solution of 0.4% TBA in 2 M acetate buffer at pH 3). The mixtures were incubated in water bath for 30 min at 90 °C. At the end of incubation, the samples were centrifuged (5 min, 10000g) to remove particulate material and 20 µL were analysed by HPLC.

The HPLC analysis was performed using a liquid chromatograph LC-10D VP, with RF-10Ax1 Fluorescence detector (Shimadzu, Kyoto, Japan). The MDA-TBA complex was fractionated on a Luna 3 μ C18 (2), 150mm x 4,60 mm (Phenomenex). The mobile phase was daily prepared and consisted of 2.5 mM sodium phosphate buffer (pH 7.0) and acetonitrile (85:15 v/v). The chromatographic run was performed at 0.8 mL/min flow rate. Fluorimetric detection parameters: excitation λ_{EX} = 515 nm and emission λ_{EM} = 543 nm. MDA identification was performed by standard coinjection.

Antioxidant activity

The antioxidant activity was determined using ABTS method as described by Pellegrini, Re, Yang, and Rice-Evans (2000). Sample preparation was carried out in triplicate as follow: 1 mL of antioxidant extract was resuspended in 10 mL of methanol; the solution was mixed for 1 min, and centrifuged for 5 min at 4000 rpm. The supernatant, after appropriate dilutions, was added to the chromogen solution for the determination of antioxidant activity.

Total polyphenols determination

Total polyphenols determination was performed with the method of Folin-Ciocalteu (Singleton & Rossi, 1965), using the same solution prepared for antioxidant activity. Samples were diluted 1:10 or 1:2 with methanol (except for clove that was diluted 1:500). 125 μ L of the antioxidant solutions were added to 500 μ L of deionised water and to 125 μ L Folin & Ciocalteu's reagent. After 6 minutes 1.25 mL of NaCO₃ 7 % and 1 mL of water were added and the samples were incubated for 90 min at room temperature and the absorbance at 760 nm was measured.

Lipid extraction

A dry-column extraction method as described by Maxwell, Marmer, Zubillaga and Dalickes (1980) was used. Briefly, aliquot of 2.5 g of freeze-dried meat was added with 20 g anhydrous Na₂SO₄ and mixed in a mortar for 1 min. 15 g Celite 545 was added and the mixture was regrind, then transferred into a glass column (2.5 cm diameter and 30 cm height) , previously loaded with 10 g CaHPO₄•2H₂O/Celite (1:9). About 250 mL of a mixture of dichloromethane / methanol 9:1 v/v containing 50 mg L⁻¹ BHT were added and 150 mL of eluate were collected. Lipid extracts coming from meat treated with different concentration of apple antioxidant were evaporated under nitrogen until constant weight and peroxides concentration was determined.

Peroxides determination

The method reported by Karppanen, Rizzo, Saari, Berg and Boström (1989) was used with some modifications. 5 mg of lipid extract were added with 1 mL of methanol and 200 μ L of a reagent prepared daily by dissolving 250 mg of dimethylphenylendiamine•2 HCl in 0.15 mL of acetic acid, 21 mL of methanol, and stored in dark at + 4 °C. Each samples were mixed and left in the dark for 90 min, than the absorbance at 560 nm was measured. A calibration curve using H₂O₂ as standard, range between 1 and 1000 μ M was prepared by adding 50 μ L of standard solution instead of lipid extract, 1 mL of methanol and 200 μ L of reagent, treated in the same way described for the samples.

Statistical Analysis

Statistical analysis was performed on the original data by Tukey test. The relations between variables were analysed using linear simple correlation. A significance level at $p < 0.05$ was considered.

RESULTS AND DISCUSSION

Apple antioxidants to prevent meat lipid oxidation during cooking

Lipid oxidation was measured by quantification of MDA and by TBA test. In Table 1 data obtained on microwave cooked beef meats treated with different concentration of apple antioxidant are shown. Results demonstrate that the oxidation levels is significantly higher in the control samples compared to those treated with apple antioxidants. The formation of MDA is also related to the antioxidant concentration: the higher the amount of catechin equivalent in the meat the lower the MDA formed after cooking.

In Figure 1 data of peroxides values (PV) are shown. No significant differences were found among control and meat added with 50 mg Kg⁻¹ CEq and 150 mg Kg⁻¹ CEq. While significantly lower ($p < 0.05$) PV were observed in sample treated with 450 mg Kg⁻¹ CEq.

Cooking induced a generalised increase in PV (Badiani, Stipa, Bitossi, Gatta, Vignola & Chizzolini, 2002), and the formation of hydroxyl radicals during meat cooking has been previously demonstrated (Kanner, 1994).

The whole set of data clearly suggests that the highest concentration of catechins (450 mg Kg⁻¹) gives the highest protection against lipid oxidation, however the concentration of TBARS is not proportional to the amount of added catechins as observed for MDA. It can be

hypothesized that oxidized catechins react with TBA thus increasing the unspecific background which is the well know problem associated with the TBARS determination (Guillér-Sans R. & Guzmán-Chozas, 1998)

The efficacy of catechins in the prevention of lipid oxidation was previously investigated by different studies. Tang et al. (2001) added to minced meat 300 mg kg⁻¹ of tea catechins and 300 mg kg⁻¹ α -tocopherol in aerosol form. They evaluated lipid oxidation after 10 days cold storage, finding also that catechins had better protective effect in comparison with α -tocopherol.

It was also suggested that catechin can chelate the heme iron and preventing the iron catalysed lipid oxidation (Record, McInerney & Dreost, 1996). Similarly Osada, Hoshina, Nakamura and Sugano (2000) reported that apple polyphenols inhibited linoleic acid oxidation in sausage; moreover they found that apple polyphenols suppressed cholesterol oxidation through their radical scavenging effects.

Commercial and SFE samples to inhibit lipid oxidation during storage

Samples obtained by SFE extraction together with rosemary and oregano based commercial antioxidants were investigated through the evaluation of antioxidant activity and total polyphenols. Results are shown in Table 2. There is a huge variation in the content of phenolic compounds, both in the commercial and in SFE extract samples. The antioxidant activity is somehow related to the concentration of phenolic compounds, however the range of variation of antioxidant activity among samples is narrower than that of phenolic compounds.

To perform the shelf life experiments on meat, four SFE extracts were selected considering those having high antioxidant activity (> 0.70 mmol equiv Trolox/100 mL) and also having high total polyphenols concentration (> 0.60 g gallic acid equivalent/100 mL). They were: anise, clove, parsley and sage. All the four commercial antioxidants were used.

To define the final concentration in the meat manufacture instructions, phenol concentration, antioxidant activity and sensorial consideration related to the pungent flavour of some samples were considered. Where possible the same final antioxidant activity was used in all samples (see Table 2, right column).

The results of the prevention of lipid oxidation in all meat samples at different storage times are summarised in Figure 2. As expected the oxidation levels, measured as MDA concentration, increased in all samples during storage. The highest lipid oxidation occurred in

the control (i.e. untreated meat). Generally, a slower rate of TBARS production in meat treated with SFE samples respect to the commercial preparations can be noted. Results show a marked difference ($p < 0.05$) for all samples between four and seven days of storage with an overall average increase of 56%. This result is in good agreement with a previous study on lipid oxidation during storage of raw beef meat (Tang et al., 2001) where an increase of 80% was reported between 3rd and 6th day of storage at 4°C. As in our study the increment of TBARS value was much lower at the beginning at the end of the storage time. This behaviour is likely related to an accelerated lipid oxidation caused by the break down of hydroperoxides (Chan & Coxon, 1987).

Meat treated with clove and parsley shown at each time the lowest value of MDA. Parsley prove to be particularly effective also for the long time storage. Meat treated with anise extract have MDA concentration significantly higher than parsley and clove. On the other hand sage and all commercial preparation are only slightly different from the control and at 14 days there are no differences at all.

Summarizing, among SFE extracts, considering the very low amount added to sample (0.02%) clove extract prove to be the most efficient. Among parsley, anise and sage, whose concentration in the meat was the same (0.6%), parsley treated meat definitively gives the lowest TBARS value.

Essential oil composition of clove, parsley, sage and anise, has been previously characterized by different studies as reported in Table 3. Total phenolic content and antioxidant activity of essential oil can be significantly different according genotypic and environmental differences within species (Srinivasan, 2005), choice of parts tested (Kumar, 1997), time of harvest (Gao, Xu & Li, 2000), determination methods (Pizzale, Bortolomeazzi, Vichi, Uberegger & Conte, 2002) and extraction procedure (Vuorela et al., 2005).

It has been reported that clove had a very strong antioxidant activity and eugenol, the main phenol compound of clove, has a very high ability in preventing lipid oxidation (Gulcin, Sat, Beydemir, Elmastas & Kufrevioglu, 2004; Lee & Shibamoto, 2001).

Ramanathan and Das (1993) studied ground and fresh spices to find efficacy in prevention of cooked ground fish lipid oxidation. The order of effectiveness for dried spices was cloves > cinnamon > cumin \geq black pepper \geq fennel.

Shan, Cai, Sun and Corke (2005) showed that clove bud extract was the most powerful phenolic antioxidant and exhibited the strongest radical scavenging activity among the 26 spices.

The various antioxidant mechanisms of clove bud extracts were attributed to a strong hydrogen-donating ability, a metal chelating ability, and their effectiveness as good scavengers of hydrogen peroxide, superoxide, and free radicals (Shan et al., 2005).

Teissedre and Waterhouse (2000) in a study on the antioxidant activity of essential oils put clove at the top of the rank of other 23 samples of essential oils. Moreover clove prove to be the more potent agent in inhibiting the copper-catalyzed oxidation of human-low-density lipoproteins (LDL) and the relative inhibition of LDL oxidation was higher for eugenol-rich clove varieties.

In earlier studies, sage and rosemary were shown to have similar patterns of phenolic compounds and their antioxidative activity have been attributed mainly to carnosic acid and rosmarinic acid (Cuvelier, Richard, & Berset, 1996; Persson et al., 2003).

Our results are in good agreement with those recently published by Vuorela et al (2005) who used rapeseed and pine bark phenols in inhibiting the oxidation of lipids and proteins in pork meat patties. They also found a maximum reduction of about 80% in lipid oxidation measures as hexanal production, moreover they found that control of lipid oxidation is associated to a reduction of protein oxidation measured as carbonyl compounds.

CONCLUSIONS

Many studies have investigated the ability of vegetable extracts in preventing lipid oxidation, but this information are not homogenous. No information on the efficacy of spice supercritical CO₂ extract in preventing meat lipid oxidation were available. To our knowledge this is the first work comparing the ability of products commercially used to prolong meat product shelf life and SFE extracts obtained by different vegetables and demonstrating the higher efficacy of the latter. The study points out the powerful efficacy of clove, but also parsley and the other SFE extract prove to be very effective in inhibiting lipid oxidation. Further studies are necessary to modulate the conditions of the SFE extraction to avoid the presence of flavour that might affect the organoleptic proprieties of processed meat. In any case we believe that there are very good market perspective for these product. In fact they will take advantage from i) the health benefits related to the consumption of this phenolic compounds ii) the use of the SFE technology which is perceived as natural and environmental friendly; iii) the efficacy which is higher than the commercial preparation present on the market.

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TABLES

Table 1. Lipid oxidation in microwave cooked meat mixed with apple antioxidants. MDA by chromatographic analysis (HPLC-FD) and TBARS by spectrophotometric analysis were measured. Values are mean \pm standard deviation; n=4. Letters show significance level at $p < 0.05$.

	[MDA] ($\mu\text{g} / \text{g fw}$)	TBARS ($\mu\text{g} / \text{g fw}$)
control	2.09 ± 0.08 ^(a)	2.85 ± 0.02 ^(a)
50 mg Kg^{-1} CEq	0.98 ± 0.11 ^(b)	2.81 ± 0.13 ^(a)
150 mg Kg^{-1} CEq	0.82 ± 0.07 ^(b, c)	2.63 ± 0.49 ^(a)
450 mg Kg^{-1} CEq.	0.77 ± 0.04 ^(c)	2.63 ± 0.08 ^(b)

Table 2. Total polyphenols and antioxidant activity of commercial preparations and SFE extracts added to meat. Data expressed as mmol of trolox equivalent/100 mL of fresh weight. Values are mean \pm standard deviation; n=3.

