

The Antioxidant Properties of Plant Phenolics in Liver Pâtés are Affected by the Composition of the Food Matrix

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ABSTRACT

The effect of plant essential oils containing phenolic diterpenes on lipid and protein oxidation and the increase of non-heme iron (NHI) during refrigeration (+4°C/90 days) of commercial liver pâtés, was studied. These results were subsequently compared to those obtained from a parallel evaluation of the same antioxidants on liver pâtés from free-range reared Iberian pigs. Liver pâtés with no added essential oil were used as controls. The effect of sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) essential oils was different depending on the type of liver pâté to which they were added. In liver pâtés from industrial genotype pigs (commercial pâtés), they acted as prooxidants, significantly increasing the generation of TBA-RS whereas no effect was detected on hexanal counts, protein oxidation and NHI content. In liver pâtés from Iberian pigs, sage and rosemary essential oils successfully reduced the generation of lipid and protein oxidation products and inhibited, in addition, the release of iron from the heme molecule. The large differences between liver pâtés from Iberian and industrial genotype pigs in terms of fatty acid composition, the levels of tocopherol and the susceptibility to undergo oxidative reactions could have an influence on the activity of the plant phenolics.

Keywords: lipid oxidation, non-heme iron, protein oxidation, rosemary, sage, tocopherol

INTRODUCTION

The understanding of the mechanisms of lipid oxidation and the factors influencing its occurrence and intensity in meat products has allowed food technologists to design strategies to control the development of oxidative reactions during meat handling, processing and storage. These strategies include the addition of additives and spices with antioxidant potential. Synthetic phenolic antioxidants have been largely used in different food products though their possible mutagenicity (Clayson *et al.* 1986) has caused the rejection by consumers. Consequently, great scientific efforts have been exerted to find natural antioxidants in plant kingdom in order to use them as an alternative to synthetic antioxidants (Kanner 1994). Sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*) are popular *Labiatae* herbs commonly used in meat and fat products to reduce the adverse effects of lipid and protein oxidation (Sebranek *et al.* 2005; Salminen *et al.* 2006; Waszkowiak and Dolata 2007). However, recent studies have reported the complexity associated to the use of herbs or plant extracts as inhibitors of oxidative reactions (Zheng and Wang 2001; Masuda *et al.* 2002; Estévez and Cava 2006). The antioxidant activity of plant phenolics are affected by many factors including the total number and location of hydroxyl groups on aromatic rings, the nature of the extracts, their concentration and the characteristics of the system in which they are added (Huang and Frankel 1997; Kähkönen *et al.* 1999; Zheng and Wang 2001; Škerget *et al.* 2005). In addition, phenolic compounds from plants can interact with other substances such as tocopherols leading to synergist effects (Wong *et al.* 1995; Škerget *et al.* 2005). Kähkönen *et al.* (1999) suggested that the antioxidant activity of plant phenolics could also be affected by the oxidation conditions and lipid characteristics of the system. Furthermore, plant phenolics have shown prooxidant properties in biological materials and food systems (Laughton *et al.* 1989; Yen *et al.* 1997). According to the previous reports and considering the large variety of

food systems and the complexity of their compositions, to approach the study of the effect of plant phenolics on some particular foods involves certain difficulties because of the inconsistency of the results.

Liver pâté is an emulsion-type cooked product made with meat, liver, adipose tissue and several additives (Estévez *et al.* 2004a). Pâté contains high levels of fat and iron which considerably increases its oxidative instability during processing and subsequent refrigerated storage. The development of lipid and protein oxidation in liver pâtés leads to heme pigment degradation and colour and texture deterioration (Russell *et al.* 2003; Estévez and Cava 2004). The addition of synthetic or natural antioxidants in liver pâtés could be an interesting option to inhibit the unpleasant effects of lipid and protein oxidation. Considering, however, that the compositional characteristics of the pâté lipids and the concentration of endogenous antioxidants, mainly tocopherols, vary considerably depending on the source of the pig tissues (Estévez *et al.* 2004a), the effect of added antioxidants in different types of liver pâtés seems to be unexpected. The present work was carried out to: i) evaluate the effect of the addition of natural (rosemary and sage essential oils) in liver pâtés produced with tissues from intensively reared commercial genotype pigs fed on a tocopherol non-supplemented mixed diet and ii) to compare the obtained effects with those found in liver pâtés manufactured with tissues from free-range reared Iberian pigs fed on natural resources (Estévez *et al.* 2006).