	Total phenols (<i>G</i> GALLIC ACID EQUIV/100 ML FW)	antioxidant activity (mmol trolox/100 mL)	% of sample added to meat balls
Commercial samples			
10	0.15 \pm 0.02	0.13 \pm 0.01	0.6
28	1.19 \pm 0.08	2.92 \pm 0.04	0.7
6	0.13 \pm 0.00	0.11 \pm 0.00	0.05
31	2.90 \pm 0.19	5.49 \pm 0.18	0.03
SFE samples			
tomato	0.19 \pm 0.11	0.44 \pm 0.02	-
anise	0.97 \pm 0.06	0.76 \pm 0.06	0.6
elder	0.06 \pm 0.12	0.03 \pm 0.00	-
grapefruit	0.11 \pm 0.38	0.03 \pm 0.00	-
sage	0.64 \pm 0.14	1.01 \pm 0.08	0.6
clove	36.47 \pm 3.45	84.54 \pm 3.31	0.02
peach	0.13 \pm 0.19	0.04 \pm 0.01	-
parsley	0.47 \pm 0.80	4.95 \pm 0.05	0.6
rosemary	0.44 \pm 1.11	0.70 \pm 0.02	-

Table 3. Essential oil and methanolic extract composition of clove, parsley, sage and anise. Reference for methanol extracts: Shan, Cai, Sun and Corke (2005).

<i>Sample</i>	<i>Essential oil composition</i>	<i>References</i>	<i>Major Phenolic Compounds of Methanolic Extracts</i>
Clove	eugenol (75.5%, mass), β -caryophyllene (12.1%, mass), eugenol acetate (11.0%, mass), and α -humulene (1.4%, mass)	Souza et al., (1992)	phenolic acids (gallic acid), flavonol glucosides, phenolic volatile oils
Parsley	1,3,8-p-menthatriene (68%), myristicin (60%), β -phellandrene (33%), apiol (22%), myrcene (16%), terpinolene and 1-methyl-4-isopropenylbenzene (13%)	Simon and Quinn (1988)	phenolic acids (caffeic acid), flavonoids, volatile oils
Sage	α -Thujone (55-18%) α -pinene (4.1-5.4%), camphene (6-7.1%), beta-pinene (9.3-14.5%), limonene (2-2.3%), 1,8-cineole (3.6-5.6%), (-)-thujone (13.2-16.1%), (+)-isothujone (6.6-7.4%), camphor (19.8-24%), α -humulene (5.1-6.8%), and manool (4.2-7.7%)	Santos-Gomes and Fernandes-Ferreira (2001)	phenolic acids (rosmarinic acid), phenolic diterpenes (carnosic acid), volatile oil
Anise	anethole (90%), γ -himachalene (2-4%), p-anisaldehyde (<1%), methylchavicol (0.9-1.5%), cis-pseudoisoeugenyl 2-methylbutyrate (3%), and trans-pseudoisoeugenyl 2-methylbutyrate (1.3%)	Rodrigues et al., (2003)	phenolic acids (protocatechuic acid), phenolic volatile oils (anethole), flavonoids

FIGURES

Figure 1. Peroxide concentration in microwave cooked meat mixed with different concentration of apple antioxidants. Results are expressed in ng H₂O₂ equivalents/g lipid extracted from sample. Values are mean ± standard deviation; n=3. Letters show significance level at *p* < 0.05.

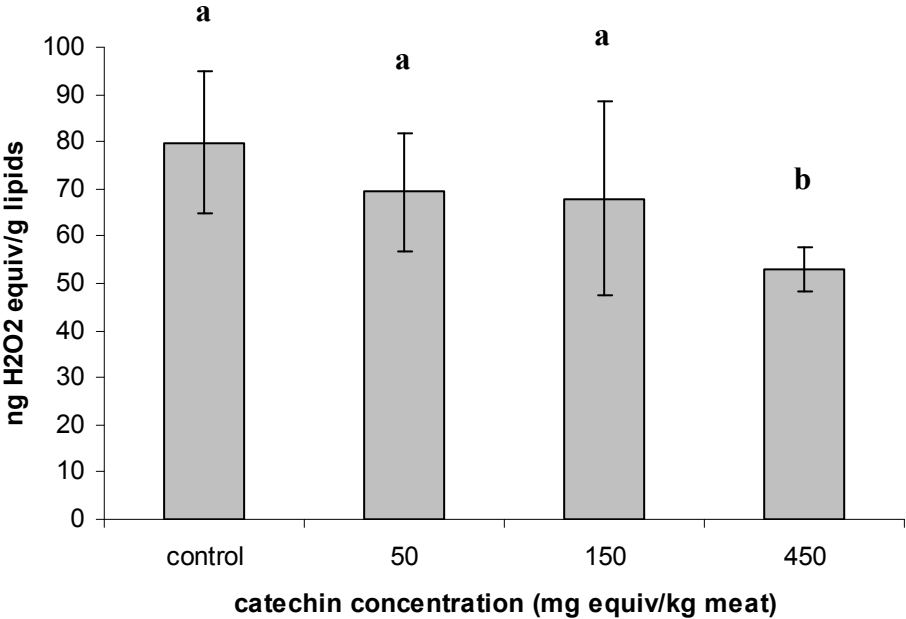
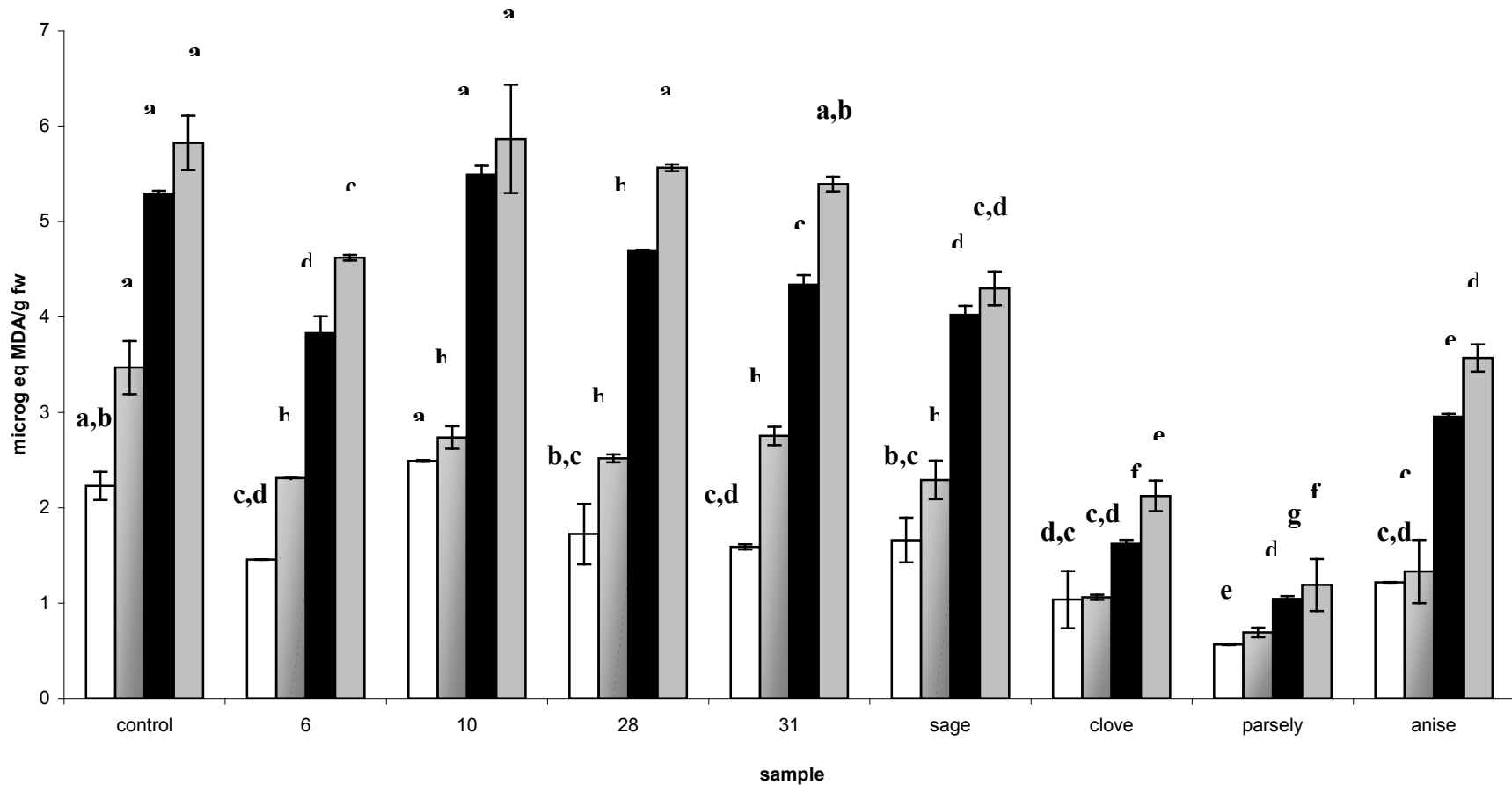


Figure 2. Effects of meat treatment with commercial antioxidants and SFE extracts from sage, clove, parsley, anise on the TBARS values at different storage times. White bar, T1; shaded bars, T4; Black bars, T9; Gray bars, T14. TBARS values are given as $\mu\text{g MDA g}^{-1}$ fresh weigh sample. Values are mean \pm standard deviation; $n=3$. Letters show significance level at $p < 0.05$ among antioxidants for each time.



CHAPTER II

Determination of MDA and TBARs in Buffalo Meat from Animals with dietary supplementation of Vitamin E

Abstract

Buffalo meat is not widely used for human consumption, but it is recently reconsidered thanks to its valuable nutritional qualities and the low cost of production. New strategies aiming to improve the quality of buffalo meat have to be applied particularly to face the problem of lipid peroxidation, one of the most important causes of meat food deterioration. The aim of this study is to evaluate the lipid oxidation of buffalo meat coming from animals fed with two different amount of vitamin E considering the concentration of malondialdehyde (MDA) as markers for lipid oxidation. Moreover it was evaluate tenderisation effect on lipid oxidation and results shown that this process led to 30% increase of the amount of MDA in the meat. Meat from buffalo fed with vitamin E had lower MDA and TBARS concentration in comparison with control. It is concluded that animal dietary supplementation with Vitamin E is a promising strategy to prevent lipid oxidation of buffalo meat.

Keywords: Buffalo meat, Lipid oxidation

INTRODUCTION

The use of buffalo meat is not still very common in Western Countries. In comparison with beef, buffalo meat is less fatty, with an higher percentage of unsaturated fatty acid and lower in cholesterol. These qualities are considered an important nutritional plus; for this reason and also for the low cost of production, buffalo meat consumption should be encouraged and new strategies aiming to improve the quality should be applied also to this product. First of all the problem of lipid peroxidation, one of the most important causes of meat food deterioration, should be face. The higher concentrations of Fe and unsaturated fatty acids in buffalo meat compared to bovine, are the main causes of the earlier qualitative deterioration. It is well known that antioxidants can extend the shelf-life of meat by reducing or preventing lipid peroxidation. Soon after death, auto-catalytic processes occur, such as lipid oxidation, that results in the production of free radicals that may be inactivated by Vitamin E. It is a lipid-soluble antioxidant able to breaks the chain of lipid peroxidation in cell membranes and prevents the formation of lipid hydroperoxides (*Halliwell, 1987; Davies, 1988*), for this reason it has been found to improve the quality of farm animal products. Feeding with vitamin E-supplemented diets reduced lipid peroxidation in turkey muscle (*Bartov, I et al., 1996, Bartov, I et al., 1983, Webb, J. E et al. 1973*), in chicken meat (*Marusich W. L et al., 1975; Galvin, K et al. 1997, Liu, C. F. et al., 1989*), in pork (*Buckley, D. J. et al., 1995*), in fish (*Frigg, M et al., 1990, Gatlin, D. M et al., 1992*), and in beef (*Lavelle, C. L. et al., 1995; Lanari, M. C. et al., 1994*), but there are not of similar researches for buffalo.

The aim of this research is to study the oxidation products of buffalo meat, monitoring the oxidation damage during tenderisation period and the efficacy of dietary vitamin E. Two different methods of MDA determination by HPLC were tested using fluorimetric and UV detection. Results were compared with TBA method.

Materials

Reagents

Trichloroacetic acid (TCA) was purposed from Carlo Erba; 1,1,3,3-tetraethoxypropane (TEP) (malondialdehyde source) and 2,4-dinitrophenylhydrazone (DNPH), butylated hydroxytoluene (BHT) were purposed from Sigma Aldrich. Sulphuric acid and HPLC

solvent were from Merck; 2-thiobarbituric acid (TBA) from Fluka. Milli-Q water was obtained with a Milli-Q Plus ultra-pure water system from Millipore (Milford, MA, USA).

Sampling

To evaluate tenderisation process on lipid oxidation, were taken samples of shoulder (*Caput longum tricipitis brachii*) (TF), loin (*Longissimus dorsi*) (LD) and buttock or rump (*Semimembranosus*) (SM) from buffalo (*Bubalus bubalis*) within 1 hr after slaughter (before tenderisation). Carcasses were stored for 4 days at 4°C, and second sampling (after tenderisation) was made.

To analyse effect of diet supplemented with vitamin E, the trial was performed on 12 buffalo calves that were 403 days old and had an average live weight of 333 kg. The animals were divided in 3 groups of 4 subjects: 1) 1500 I.U. of vitamin E/die; 2) 600 I.U. of vitamin E/die; 3) control. Buffaloes were controlled until the live weight for slaughter (426 kg) and received the same diet (mixed hay and concentrate 38%/62%) ad libitum. All buffalo calves were slaughtered between 102 and 123 days from the beginning of the trial. The carcasses were kept at 8°C for four days after slaughter and processed and cut. It was used same muscle samples previous cited. Meat was freeze dried, grounded and stored at -20 °C until using.

METHODS

TBA analysis

The method published by *Maraschiello C. et al*, (1999) was used. Briefly meat aliquot (0.5 g) was weighed, added of 10 mL of deionised water and samples were vigorously mixed (1 min). Then aliquot (2.5 mL) of 25 % TCA was added. The samples were stored for 15 min at 4 °C, then they were mixed and centrifuged (5 min, 4000 rpm, at 4 °C). Supernatant aliquots (3.5 mL) were added to 1.5 mL of 0.6 % TBA and incubated for 30 min at 70°C. After incubation, the absorbance at 532 nm was measured against a blank consisting of 2.5 mL of ultrapure H₂O, 1 mL 25% aqueous TCA, and 1.5 mL 0.6 % TBA.

MDA determination by HPLC-FD

For fluorometric detection of MDA, the method proposed by *Bergamo et al., 1998*, was used with some modifications.

Briefly 1 g of freeze dried meat was added with a solution of 4.75 mL of Milli-Q water and 0.25 mL of ethanolic BHT (1000 ppm final concentration). The samples were vigorously mixed and sonicated for 30 min. Samples were centrifuged at 4000 rpm for 5 min and 500 μ L of aqueous phase was taken and added to 500 μ L of ice-cold 10% TCA. Proteins were removed by centrifugation (5 min, 10000g); 300 μ L were taken from the supernatant and added to 700 μ L of TBA mix (solution of 0.4% TBA in 2 M acetate buffer at pH 3). The mixtures were incubated for 30 min at 90 °C. At the end of incubation, the samples were cooled, centrifuged (5 min, 10000g) to remove particulate material and, finally, sample aliquots (20 μ L) were analysed by HPLC.

The HPLC analysis was performed using a liquid chromatograph LC-10D VP, with RF-10Ax1 Fluorescence detector (Shimadzu, Kyoto, Japan). The MDA-TBA complex was fractionated on a Luna 3 μ C18 (2), 150mm x 4,60 mm (Phenomenex). The mobile phase was daily prepared and consisted of 2.5 mM sodium phosphate buffer (pH 7.0) and acetonitrile (85:15 v/v). The chromatographic fractionation was performed at 0.8 mL/min flow rate; excitation was $\lambda_{EX} = 515$ nm and emission $\lambda_{EM} = 543$ nm.

MDA peak identification was done in base of retention time of standard. MDA concentration is expressed in μ g MDA / g dw.

MDA determination by HPLC-UV

For UV detection of MDA, the method proposed by *Mateos et al., 2004*, with some modifications was used.

For sample extraction, 1 g of freeze dried buffalo meat were added with 5 mL of deionised water. The sample were centrifuged (5 min, 4000 rpm) and 1 mL of surnatant was moved into microcentrifuge tubes containing 700 μ L of ice-cold 5% TCA and 300 μ L of hexane. Solutions were centrifuged (5 min, 10000 rpm), hexane phase was eliminated. Surnatant was removed and 100 μ L of DNPH reagent (DNPH 5 mM in HCl 2 M) was added. Mixtures were incubated for 60 min at room temperature in the dark. At the end of time, the samples were cooled and 50 μ L were analysed by HPLC.

The HPLC analysis was performed on a liquid chromatograph LC-10D VP, at the diode array detector SPD-M10A VP and the system controller SCL-10A VP (Shimadzu, Kyoto, Japan). The MDA-DNPH complex was fractionated on a Spherclone C₁₈ column (4.6 x 250 mm) with a 5 µm particle size (Phenomenex, Milford, MA). The mobile phase was isocratic with a mixture of 0.2% (v/v) acetic acid in deionised water and acetonitrile (62:38, v/v) at a flow rate of 1 mL/min at room temperature. The detection was performed at a wavelength of 310 nm.

Statistical Analysis

Statistical analysis of data was performed on the original data by Tukey's test . The relations between variables were analyzed using linear simple correlation.

It was considered a significance level at $p < 0.05$.

RESULTS AND DISCUSSIONS

Effect of tenderization on lipid oxidation products

Meat is commercialised after a period of tenderisation, which is the process that leads to improvement in tenderness. Tenderisation occurs as the structural proteins degradation (i.e. proteolysis), moreover during this period lipolytic processes occur and all these phenomena influence sensorial proprieties of meat. These properties concern both consumer acceptance and technological aspects such as colour, water holding capacity and texture. Aim of this work was analyse effect of tenderisation on lipid oxidation.

Results of spectrophotometer and chromatographic analysis (with FD, chromatogram in Figure 1) are shown in Table 1.

After tenderisation there was a meaningful increase of MDA concentration, that is in according to proteolytic processes that start after animal death. In mean, before tenderisation MDA value (HPLC results), it's reported a mean increase of 31%. This results confirm previous study on buffalo meat oxidation reported by *Sahoo & Ajaneyulu (1997)*, which found that buffalo meat had a linear increase of TBARS from 0 to 10 day since slaughtering. These authors reports an increase of 32% after 4 days of storage at 4°C, considering 24 hours for tenderisation.

In Figure 2, it is possible to evaluate the correlation between results obtained by HPLC and spectrophotometer.

Correlation coefficient was in mean +83%.

In according to literature dates (*Guillén-Sans R. et al., 1998*), results with spectrophotometric test gave higher values than HPLC one, because of formation of other substances that are absorbed at same wavelength of TBA-MDA adduct.

Effect of dietary vitamin E supplementation on lipid oxidation products of the meat

MDA was determined by HPLC as its 2,4-dinitrophenylhydrazone derivative (MDA-DNPH). In **Figure 3** is shown typical HPLC chromatogram. Dates coming from both HPLC detector method (FD and UV), showed the same precision and reproducibility.

Correlation between oxidation analysis with spectrophotometer method and chromatographic method was in mean +93%.

Lipid oxidation for muscle samples, which coming from dietary Vitamin E supplementation, had a meaningfully lower value in comparison with control (**Table 2**).

It has been observed that there were not significant differences between the dose 600 IU/die and dose of 1500 IU/die when MDA values of TBA test were considered. Instead there were significant ($p < 0.05$) differences in the susceptibility to lipid oxidation among muscle evidenced by HPLC analysis.

For ideal concentration, Faustman et al. (1989) proposed 3 mg of α -tocopherol/g of fresh GM (*gluteus medius*), the minimum muscle α -tocopherol concentration that provides for near maximal suppression of lipid oxidation in fresh meat. Arnold et al. (1993a) analysed a larger data set and proposed critical concentrations for LL (*longissimus lumborum*) and GM of 3.3 and 3.8 mg/g of fresh muscle, respectively.

To achieve an ideal concentration of 3.5 mg/g, *Liu et al., 1995* recommended a supplementation strategy of 500 IU of supplemental vitamin E/steer daily for 126 d, whereas *Arnold et al. (1993b)* suggested 1.300 IU/ steer daily for 44 d.

Evaluating results of HPLC analysis, mean lowering in perceptual between control and 600 IU/die and 1500 IU/die of vitamin E supplementation were: 37% and 61%.

CONCLUSIONS

Results show that dietary supplementation with vitamin E decreases the lipid oxidation. In consideration of major susceptibility of buffalo meat, results of this study seems to agree a dose of 1500 IU/buffalo die for about 100 days.

Since one of the characteristics that is responsible for the low diffusion of buffalo meat is represented by its earlier (4-5 days after cutting) darkening compared to bovine meat, it is possible to suppose that vitamin E can prolonged the shelf-life of the meat, with a cost of the treatment with 1500 I.U. of vitamin E of 0.86 and 0.30 € respectively for kg of meat and of live weight.

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TABLES

Table 1. MDA concentration BEFORE and AFTER tenderisation. LD: *Longissimus dorsi*; TB: *Caput longum tricipitis brachii*; SM: *Semimembranosus*. MDA concentration in $\mu\text{g} / \text{g}$ fresh weight, evaluated by HPLC-FD and by TBA test. Values are mean \pm standard deviation; n=3. Letters show significance level at $p < 0.05$

sample	Before tenderisation		After tenderisation	
	HPLC-FD	TBA test	HPLC-FD	TBA test
LD	n.d.		0.62 ± 0.03	1.50 ± 0.13
TB	0.33 ± 0.06	1.33 ± 0.17	1.44 ± 0.12	2.43 ± 0.15
SM	0.21 ± 0.10	0.87 ± 0.21	0.50 ± 0.12	2.44 ± 0.57

Table 2. MDA concentration in animals fed with vitamin E supplemented diet. LD: *Longissimus dorsi*; TB: *Caput longum tricipitis brachii*; SM: *Semimembranosus*. MDA concentration in $\mu\text{g} / \text{g}$ fresh weight, evaluated by HPLC-UV and by TBA test. Values are mean \pm standard deviation; n = 4. Letters show significance level at $p < 0.05$

sample	TB		LD		SM	
	TBA test	HPLC-UV	TBA test	HPLC-UV	TBA test	HPLC-UV
control	0,615 \pm 0,048 ^(a)	0,399 \pm 0,009 ^(a)	0,875 \pm 0,184 ^(a)	0,308 \pm 0,024 ^(a)	0,675 \pm 0,023 ^(a)	0,716 \pm 0,028 ^(a)
600 I.U. of vitamin E/die	0,447 \pm 0,028 ^(b)	0,247 \pm 0,027 ^(b)	0,310 \pm 0,078 ^(b)	0,222 \pm 0,115 ^(a,b)	0,469 \pm 0,088 ^(b)	0,391 \pm 0,171 ^(b)
1500 I.U. of vitamin E/die	0,423 \pm 0,126 ^(b)	0,198 \pm 0,016 ^(c)	0,327 \pm 0,064 ^(b)	0,123 \pm 0,008 ^(b)	0,422 \pm 0,119 ^(b)	0,107 \pm 0,024 ^(c)

FIGURES

Figure 1. Sample meat chromatogram obtained by HPLC-FD. MDA-TBA adduct at $T_r = 24.467$

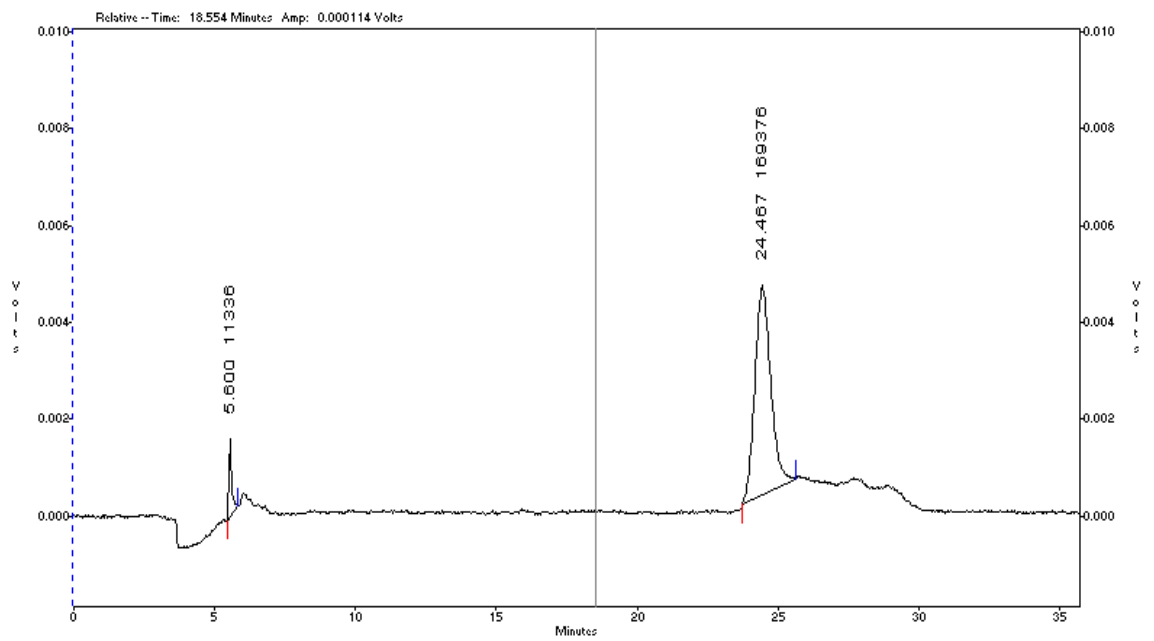


Figure 2. Correlation between oxidation analysis with spectrophotometric and chromatographic method (HPLC/FD). LD: *Longissimus dorsi*; TB: *Caput longum tricipitis brachii*; SM: *Semimembranosus*. (results after tenderisation). Values are mean \pm standard deviation; n=3.