MATERIALS AND METHODS

Raw material

Seven white pigs derived from industrial genotypes (Large-white × Landrace) were intensively reared under controlled conditions in a typical industrial livestock farm. The animals were fed on a mixed diet and slaughtered at ~85 kg live weight when 7 months old.

Seven Iberian pigs commonly produced in the South-West of Spain and belonging to Iberian pig pure breed were free-range reared and fed on natural resources (grass and acorns) following traditional livestock farming procedures for Iberian pigs. Iberian pigs and industrial genotype pigs were slaughtered at the same slaughterhouse one week apart. After slaughter, back fat, muscle *quadriceps femoris*, and liver were removed from carcasses, vacuum packaged and stored at -80°C until the manufacture of pâté.

Manufacture of liver pâté

The experimental liver pâtés were manufactured in a pilot plant. Depending on the origin of the raw material two types of liver pâtés were produced: liver pâtés from intensively reared industrial genotype pigs and liver pâtés from free-range reared Iberian pigs. Muscles *quadriceps femoris*, livers and adipose tissues from seven animals from each pig breed were used. The same formulation was used for all liver pâtés except for the addition of antioxidants. In the basic formulation the ingredients were as follows per 100 g of manufactured product: 28 g liver, 40 g subcutaneous fat, 5 g muscle, 23 g distilled water, 2 g sodium caseinate, and 2 g sodium chloride. The concentrations of all added additives and antioxidants were calculated on the basis of total ingredients. Sodium di- and tri-phosphates (0.3%), sodium ascorbate (0.05%), and sodium nitrite (0.03%) (All from ANVISA additives, Madrid, Spain) were included. Depending on the experimental batch, different essential oils (Soria Natural S.L., Soria, Spain) were added to liver pâtés from both Iberian and industrial genotype pigs: rosemary extract (ROSE) (0.1%) and sage extract (SAGE) (0.1%). According to McCarthy *et al.* (2001), the essential oils might show a clear effect at this concentration. Control pâtés (CON) containing no added antioxidants were also prepared. The protocol followed for the manufacture of liver pâtés has been explained elsewhere (Estévez *et al.* 2004a). After the manufacture, liver pâtés were stored at +4°C for 90 days in the dark. Liver pâtés were analysed at 0, 30, 60, and 90 days for TBA-RS numbers, protein carbonyls, concentrations of NHI and hexanal counts. At sampling times, samples were stored at -80°C until the other analytical experiments were conducted.

Analytical methods

Compositional analysis of liver pâté

The method of Bligh and Dyer (1959) was used for the extraction and quantification of the fat from liver pâtés.

Fatty acid methyl esters (FAMES) were prepared by acidic esterification in presence of sulphuric acid, following the method of López-Bote *et al.* (1997). FAMES were analysed using a Hewlett Packard, mod. HP-5890A, gas chromatograph, equipped with a flame ionisation detector (FID). The derivatives were separated on a FFAP-TPA fused-silica column (Hewlett Packard 30 m long, 0.53 mm internal diameter and 1.0 µm film thickness). The injector and the detector temperature were held at +230°C. Oven temperature was maintained at +220°C. The flow rate of the carrier gas (N₂) was set at 1.8 ml/min. Identification of FAMES was based on retention times of reference compounds (Sigma). The quantification of fatty acids was carried out by using C¹³ as an internal standard. Results are expressed as g fatty acid/100 g total fatty acid analysed.

Tocopherol content

α-tocopherol was extracted from porcine tissues according to the method described by Rey *et al.* (1997). The analysis was carried by reverse phase HPLC (HP 1050, with a UV detector, HPIB 10) (Hewlett-Packard, Waldbronn, Germany). Results are expressed as µg tocopherols/g tissue.

TBA-RS measurement

Malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBA-RS) were determined using the method of Rosmini *et al.* (1996). Results were expressed as mg MDA/kg liver pâté.

Hexanal analysis

The SPME fibre, coated with a divinylbenzene-carboxen-poly (dimethylxilosane) (DVB/CAR/PDMS) 50/30 µm, was preconditioned prior analysis at +220°C during 45 min. The HS sampling was performed following a method previously described (Estévez *et al.* 2004b). One g of liver pâté was placed in 2.5 ml vials and the SPME fibre was exposed to the headspace of the pâté while the sample equilibrated during 30 minutes was immersed in water at +50°C. Analyses were performed on a HP5890GC series II gas chromatograph (Hewlett-Packard, USA) coupled to a mass-selective detector (Agilent model 5973). Volatiles were separated using a 5% phenyl-95% dimethyl polysiloxane column (Restek, USA) (30 m x 0.25 mm i.d., 1.0 mm film thickness). The carrier gas was Helium at 18.5 psi, resulting in a flow of 1.6 ml/min at 40°C. The SPME fibre was desorbed and maintained in the injection port at 220°C during the whole chromatography run. The injector port was in the splitless mode. The temperature program was isothermal for 10 min at +40°C and then raised at the rate of +7°C/min to 250°C, and held for 5 min. The GC/MS transfer line temperature was +270°C. The mass spectrometer operated in the electron impact mode with an electron energy of 70 eV, a multiplier voltage of 1650 V and collecting data at a rate of 1 scan/s over a range of 40 to 300 m/z. Hexanal was identified by comparing its retention time with that from the standard compound (Sigma-Aldrich, Steinheim, Germany). Results from the volatiles analysis are provided in area units (AU).

Protein oxidation measurement

Protein oxidation as measured by the total carbonyl content was assessed following the 2,4-dinitrophenylhydrazine (DNPH) coupling method described by Oliver *et al.* (1987). DNP hydrazones were quantified by measuring absorbance values at 370 nm. Protein concentration was determined by spectrophotometry at 280 nm using bovine serum albumin (BSA) as standard. The amount of carbonyls was expressed as nM carbonyls/mg protein.

Iron analysis

Nonheme iron (NHI) content was determined by spectrophotometry following the method described by Rhee *et al.* (1987). The amounts of iron were expressed as µg iron/g pâté.

Data analysis

Means and deviations from 5 measurements within each batch were obtained from all analytical experiments. Results from the experiments were used as variables and analysed by using an Analysis of Variance (ANOVA) from SPSS software in order to assess the effect of the addition of antioxidants and the effect of refrigerated storage on liver pâtés. When statistically significant differences were found, Tukey's test was performed. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

In a previous work (Estévez *et al.* 2006) the effect of the addition and sage and rosemary essential oils on the oxidative stability of liver pâtés produced with tissues from free-range reared Iberian pigs was reported. **Fig. 1-4** show the comparison between the activities of the sage and rosemary essential oils in liver pâtés from Iberian and industrial genotype pigs. The effect of the essential oils seems to be influenced by the characteristics of the liver pâté. The addition of sage and rosemary essential oil enhanced the generation of TBA-RS in liver pâtés from industrial genotype pigs at day 90 whereas significantly reduced TBA-RS numbers in liver pâtés from Iberian pigs (**Fig. 1a vs. 1b**). In liver pâtés from industrial genotype pigs, the essential oils had no effect on the hexanal counts while greatly influenced on liver pâtés from Iberian pigs significantly reducing the generation of hexanal (**Fig. 2a vs. 2b**). Similar results were obtained on protein oxidation since no effect of essential oils was observed in liver pâtés from industrial genotype pigs