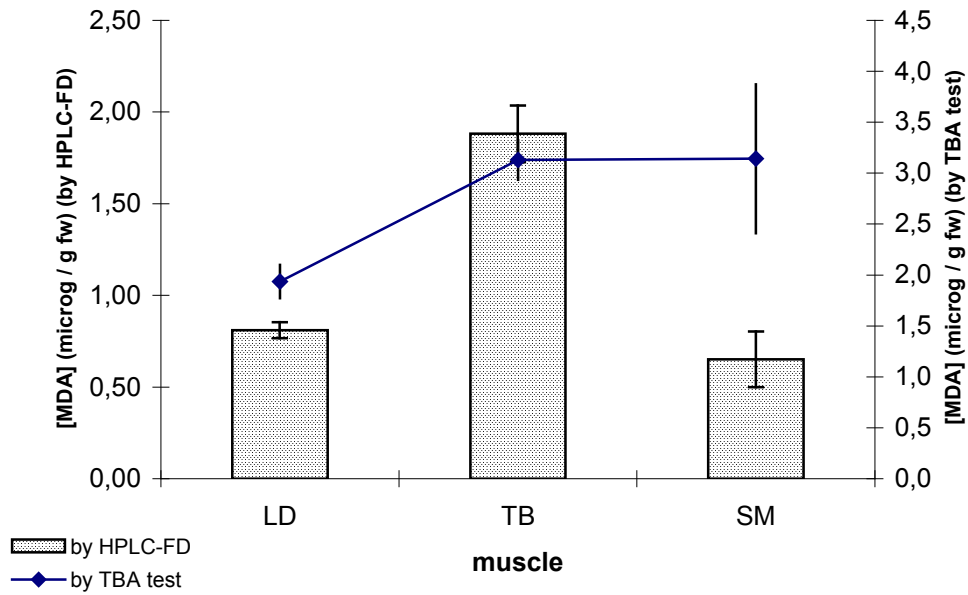
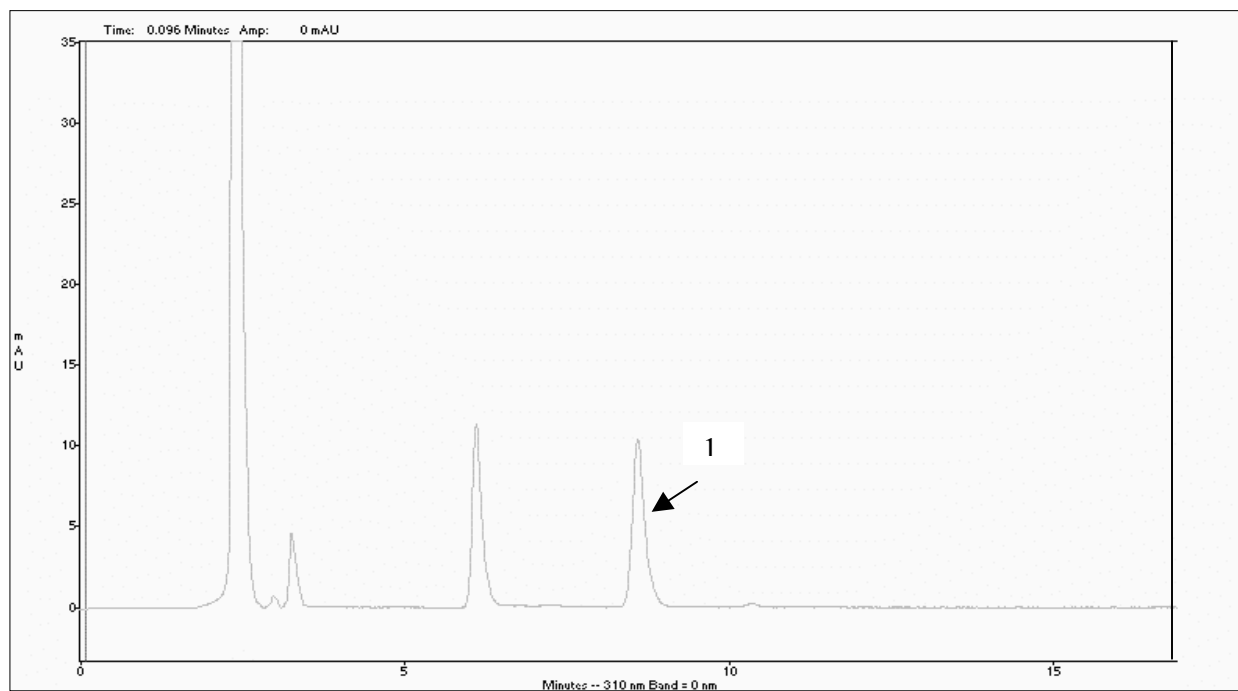


Figure 3. HPLC-UV chromatogram at 310 nm of standard solution. Peak 1 at 8.0 min corresponds to MDA - DNPH.



CHAPTER III

DEVELOPMENT OF ANALYTICAL PROCEDURES TO STUDY CHANGES IN THE MEAT PHOSPHOLIPIDS COMPOSITION CAUSED BY INDUCED OXIDATION

Abstract

Lipid peroxidation is an important deterioration problem in meat products. Aim of this study was the development of a model system and analytical procedures to study changes in meat lipid composition. For this purpose different techniques (liquid-, dry column-, accelerated solvent extraction) have been investigated to find a suitable lipid extraction system from cow meat and to induce lipid oxidation by using *tert*-butyl hydroperoxide, 2,2'-Azobis(2-methylpropionamide) dihydrochloride, Fe²⁺ and Cu²⁺ salts. Accelerated solvent extraction (ASE) gave results not significantly different from other extraction methods, but had the advantage to be a rapid and solvent saving procedure. Analytical techniques used to analyse lipid oxidation products included GC-FID, HPLC with Corona Charged Aerosol Detector (CAD), MDA determination and the spectrophotometric measurement of peroxide levels. The comparison among different extraction methods showed that liquid extraction gave the lowest raw weight of lipid extract, while not meaningful difference were between ASE and dry column extraction. Separation method of phospholipids fraction by silica column demonstrated to be able to elute and separate PL standards mixture. For oxidation induction was chosen method by AMPA to be fast and not producing artifact products. The oxidation gave changes correlated with an increase of PV, MDA and aldehydes while a decrease or disappearance of unsaturated fatty acid was observed. Results demonstrated that the model used in this work is useful for studying phospholipids oxidation in meat and the use of the innovative detector CAD has proved to be a good complementary technique in investigating lipid oxidation products.

Key words: lipid oxidation, phospholipids

INTRODUCTION

Lipid oxidation is a degradation chain reaction of polyunsaturated fatty acids, which are easy target of free radicals attack. It is one of main causes of quality deterioration in meat and several methods have been developed in order to measure its level. Traditionally, the extent of oxidation is determined by the peroxide value or by TBA test, but these methods are known to have questionable accuracy and the results vary with details of the procedure [1]. However, the assay of only one or few of the oxidised by-products (TBAR, MDA or hexanal, etc.), as it is usually done, will not give a view of the real oxidation level of the sample investigated.

This study hypothesizes that the quality and safety of the meat foodstuff can be estimated by using characteristic products of lipid peroxidation as marker compounds. Investigation on these compounds needs a model system to characterise the substances resulting from induced lipid oxidation in meat. For this reason aim of this study was the development of a model system to study changes in meat phospholipids components, considered the most susceptible to oxidation. During the work plan, particular attention was given to the following steps: extraction method of lipids; lipids separation; HPLC analysis of phospholipids (PL); method for inducing lipid oxidation.

In the assessment of lipid oxidation in foods, when the amount of lipids is small and/or they are strongly bound to non-lipid components, extraction procedure is a remarkable step. To minimize lipid alterations during the separation step, a compromise must be reached instead of striving for a full lipid extraction, using drastic conditions [2]. Usually, the extraction is performed with a mixture of polar and non polar solvents. Very popular are the procedures described by Folch [3] and Bligh & Dyer [4]. In addition, in literature are reported other methods, among these: accelerated solvent extraction (ASE) [5] and dry column extraction [6]. A study on oilseeds [5] shows that there are no significant differences of oil contents among the first two methods cited.

After extraction, since meat lipid extracts contain several different classes or groups of compounds, it is convenient to separate the individual lipid classes first, and then, after a preliminary fractionation has been carried out, to separate the components of the phospholipid group. Separation of lipids is usually performed by using silica gel column, but Kaluzny *et al.* [7] were among the first to apply SPE cartridges packed with aminopropyl bonded phases for separating groups of lipid classes [8]. In this study silica and aminopropyl bonded phases

columns were compared in order to evaluate the more suitable column. HPTLC analysis proved to be a valid support for qualitative and rapid determinations of compounds to be analysed.

During last years the evaporative light-scattering detector (ELSD) was the detector commonly used in lipid analysis, with the advantages of being not sensitive to the flow rate of solvent and to be able to quantify any solute less volatile than solvents.

When using ELSD, however, the sample has to be heated and the instrument has significant limitations in precision, sensitivity, dynamic range, and the linearity of calibration curves [9, 10, 11]. ELSD often generates very different responses for compounds of the same molecular weight. This means that it cannot accurately detect the quantities of the separated compounds. Dixon and Peterson (2002) [10] developed a new, more sensitive version of ELSD that overcame many of these problems. Gallagher (2005) [12] found that this measurement of quantity was independent of chemical structure, in that different compounds of the same molecular weight generated similar responses. In this new version, which the researchers termed aerosol charge detection (CAD), the aerosol particles are not detected by light scattering but instead are given an electrical charge by passing them close to a stream of charged nitrogen. The charged aerosol particles are then detected. The detection principle involves the charging of aerosol particles via corona discharge with subsequent electrometer-based measurement and thus it has some similarity with atmospheric-pressure chemical ionization (APCI) MS. However, CAD operates by detecting charged *particles* that have a selected range of mobility, rather than by measuring individual *gas-phase ions* that are differentiated on the basis of the m/z [13].

The extent of oxidation depends on the nature of the unsaturation, the strength of the oxidant and the length of the exposure. Lipid oxidation methods performed by using metal ions and radical initiators were compared. To investigate the mechanism of action, the oxidation was induced in individual standard of lipids and the samples were analysed for their oxidation products at different temperatures and time intervals.

Summarising, aims of the present work were: to test a suitable method of extraction, separation and assay of induced oxidation of phospholipids in cow meat samples; to study the oxidation products of the phospholipidic fractions obtained from cow meat.

MATERIALS AND METHODS

Samples

The cow's *longissimus dorsi*, was ground (Homogenizer 1094, Tecator, France) few hours after slaughtering and stored as meat balls (about 5-10 g) at $-70\text{ }^{\circ}\text{C}$ until use. A part of samples was freeze-dried (Freeze Dryer Modulyo, Edwards, England).

Reagents

L- α - Phosphatidylcholine (PC), L- α -phosphatidylethanolamine (PE), L- α -lisophosphatidylethanolamine (LPE), L- α -phosphatidyinositol (PI), L- α -phosphatidylserine (PS), L- α -lisophosphatidylcholine (LPC), sphingomyelin (SP), phosphatidic acid (PA), cerebrosides (CER) and cardiolipin (CARD) were purchased from Larodan, Malmö, Sweden.