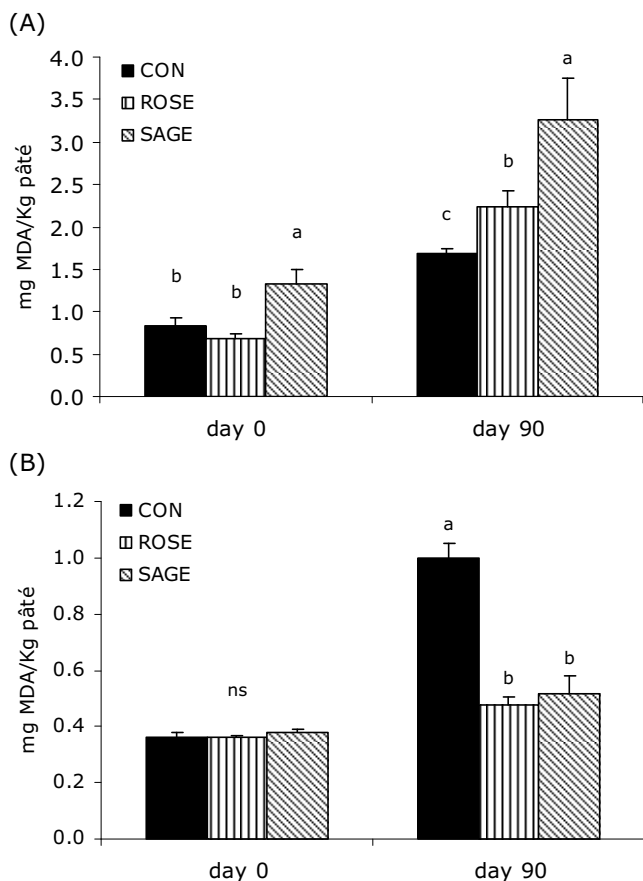


Fig. 1 TBA-RS numbers in liver pâtés from white (A) and Iberian pigs (B) treated with sage and rosemary essential oils. (Significant differences, $p < 0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

and the same essential oils significantly inhibited the generation of protein carbonyls in liver pâtés from Iberian pigs at day 90 (Fig. 3a vs. 3b). This contradictory effect was also observed regarding the release of iron from the heme molecule since control and treated pâtés from industrial genotype pigs contained similar amounts of NHI and the addition of sage and rosemary oils in liver pâtés from Iberian pigs significantly reduced the release of iron from the heme molecule (Fig. 4a vs. 4b).

Results from the present work suggest that the activity of the rosemary essential oil was dependent on the compositional characteristics of the food matrix. In fact, the effect of plant phenolics has been considered to be influenced by the compositional characteristics of the food system and the presence of other active substances (Huang and Frankel 1997; Yen *et al.* 1997). In this sense, Wong *et al.* (1995) and Fang and Wada (1993) reported possible interactions between phenolic compounds from sage and rosemary essential oils and tocopherols, leading to different effects depending on the particular amounts of each of these substances in the food system. In the present work, muscles, adipose tissues and livers used for the production of liver pâtés from Iberian pigs contained significantly higher amounts of α -tocopherol than those from industrial genotype pigs (Fig. 5). The high levels of tocopherols in tissues and elaborated products from free-range reared Iberian pigs is explained by the intake of natural resources (mainly grass and acorns) during the outdoors rearing of Iberian pigs (Estévez *et al.* 2004a). Therefore, the presence of a certain amount of endogenous antioxidants (tocopherols) in the raw material and manufactured product could influence on the activity of exogenous active extracts, leading to overall antioxidant or pro-oxidant effects. In this sense, similar effects have been reported in foods when mixtures of two antioxidants were joined at different proportions. For ins-

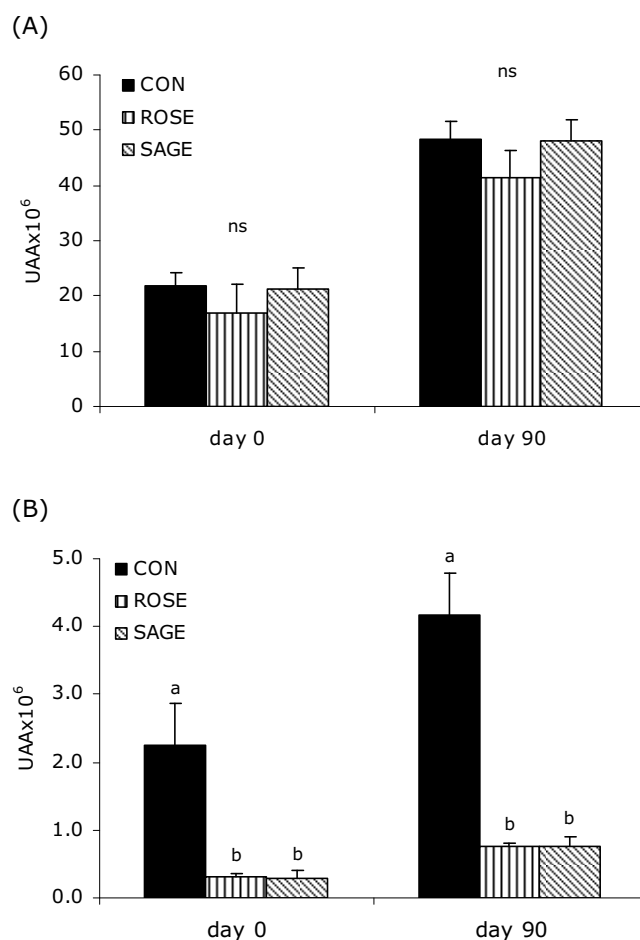


Fig. 2 Hexanal counts in liver pâtés from white (A) and Iberian pigs (B) treated with sage and rosemary essential oils. (Significant differences, $p < 0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

tance, β -carotene acted as a prooxidant in refrigerated stored chicken when the tissue vitamin E was low, whereas it showed antioxidant effects at higher concentrations of vitamin E (Esteve-Garcia *et al.* 1998) which is in clear coincidence with the present results.

In addition, when testing natural antioxidants it is important to consider the system composition in terms of lipid substrate and degree of unsaturation (Frankel and Meyer 2000). In accordance to Huang and Frankel (1997), whether phenolic compounds act as antioxidants or prooxidants appears to be dependant on the lipid characteristics of the model system. These authors reported antioxidant activities of tea catechins in corn oil triglycerides whereas in oil in water emulsions, these compounds were all prooxidants. Liver pâtés from Iberian pigs had significantly higher percentages of oleic and total monounsaturated fatty acids (MUFA) than liver pâtés from industrial genotype pigs which contained higher proportions of saturated (SFA) and polyunsaturated fatty acids (PUFA) (Table 1). The analysis of the fatty acid composition of the tissues used for liver pâtés manufacture revealed similar results (Estévez *et al.* 2004a). The high levels of oleic acid in tissues and meat products from Iberian pigs is due to the deposition of high amounts of such fatty acid derived from the intake of acorns. The fatty acid profile of the tissues and liver pâté from industrial genotype pigs reflected the fatty acid composition of the mixed diet on which industrial genotype pigs were fed (Estévez *et al.* 2004a). The different fatty acid composition between liver pâtés (Table 1) affects the physical state of the lipids that could have affected the dispersion and antioxidant activity of the sage and rosemary essential oils leading to different effects. As long as liver pâtés from Iberian and industrial genotype pigs showed similar proximate