Sodium methoxide 0.5 M in methanol, Iron (II) sulfate heptahydrate (FeSO_4), Celite 545, hydrogen peroxide 30%, N-n-dimethyl-p-phenylenediamine•2 HCl (DMPH) from FLUKA, Buchs, Switzerland.

Chlorogenic acid, caffeic acid, (+)-catechin, NaH_2PO_4 , butylated hydroxytoluene (BHT), (KCl), Na_2SO_4 , $\text{CaHPO}_4\cdot 2\text{H}_2\text{O}$, iodine, CuSO_4 , *tert*-butyl hydroperoxide (TBPH), EDTA, NaOH, 2-thiobarbituric acid (TBA), malondialdehyde tetrabutyl ammonium (MDA source), Trichloroacetic acids (TCA), 1- α -naphthol, primuline, ninhydrin, 2,2'-Azobis(2-methylpropionamide) dihydrochloride, (AMPA), 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma-Aldrich, Stockholm, Sweden.

Diatomaceous earth from Dionex (Leeds, United Kingdom); boron trifluoride in methanol (complex) (BF_3) from BDH, (Kebo Lab, Espoo, Finland). Trimyristin, tripalmitin, tristearin, triolein, trilinolein from Nu-Chek-Prep, Inc. (Elysian, MN, USA).

FAME standards came from Inc. (Elysian, MN, USA). They were methyl esters of follow fatty acids: tetradecanoic (myristic) (C14:0); pentadecanoic (C15:0); hexadecanoic (palmitic) (C16:0); heptadecanoic (margaric) (C17:0); 10-heptadecenoic (C17:1); 12-hydroxystearic (stearic) (C18:0); 9-octadecenoic (oleic) (C18:1); 9-12 octadecadienoic (linoleic) (C18:2); 9-12-15 octadecatrienoic (C18:3); 8-11-14 eicosatrienoic (homogamma linolenic) (C20:3); 5-8-11-14 eicosatrienoic (arachidonic) (C20:4); docosanoic (behenic) (C22:0); 7-10-13-16 docosatetraenoic (C22:4); tetracosanoic (lignoceric) (C24:0).

Aldehydes standards: butyraldehyde; valeraldehyde (pentanal); 1- heptaldehyde; decanal from Fluka, (Buchs, Switzerland); hexanal; trans-2-octenal; trans-2-nonenal from Aldrich-Chemie, (Steinheim, Germany). HPLC-grade solvents were purchased from Merck, Darmstadt, Germany, otherwise is specified. Milli-Q water was obtained with a Milli-Q Plus ultra-pure water system from Millipore (Milford, MA, USA).

Extraction method

The amount of sample used was 5 g for fresh and 2.5 g for freeze-dried meat. All extracts from different extraction method were evaporated under nitrogen at 21°C (Reacti-Therm heating module, PIERCE, Rockford, IL., USA) until constant weight; the evaporation of water for fresh samples was accelerated by adding acetone.

Liquid extraction

It has been used liquid method extraction according to Bligh–Dyer [4], with some modifications suggested by [2] and [17].

Samples were homogenized for 2 min in 50 mL methanol by Ultra-Turrax (Ultraturrax T25 BASIC, Ika Labortechnik, Helsinki, Finland); 100 mL chloroform were added and again homogenized, then shaken on a mechanical shaker for 30 min in the dark and at 4°C. The mixture was then filtered and the residue re-suspended in chloroform-methanol (2:1 v/v) and homogenized again. After 30 min of extraction shaking in the dark and at 4°C, the combined filtrates were transferred to a graduated cylinder and were added with one fourth of the total volume of 0.88% KCl. The upper aqueous layer was drawn off; one fourth of the volume of the lower layer of chloroform-methanol-saline solution (1:1, v/v) was washed again with saline solution. The bottom layer containing the purified lipid was filtered and the solvent removed by a rotary film evaporator. The lipid extract was redissolved in dichloromethane and dried under nitrogen until constant weight, at 21°C (Reacti-Therm heating module, PIERCE, Rockford, IL., USA).

Dry-column extraction

A dry-column extraction method according to Maxwell et al., 1980 [6] was used. The method is simple, rapid and solvents saving.

Accelerated solvent extraction

Lipids extraction was performed on fresh and freeze-dried meat samples with accelerated solvent extraction (ASE) according to [18] with some modifications. The accelerated solvent

extraction (ASE) instrument was an ASE Dionex (Oriola Oy Prolab, Espoo, Finland). Samples were supplemented with the same amount of diatomaceous earth and manually mixed in mortar. A cellulose filter was placed on the bottom of the extraction tube, covered with the sample mixed with glass balls. The solvent was dichloromethane/methanol (9:1 v:v) and the extraction programme was T= 100 °C, pressure 1500 psi, 2 cycles of 5 minutes, purge 60% with 22 mL collector tube, final time to fill tube containing extract time 90 sec.

Separation methods

The methods by M.A. Kaluzny, et al., [7] and Avalli and Contarini [19] were used for lipid separation.

According to method [7] it was used an aminopropyl column (Bond Elut 500 mg NH₂, Varian), while the method of Avalli and Contarini, [19] reports the use of silica gel bonded column (Supelclean LC-SI, 6-ml volume, 1 g sorbents, Supelco). The amounts of lipids loaded into the column were: 10 mg of lipid in 0.5 mL of chloroform for the aminopropyl and 200 mg of lipids in 0.5 mL chloroform/methanol 2:1 for silica column, respectively.

HPTLC method

HPTLC analysis were performed by HPTLC plates 10x10 cm with Silica gel 60 F₂₅₄ with concentrating zone 10x2.5 cm (Merck, Darmstadt, Germany).

The following mobile phases were tested: chloroform-methanol-water (65:25:5 v/v/v) [20]; chloroform-methanol- ammonia (65:25:5 v/v/v) [21]; chloroform-acetone-methanol- acetic acid (6:8:2:1 v/v/v/v) [21]. For lipids detection it was used primuline spray solution (5 mg in 100 mL acetone/water 80:20, v/v) (detection at wavelength of 366 nm) or iodine vapours [22].

FAME preparation

The following methods were tested: hydrolysis with NaOH 1N; hydrolysis with NaOH 2N; esterification with BF₃ in MeOH; esterification with a mixture of BF₃/MeOH and benzene; Transesterification with sodium methoxide.

Hydrolysis and esterification

A standard mixture of homotriacylglycerol (trimyristin, tripalmitin, tristearin, triolein, trilinolein), and a meat extract sample of about 5 mg, was used in the tests for identifying the most suitable hydrolysis and esterification method. The samples were treated according to [23] with modifications. They were hydrolysed by adding 0.5 mL of NaOH 1N (or 2N) in

benzene/methanol (4:6 v/v) and 2 drops of phenolphthalein as indicator. After being flushed with nitrogen and mixed by vortex they were heated at 100°C for 30 min. The samples were cooled, acidified by adding 0.5 mL of HCl 1N (or 2N) and 1 mL benzene, mixed and centrifuged at 4000 rpm for 5 min at +10°C. The upper phase containing the hydrolyzed fatty acids was transferred into a vial, added with 1 mL BF₃ in methanol or with 1 mL of a mixture of BF₃, benzene, methanol (5:4:11, v/v/v). The samples were flushed with nitrogen and mixed, left 30 min at 100°C for esterification. FAME were extracted by adding 1 mL of water and 1 mL of hexane and centrifuged at 4000 rpm for 5 min at +10°C. The upper phase was transferred to another tube, dried with nitrogen as above described, re-dissolved in 100 µL of hexane and 2 µL of the sample were injected into the GC-FID. When necessary, the sample had to be diluted ten fold with hexane.

Transesterification

The method of Seppänen-Laakso et al., 1990 [24], was used. Briefly, 5 mg of total lipid extract were dissolved in 0.5 mL of petrol ether and added with 0.5 mL of 0.5 M sodium methoxide in methanol. After reacting 5 min at 40°C, sample was supplemented with NaHPO₄ 15% (about 2 mL) until pH 7 (control pH value). FAME were extracted with 1 mL of petrol ether then centrifuged at 4000 rpm for 3 min; the upper phase was dried and re-dissolved in 25 µL of hexane. 2 µL were injected into the GC-FID.

GC-FID conditions. The analyses were carried out on a HP 5890A Series II GC, using Empower software. The GC was equipped with a fused silica column, length 60 m, ID 0.25 mm, coating phase 0.25 µm (INNOWAX Agilent, USA). Ahead the column of 60 m, was placed a column of 30 m, ID 0.32 mm, coating phase 0.5 µm (INNOWAX Agilent, USA). The oven was programmed from 70°C to 175°C at a rate 6°C/min, from 175 °C to 240°C at a rate 4°C/min, and left at this temperature for 30 min. Carrier gas Helium (1.5 mL/min), make up gas was nitrogen (33 mL/min), split ratio: 1:25, flame ionization detector (FID) temperature 300°C, injector temperature 260 °C. Identification of FAME was based on the retention time of reference standards and their identity was confirmed by GC-MS analysis (HP5971 mass selective detector).

HPLC analysis of phospholipids

HPLC-CAD analysis was carried out by using a HPLC Waters instrument (Manchester, England) equipped with 515 & 510 pumps, an Employer gradient system, and a Water 717

with autosampler. The analytical column (250mm × 4.6mm I.D., 5 µm) was a silica normal-phase Zorbax Rx-SIL (Agilent-Technologies, Espoo, Finland). Chromatographic separation was carried out by using a linear binary gradient according to the following programme: t_0 min: 0%B, t_{12} 30%B, t_{25} 70%B and finally isocratic conditions (100%B) for 4 min. Total chromatographic run time was 40 min per sample, which consisted of a 25 min analysis, 10 min to restore initial conditions and 5 min for re-equilibration. Eluent A consisted of dichloromethane–methanol–ammonium hydroxide (75:24:0.5, v/v/v) and eluent B of dichloromethane–methanol–ammonium hydroxide–water (43:43:1:13, v/v/v) with a flow rate of the eluent of 1.0 mL/min. A Corona Aerosol Detector (CAD) (ESA Inc., Chelmsford, Massachusetts, USA) was used. The sensitivity was 200 pA and the column temperature was 30 °C.

Total phospholipids were calculated by adding up PI, PC and PE amounts, and reported as µg/total phospholipid fraction (weighted after SPE).

Aldehydes analysis

The method proposed by [25] was used but modifications were adopted. 1 g of 2,4-DNPH reagent was weighted into a 500 ml flask, added with about 400 mL of distilled water, 5 mL 37% HCl and 5 ml 85% orthophosphoric acid and brought to volume with water. The suspension was sonicated after each preparation and before using it. The reagent may be stored at 4 °C in the dark for 1 month. For derivatisation of standard mixture, 1 mL of standard solution was used. The mixture consisted of: butanal, pentanal, hexanal, heptanal, trans-2-octenal, trans-2-nonenal and decanal, with a concentration of 400 µg/mL in ethanol and could be stored in the dark at 4°C for 1 month. A working solution with a concentration of 4 µg/mL may be stored in the dark at 4°C for 1 week.

10 mL of 2,4-DNPH reagent were added to sample extract (see paragraph “oxidation methods”) or to 1 mL of standard solution, and mixed for 30 min at room temperature in the dark.

10 ml of hexane were added, the sample was shaken for 15 min and the hexane phase was transferred into a new vial. This step was replicated twice. The formed dinitrophenylhydrazones were purified with 5+5 mL water by mixing with vortex.

2 g of anhydrous sodium sulphate were added and the mixture was left 15 min in the dark. The extract was passed through a Silica column (Bond Elut 500 mg Si, Varian, Palo Alto, Ca, USA). The column was conditioned with 2 mL n-hexane, the sample was passed through and

the aldehydes were eluted with 2 mL of 50% acetonitrile, recovering the eluent in 5 mL measuring test tube. The residual hexane was evaporated with nitrogen, as above mentioned, and the extract was filtered with 0.45 μm filter.

The elution program was made with water and acetonitrile. The gradient began at 60% and ended at 90% acetonitrile in 10 min. The chromatographic conditions were: flow rate, 1.0 mL/min; column temperature, 40°C; injection volume, 50 μL ; wavelength of detection, 360 nm. The HPLC was Agilent 1100 Series, the column Symmetry C18 3.5 μm , 150 x 2.1 mm (Waters).