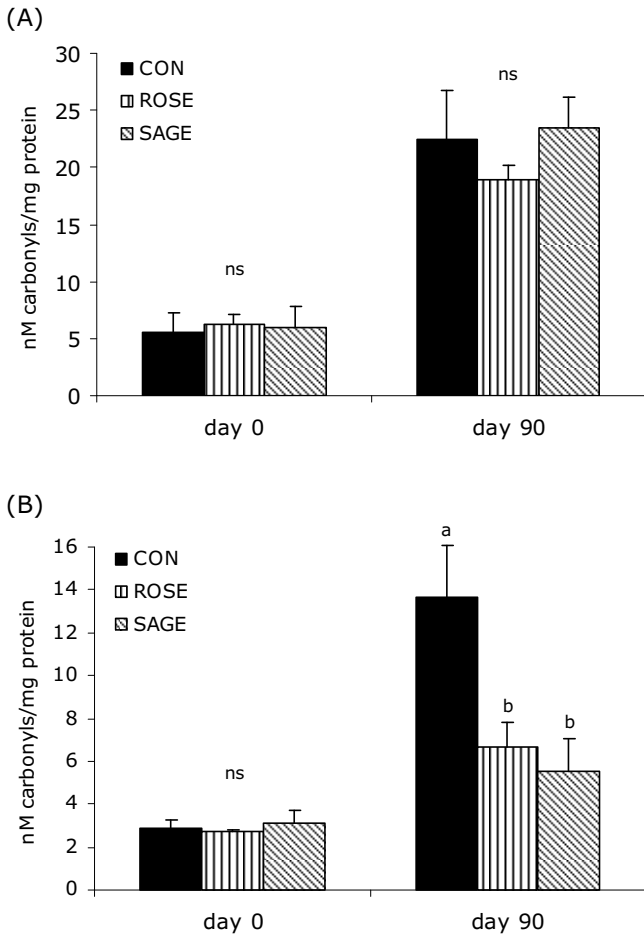


Fig. 3 Protein carbonyls in liver pâtés from white (A) and Iberian pigs (B) treated with sage and rosemary essential oils. (Significant differences, $p < 0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

compositions (data not shown), the influence of other major component of liver pâtés supporting the contradictory effect of added antioxidants is unlikely.

Finally, the activity of the rosemary essential oil could have been affected by the initial oxidation state of the liver pâtés in which it was added (Frankel and Meyer 2000). In systems with higher oxidative instability, the activity of plant phenolics could be reduced since phenolic compounds can be oxidised and the oxidation products could act as prooxidants promoting oxidative reactions (Huang and Frankel 1997). The high oxidative instability in liver pâtés from industrial genotype pigs would explain the prooxidant effect of the essential oils regarding TBA-RS numbers and the lack of effect on protein oxidation whereas in Iberian liver pâtés, which showed considerably higher oxidative stability, the essential oil acted as a potent antioxidant. Furthermore, the oxidation of phenolics in Iberian pâtés could have been inhibited by the presence of high levels of tocopherols with which plant phenolics interact leading to regeneration and synergist effects (Wong *et al.* 1995; Hopia *et al.* 1996; Zhu *et al.* 1999). The results obtained in the present work are in agreement with those obtained in a previous study in which a rosemary essential oil (600 ppm) showed an antioxidant effect when added on frankfurters from Iberian pigs and exhibited the opposite (prooxidant) effect in frankfurters from industrial genotype pigs (Estévez and Cava 2006). Differences between liver pâtés from Iberian and industrial genotype pigs reported in that study in terms of fatty acid composition and tocopherol contents are consistent with those reported in the present study which support the hypothesis and mechanisms suggested.