The calibration curves were made diluting working solution from 1/100 until 1/10000. The linearity of the method was tested by derivatising the standard solution at different concentrations. The linearity range was found to be within the concentration of 0.02 and 4.8 $\mu\text{g/mL}$, where the correlation coefficients of linear regression curves for each analyte were >0.99 . The linearity of butanal, pentanal, hexanal, heptanal and octanal was, however, good up to 20 $\mu\text{g/mL}$ with the same correlation coefficient. The limit of detection for individual aldehydes was found to be in the range of 5-37 ng/mL, when injecting a standard solution.

MDA analysis

The method proposed by Bergamo et al., 1998 [26], was used with modifications. About 1 mg of meat extract PLs were added to 0.235 mL of Milli-Q water and 0.015 mL of BHT (1 g/L) in ethanol, with 0.250 mL TCA 10% and 1 mL of solution of 0.4% TBA in 2 M acetate buffer at pH 3 (TBA mix). The mixture was incubated for 30 min at 90°C. After incubation, the samples were cooled and filtered by Millipore HV filter to remove particulate material and finally, sample aliquots (20 μL) were injected onto the HPLC system. The HPLC analysis was performed using a Waters 290-Alliance; detector Waters 474 scanning fluorescence (excitation $\lambda_{\text{EX}} = 515$ nm and emission $\lambda_{\text{EM}} = 543$ nm). The MDA-TBA complex was separated on a Symmetry C18 3.5 μm , 150 x 2.1 mm (Waters) column. The mobile phase consisted of 5 mM sodium phosphate buffer (pH 7.0) and acetonitrile (85:15 v/v). Sample aliquots of 20 μL were injected, and the chromatographic analysis was performed at 0.3 mL/min flow rate. The MDA concentration was calculated on the basis of the calibration curve. The MDA standard solutions were prepared according to Mateos et al. [27].

Peroxides analysis

The method of Karppanen et al., 1989 [28] was used, with some modifications. About 5 mg of meat extract PLs was dried, added with 1 mL of methanol and 200 μ L of DMPH reagent. This reagent was prepared daily by dissolving 250 mg of DMPH in 0.15 mL of acetic acid, 21 mL of methanol, and stored in the dark and at + 4 °C. Each sample was mixed and left in the dark for 60 and 90 min and then compared with a blank solution at 520 and 560 nm. The values were compared with those of a calibration curve made with standard solutions of H₂O₂. Calibration curve was prepared with 50 μ L of standard solution at concentration from 12.5 μ M to 250 μ M, 1 mL of methanol and 200 μ L of DMPH reagent, treated in the same way as the sample and prepared at the same time.

Oxidation methods

FAMES oxidation

In order to use the most suitable oxidation procedure, some oxidation methods were tested [29, 30, 31, 32] and modified.

These tests were carried out by using a standard mixture of FAMES. It was composed by methyl esters of following fatty acids: C14:0; C15:0; C16:0; C17:0; C17:1; C18:0; C18:1; C18:2; C18:3; C20:3; C20:4; C22:0; C22:4; C24:0.

The concentration of standard FAMES was 20 μ g /mL in hexane. 200 μ L of mixture was put in a tube, and the solvent was evaporated under nitrogen. For each A, B, C, D methods, 4 replicates were left for 48 hours at room temperature with shaking. Sample was added with 1 mL methanol .

Oxidation Method A. Add the sample with 10 mL CuSO₄ 33 μ M and 1.7 mL of H₂O₂ 30%

Oxidation Method B. Add the sample with 10 mL CuSO₄ 33 μ M

Oxidation Method C. Add the sample with 5 mL FeSO₄ and 5 mL ascorbic acid 20 mM

Oxidation Method D. Add the sample with 10 mL FeSO₄ 10 μ M and 1 mL TBPH 50% in water

Oxidation Method E. Add 10 mL of AMPA 10 mM dissolved in water. Reaction at 40°C, the oxidation was stopped at 3 hours.

100 µL EDTA 2% in water and 10 µL BHT 2% in methanol, were used for stopping the oxidation induced by metals, while 10 µL of BHT 2% was used in the method using AMPA. A blank sample was prepared for every test.

Comparison among induced lipid oxidation method was made by GC analysis of the FAME.

Phospholipids oxidation

For PL samples was used “Oxidation Method E”. 10 mg of meat extract phospholipids were used. The oxidation process was stopped after 1,5 and 3 hours and the samples were extracted with 5+5 mL of chloroform/methanol (2:1, v/v), divided in equal amount for FAME, MDA and peroxides evaluations and evaporated under nitrogen.

For aldehydes analysis, 10 mg of meat extract phospholipids were used for oxidation. The samples were extracted with 5+5 mL of chloroform/methanol (2:1, v/v) and analysed right away for the presence of aldehydes.

Statistical analysis

Statistical analysis of data was performed on the original data by Tukey’s test . It was considered a significance level at $p < 0.05$. The relations between variables were analysed using linear simple correlation.

RESULTS AND DISCUSSION

Extraction method

The optimal conditions were optimised for the determination of the fat content starting from methods reported in literature, with the aim to extract the highest percentage of fat from the sample, in the same time avoiding as much as possible oxidation. The raw weight of the extracts obtained was compared in order to select the best quantitative extraction method. The results are shown in [Figure 1](#). They are the average values of triplicate measurements. Fat content obtained from liquid extraction resulted to be significantly different in comparison with dry extraction and ASE. While there was no real difference between the results found with these two methods, both had values significantly ($p < 0.05$) higher than the first one. The extraction with ASE is more convenient because it is rapid, solvent saving and, in the same time, it protects sample from oxidation occurring under nitrogen.

The extraction of freeze-dried samples avoids getting too much meat pigments and the sample is easier to dry. Furthermore HPTLC analysis and HPLC analysis showed a more efficient extraction from freeze-dried sample for phospholipids fraction (data not shown).

Lipid separation

Fundamental differences were found between the use of SPE by aminopropyl and silica columns. A mixture of three standards (PI, PS, PC) was tested. The use of SPE with aminopropyl bonded phases, according to the method of Kaluzny did not elute L- α -phosphatidylinositol and L- α -phosphatidylethanolamine.

The results of chromatogram and HPTLC analyses are shown in [Figure 2](#) and [Figure 3](#).

The separation of 200 mg of meat extract lipids according to [19], gave an amount of phospholipids of $14.5 \text{ mg} \pm 3.8$ ($n=20$).

Thin – layer chromatography (TLC)

For our aims, primuline proved to be a better TLC detection reagent than iodine.

In the chromatographic analyses of phospholipids different mobile phases were tested: chloroform-methanol-water phase and chloroform-methanol-ammonia. The mobile phase with ammonia shown a better separation, therefore it was used in our tests.

HPLC analysis of phospholipids

In [Figure 4](#) is shown a chromatogram obtained from PL standard mixture.

Baseline was as high as water concentration during elution gradient, therefore CAD response was dependent upon eluent composition, this agrees with [13].

The values obtained for the limit of detection (LOD) and the limit of quantification (LOQ), based on the standard curve, are shown in [Table 1](#).

In meat samples were found: phosphatidylinositol, phosphatidylethanolamine and phosphatidylcholine (concentration of injected sample 2 mg/mL) ([Figure 5](#)).

The concentration of phospholipids in the lipid extract, calculated as sum of three monitored peaks, was $0.98\% \pm 0.17$ (w/w \pm std dev), calculated on 10 different separation replicates.

FAME preparation

Several methods are available for preparation of FAME for analysis by gas chromatography, but reliable results may be obtained if is chosen the one most suitable for the matrices to be analysed. Hydrolysis with NaOH at different concentrations and esterification with BF_3 in methanol, under different conditions to prepare FAME from standards mixture and lipid sample extracts did not give significant differences. Trans-esterification reaction of lipid samples indicate that part of fatty acids was missing probably the time of reaction was not enough long to allow hydrolysis and esterification of analysed compounds.

Oxidation

To choose the oxidation method it was considered the fatty acid profile at different oxidation times.

Each evaluated oxidation method required a blank sample which didn't give any peak in the GC analysis. The recovery of the lipid standard mixture was calculated by mixing it with the oxidation reagent and extracting it as previously described. Mean of recovery percent for every FAME was 89.7 ± 16.0 (w/w \pm std dev).

Oxidation method with AMPA resulted to be the most appropriate for our aim. In fact, it was fast and gave a chromatogram without by-products of secondary reactions. Furthermore, the method with TBHP induced a more intense oxidation, but in the chromatogram ([Figure 6](#)) appeared artefact peaks.

In [Figure 7](#) is shown the chromatogram of oxidation method B, which is similar to the chromatogram of the oxidation methods using only metal ions. The comparison between oxidation by AMPA and that by TBHP showed a similar difference, from the control, in fatty acids composition at time 0 and final time but for AMPA, the reaction was completed in 3 h, while TBHP needed 48 hours.

To evaluate the pH effect on AMPA's reaction, two different pH were tested: phosphate buffer 10 mM at pH 5.5 and pH 7.0. At pH 7.0 the oxidation of a fatty acid standard mixture (C14:0; C15:0; C16:0; C17:0; C17:1; C18:0; C18:1; C18:2; C18:3; C20:3; C20:4; C22:0; C22:4; C24:0), was more effective.

It was studied the induced oxidation with AMPA on a standard mixture of PLs to select time reactions which give significant differences in fatty acids composition, aldehydes, peroxides and MDA in the period of 6 hours. Investigated times were: time 0; 45 min; 90 min; 135 min; 3 h and 6 h. Significant differences ($p < 0.05$) were found at time 90 min and at 3 h, therefore phospholipids samples were analysed at these 3 times, plus time 0, and the oxidation with AMPA was induced at these times.

The phospholipids amount got by HPLC-CAD for samples treated at different oxidation times are shown in [Table 2](#). Although there was a trend to decrease, significant differences in the phospholipids amounts were not found.

The fatty acids profile obtained from the phospholipids treated with AMPA was evaluated considering the following fatty acids methyl esters: C14:0; C15:0; C16:0; C17:0; C17:1; C18:0; C18:1; C18:2; C18:3; C20:3; C20:4; C22:0; C22:4; C24:0.

Three hours after oxidation by AMPA, the following FAME were not found in the sample: C18:3; C20:3; C20:4; C22:0; C22:4; C24:0, while were detected C14:0 and C15:0, which were not presented at time 0.

The profile of the aldehydes generated during the FAMES oxidation is shown in [Table 3](#). The samples were analysed for the presence of the following aldehydes: butyraldehyde; valeraldehyde (pentanal); hexanal and 1-heptaldehyde. A significant increase ($p < 0.05$) of butyraldehyde and valeraldehyde was found after three hours of oxidation, while at 90 minutes it was detected hexanal, significantly lower in comparison with 180 minute of oxidation and 1-heptaldehyde was present only after 180 minutes.

The effect of the oxidation induced by AMPA on PLs, measured by MDA concentration is shown in [Figure 8](#). Increasing the oxidation time increased the MDA concentration in the sample, but significant differences were found when the reaction was carried on longer than 90 and 180 minutes.

Induced oxidation in PLs caused an increase of peroxides value at 90 and 180 min. The results are reported in [Figure 9](#).

The correlation calculated by r-value, among considered variables, is shown in [Table 4](#). It was found that an increase in aldehydes, MDA and peroxides corresponds to a decrease in phospholipids concentration. Moreover, there was an inverse correlation between C18:3, C20:3, C20:4, C22:0, C24:0 and C14:0, C15:0 ([Table 5](#)). These fatty acids resulted to have a high positive correlation ($r > 0.95$) with aldehydes.

CONCLUSIONS

In the work to produce high-quality food products, some emphasis is also needed on the development of fast and reliable methods for the measurement of early lipid oxidation. A method that can detect low level of lipid oxidation products and predict the development of unpleasant sensory attributes would a valuable tool for research as well as quality control purposes.

A chemical analytical method capable of a satisfactory evaluation of the level of lipid oxidation in meat-based products can be achieved by using the proposed model system to characterise the substances resulting from induced lipid peroxidation in meat.

The results of this study will provide the criteria to be used when evaluating the quality of meat-based foodstuffs, concerning lipid oxidation levels, which will contribute to improve the consumer safety. Moreover during these study an innovative detector has been studied, testing its efficacy for phospholipids analysis.

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TABLES

Table 1. The limit of detection (LOD) and the limit of quantification (LOQ), based on phospholipids standard curve obtained by HPLC-CAD. Results are expressed in $\mu\text{g/mL}$ solution. Cerebrosides (CER); cardiolipin (CARD); L- α -phosphatidyinositol (PI); L- α -phosphatidylethanolamine (PE); L- α -phosphatidylserine (PS); L- α -lisophosphatidylethanolamine (LPE); phosphatidic acid (PA); L- α - Phosphatidylcholine (PC), sphingomyelin (SP); L- α -lisophosphatidylcholine (LPC).

Phospholipids	LOD $\mu\text{g/mL}$	LOQ $\mu\text{g/mL}$
CER	7.85	23.55
PI	10.35	31.35
PE	13.12	39.37
SP	15.75	47.25
PS	12.80	38.40
PC	13.95	41.85
LPC	17.55	52.65

Table 2. Phospholipids (PL) amount obtained by HPLC-CAD. Results are expressed in $\mu\text{g}/\text{mg}$ lipid. Values are mean \pm standard deviation; $n = 4$; (a) = level of significance at $p < 0.05$.

time of oxidation	PL
0	1,02 \pm 0,03 (a)
90 min	0,92 \pm 0,10 (a)
180 min	0,86 \pm 0,08 (a)

Table 3. Assay of aldehydes generated by PLs oxidation induced by AMPA. C4: butyraldehyde; C5: valeraldehyde (pentanal); C6: hexanal; C7: 1-heptaldehyde. Values are mean \pm standard deviation; n = 4. The letters show significance level at $p < 0.05$.

	C4 (ng/mg PL)	C5 (ng/mg PL)	C6 (ng/mg PL)	C7 (ng/mg PL)
time 0	143,1 \pm 23,0 (b)	41,4 \pm 15,9 (b)	(c)	(b)
90 min	158,6 \pm 41,8 (b)	36,4 \pm 18,1 (b)	19,7 \pm 9,1 (b)	(b)
180 min	465,8 \pm 19,5 (a)	126,7 \pm 38,4 (a)	35,4 \pm 6,9 (a)	57,2 \pm 12,0 (a)

Table 4. Correlation Coefficient (r-value) of fatty acids, phospholipids (PL), aldehydes (ALD), MDA, peroxides (PER), generated by phospholipids treated with AMPA for 3 hours.

	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	C18:3	C20:3	C20:4	C22:0	C24:0
PL	-0,68	-0,74	-0,41	-0,17	-0,71	0,71	-0,36	-0,24	0,34	0,68	0,67	0,68	0,68	0,67
ALD	0,99	0,95	0,90	0,73	0,95	-0,53	0,87	0,78	-0,13	-0,52	-0,52	-0,52	-0,52	-0,52
MDA	0,68	0,87	0,59	0,19	0,88	-0,92	0,51	0,29	-0,76	-0,91	-0,91	-0,91	-0,91	-0,91
PER	0,67	0,88	0,54	0,16	0,87	-0,92	0,45	0,25	-0,67	-0,92	-0,92	-0,92	-0,92	-0,91

Table 5. Correlation Coefficient (r-value) of fatty acids, obtained from phospholipids treated with AMPA for 3 hours.

	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	c18:2	C18:3	C20:3	C20:4	C22:0	C24:0
C14:0	1	0,93	0,79	0,47	0,92	-0,53	0,77	0,55	-0,25	-0,53	-0,53	-0,53	-0,53	-0,53
C15:0	0,93	1	0,76	0,39	0,99	-0,73	0,70	0,47	-0,38	-0,75	-0,75	-0,75	-0,75	-0,75
C16:0	0,79	0,76	1	0,87	0,81	-0,23	1,00	0,92	0,03	-0,20	-0,20	-0,20	-0,20	-0,20
C16:1	0,47	0,39	0,87	1	0,45	0,21	0,90	0,99	0,47	0,26	0,26	0,26	0,26	0,26
C17:0	0,92	0,99	0,81	0,45	1	-0,73	0,76	0,54	-0,40	-0,71	-0,71	-0,71	-0,71	-0,71
C17:1	-0,53	-0,73	-0,23	0,21	-0,73	1	-0,15	0,12	0,82	0,94	0,94	0,94	0,94	0,93
C18:0	0,77	0,70	1,00	0,90	0,76	-0,15	1	0,94	0,09	-0,11	-0,11	-0,12	-0,12	-0,11
C18:1	0,55	0,47	0,92	0,99	0,54	0,12	0,94	1	0,38	0,17	0,17	0,16	0,16	0,17
c18:2	-0,25	-0,38	0,03	0,47	-0,40	0,82	0,09	0,38	1	0,73	0,73	0,74	0,74	0,73
C18:3	-0,53	-0,75	-0,20	0,26	-0,71	0,94	-0,11	0,17	0,73	1	1,00	1,00	1,00	1,00
C20:3	-0,53	-0,75	-0,20	0,26	-0,71	0,94	-0,11	0,17	0,73	1,00	1	1,00	1,00	1,00
C20:4	-0,53	-0,75	-0,20	0,26	-0,71	0,94	-0,12	0,16	0,74	1,00	1,00	1	1,00	1,00
C22:0	-0,53	-0,75	-0,20	0,26	-0,71	0,94	-0,12	0,16	0,74	1,00	1,00	1,00	1	1,00
C24:0	-0,53	-0,75	-0,20	0,26	-0,71	0,93	-0,11	0,17	0,73	1,00	1,00	1,00	1,00	1

FIGURES

Figure 1. Comparison among different lipid extraction methods. Liq. Ex.: liquid extraction; Dry Ex. : dry column extraction ; ASE : Accelerated solvent extraction. White bar, fresh meat T1; Grey bars, freeze dried meat. Results are expressed in % fat (w/w) for fresh weight. Values are mean \pm standard deviation; n=4. Letters show significance level at $p < 0.05$.

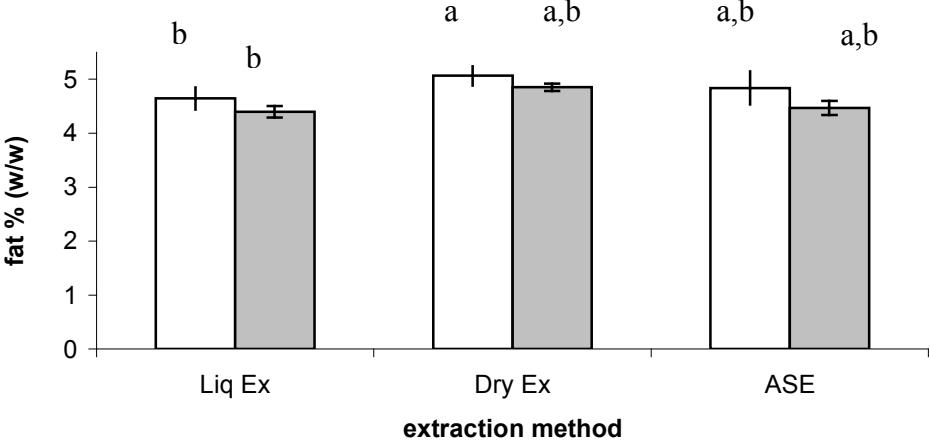


Figure 2. SPE separation: 1 = std L- α -phosphatidylinositol; 2 = std L- α phosphatidylcoline; 3 = std L- α -phosphatidylethanolamine. After SPE with aminopropyl column (eluent MeOH): 4 = std L- α -phosphatidylinositol; 5 = std L- α -phosphatidylcoline; 6 = std L- α -phosphatidylethanolamine.

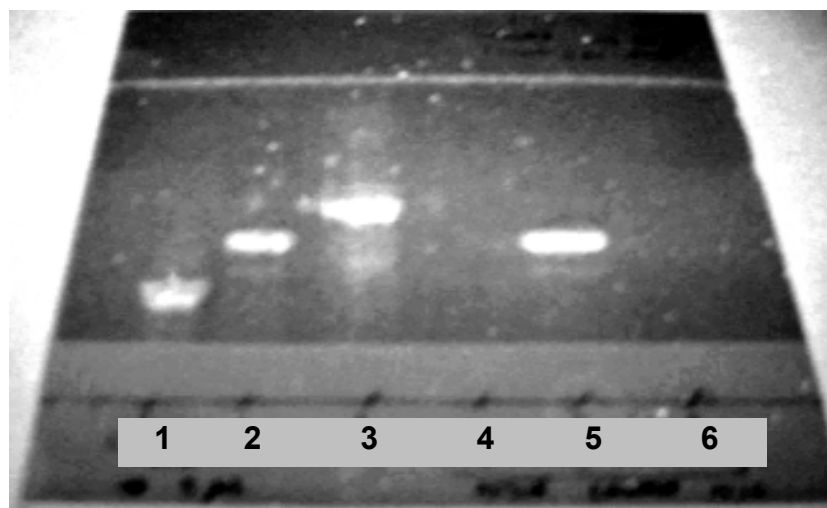


Figure 3. Left, Chromatograms comparison: black line: std after elution with aminop. column with MeOH; blu line: std elution from aminopropylic column with MeOH and water phase; green line: std elution from silica column with MeOH and water phase. Right, TLC plate: 1=std (L- α -phosphatidylinositol;L- α phosphatidylcoline; L- α phosphatidylethanolamines) 2=std after elution with aminop. column with MeOH 3=std elution from aminopropylic column with MeOH and water phase 4 = std elution from silica column with MeOH and water phase.

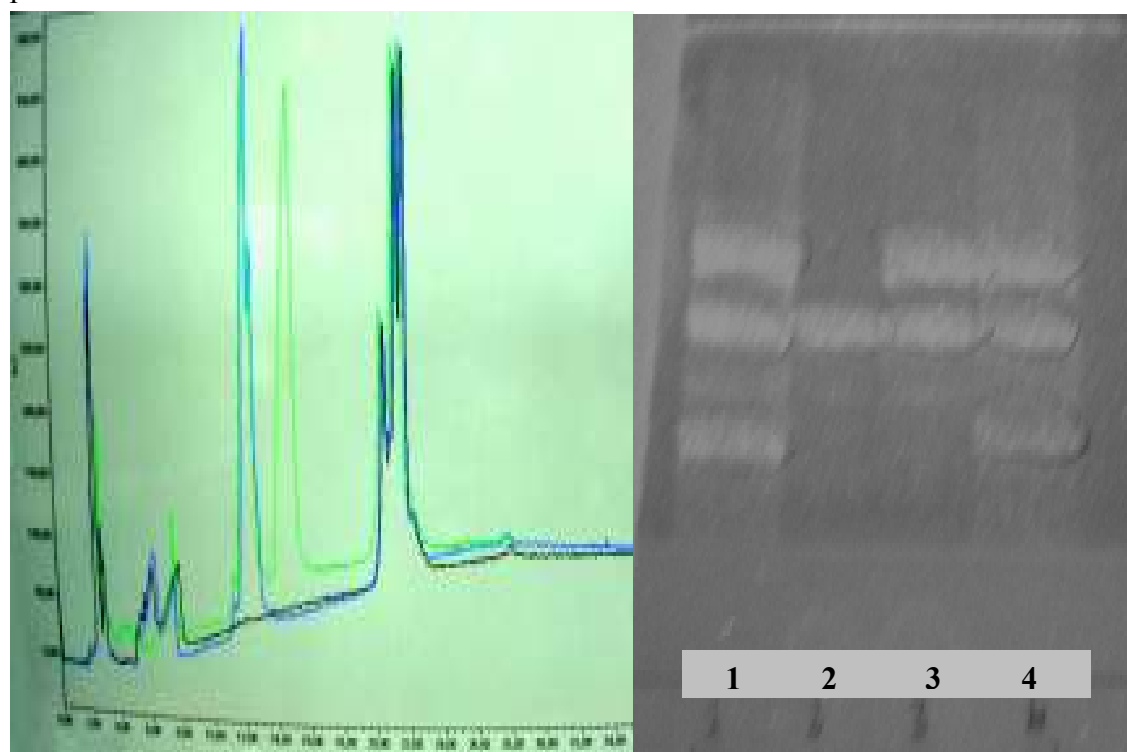


Figure 4. Chromatogram of phospholipid standards by HPLC-CAD. Cerebrosides (CER); cardiolipin (CARD); L- α -phosphatidyinositol (PI); L- α -phosphatidylethanolamine (PE); L- α -phosphatidylserine (PS); L- α -lisophosphatidylethanolamine (LPE); phosphatidic acid (PA); L- α -Phosphatidylcholine (PC), sphingomyelin (SP); L- α -lisophosphatidylcholine (LPC).

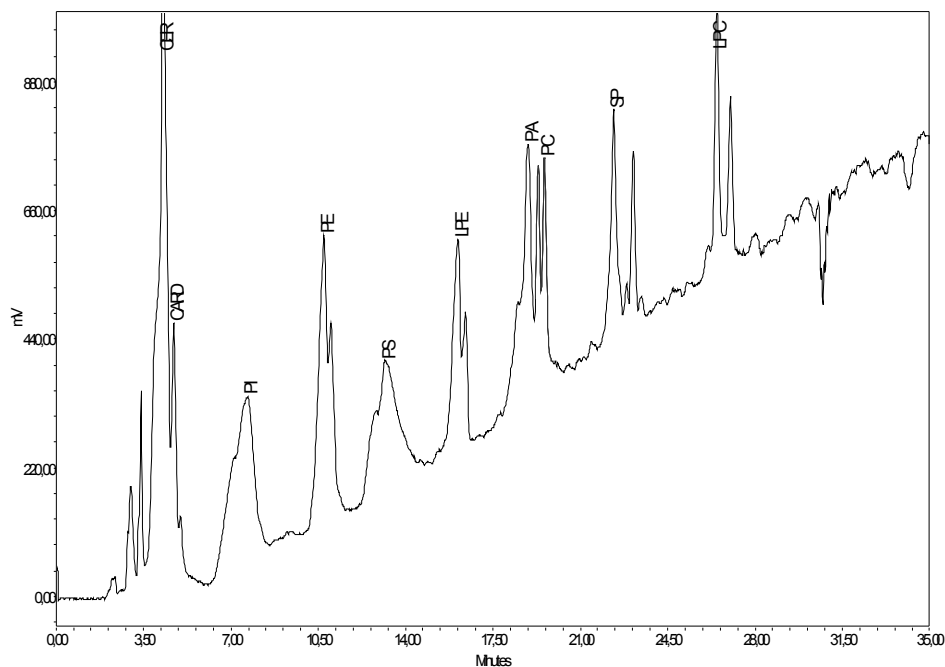


Figure 5. Chromatogram of phospholipids fraction of meat lipid extract. L- α -phosphatidyinositol (PI); L- α -phosphatidylethanolamine (PE); L- α -phosphatidylcholine (PC)

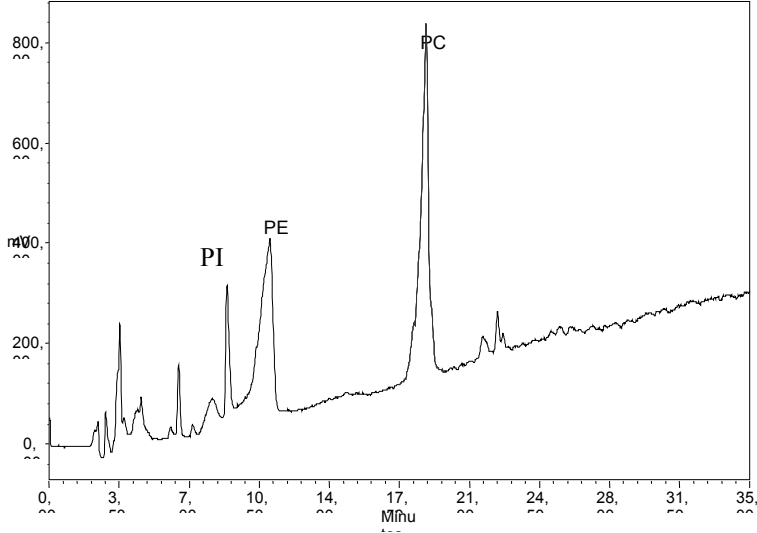


Figure 6. GC-FID chromatogram of FAME after oxidation with Method D (Sample added with 10 mL FeSO₄ 10 μM and 1 mL TBPH 50% in water). Between 10 and 20 minutes artefact peaks.

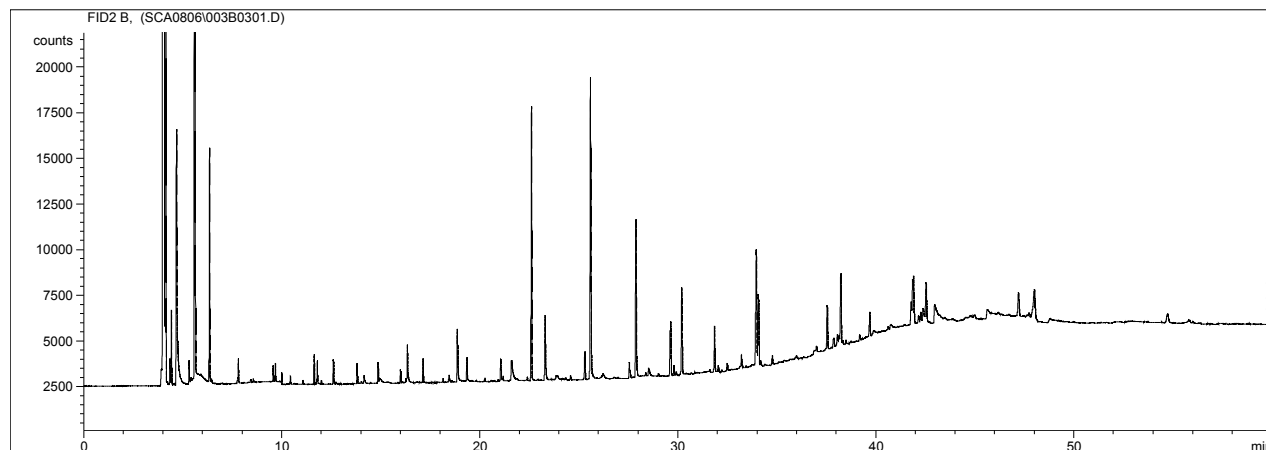


Figure 7: GC-FID chromatogram of FAME after oxidation with Method B (Sample added with 10 mL CuSO₄ 33 μM)

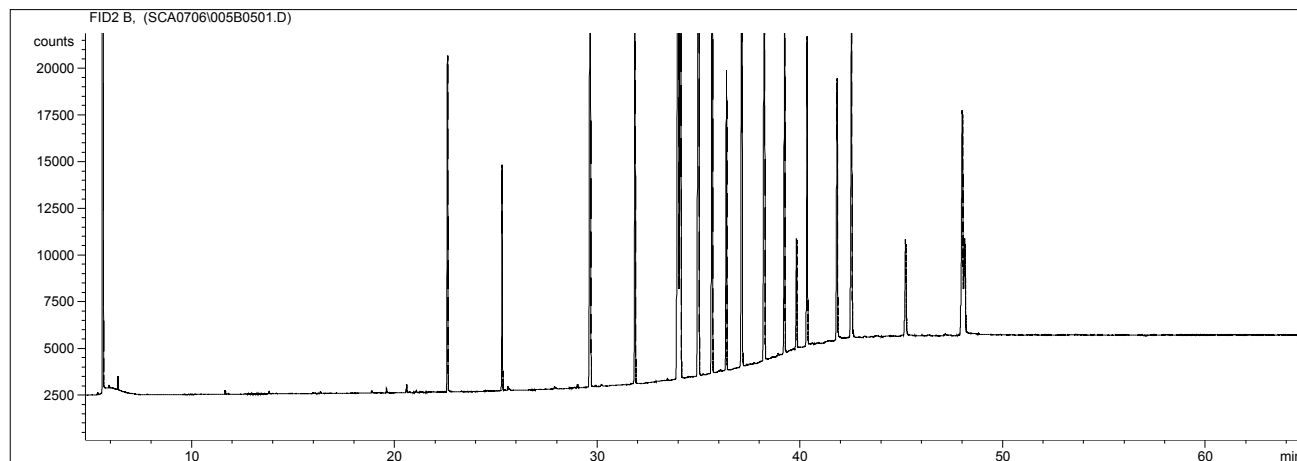


Figure 8. Assay of MDA levels generated by PLs oxidation induced by AMPA. The values are mean \pm standard deviation; n = 4. The letters show significance level at p < 0.05.

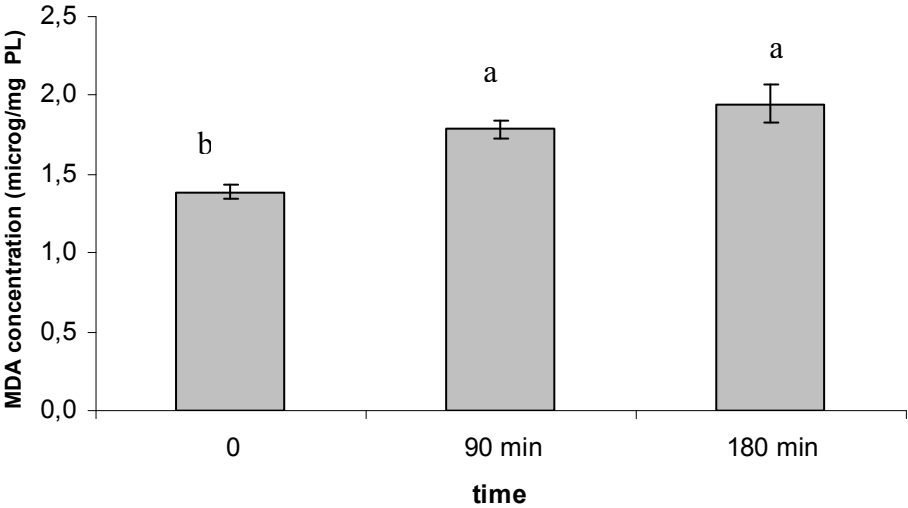
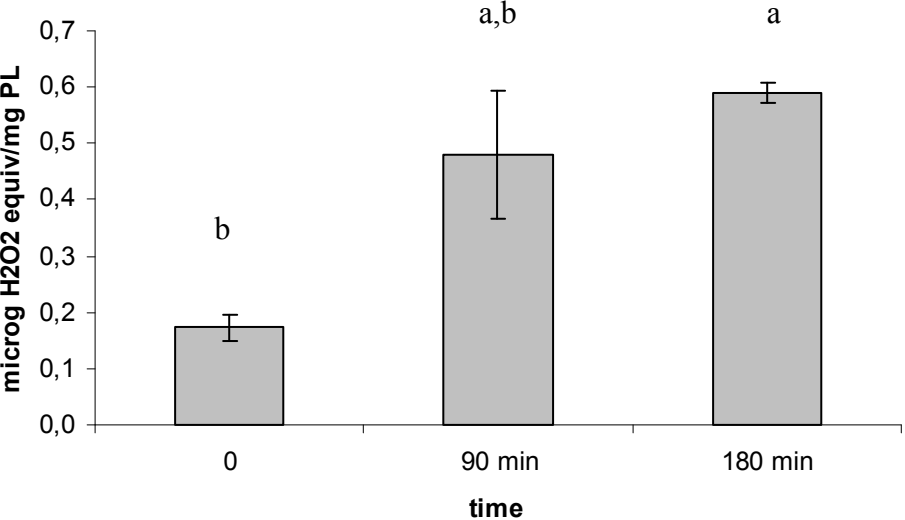


Figure 9. Peroxide levels generated by oxidation of PLs, induced by AMPA. The values are mean \pm standard deviation; n = 4. The letters show significance level at $p < 0.05$.



CONCLUSIONS

One of the main aims of the present study was to investigate natural antioxidants prevention against lipid oxidation in meat. Oxidation products generated during meat storage have previously been observed in many studies. However, data from lipid oxidation in meat added of antioxidant extracts obtained by supercritical fluid extraction are limited.

In the present work it was demonstrated that meat added with commercial antioxidant preparation, usually used by meat industry, are less efficacious against lipid oxidation.

Oxidation status in meat samples were studied by suitable modifications of methods previously described. The study of MDA was performed as MDA adduct.

Moreover oxidation products were characterized in detail using other analytical techniques such as peroxides values and TBA test.

Another aim of this study was to evaluate an analytical method for the determination of MDA in meat. The method consists in an MDA adduct by DNPH reaction, by simple but efficient technique. Good recovery, linearity, repeatability and low detection limit were reported.

Further, the oxidation status of buffalo meat was evaluated considering a diet with vitamin E supplementation. The levels of oxidation products was major in meat coming from animal with diet without vitamin E. These results were basically in line with previously reported data, where other kinds of meat have been studied

As mentioned earlier, many studies have reported oxidation products in meat, while data concerning a model system to study this process are limited. The present study indicates that corresponding products are formed from oxidation induction by AMPA. If this model system is further developed, oxidation products of meat can be isolated and studied, it is a matter of health concern.

The high exposure of meat lipid oxidation products, and the fact that these compounds, like COP, might have other biologically effects, warrant further studies.

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