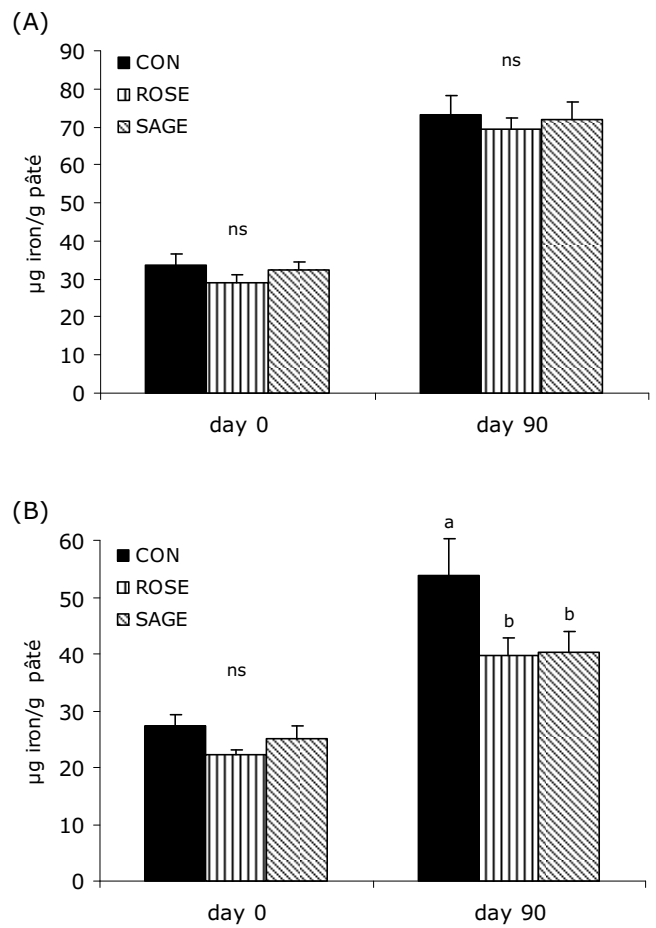


Fig. 4 NHI content in liver pâtés from white (A) and Iberian pigs (B) treated with sage and rosemary essential oils. (Significant differences, $p < 0.05$, between antioxidant groups within a day of storage are denoted by different letters; ns: non significant).

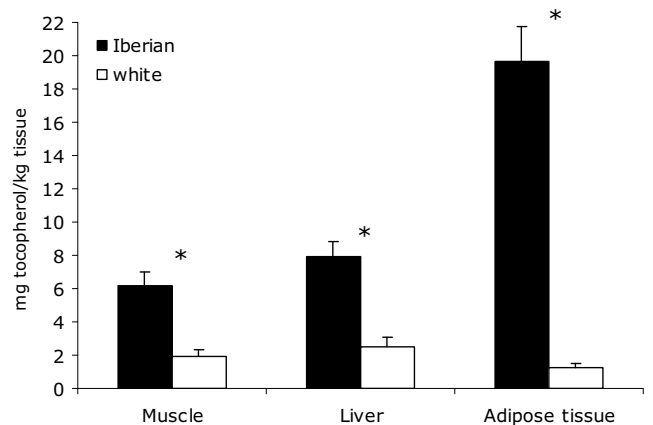


Fig. 5 Tocopherol content in tissues from extensively reared Iberian pigs and intensively reared white pigs (Significant differences, $p < 0.001$, between groups within a tissue are denoted with an asterisk).

CONCLUSION

According to the present results, the effect of plant phenolics from sage and rosemary essential oils added to liver pâtés is unpredictable depending on the characteristics of the liver pâté. Therefore, the use of plant materials with antioxidant properties in commercial liver pâtés should be preceded by systematic studies to assure the desirable antioxidant effects. In the absence of the knowledge of precise mechanisms of interaction between the essential oil components and the liver product, further experiments would be required to shed light on the specific interactions between plant extracts and food components and to evaluate the influence of the chemical composition of tissues in terms of

Table 1 Fatty acid composition (means \pm standard deviation) of liver pâtés from extensively reared Iberian and intensively reared white pigs.

	Iberian	White	p ¹
C16:0 ²	20.69 \pm 0.08	22.65 \pm 0.05	<0.001
C18:0	10.58 \pm 0.00	13.40 \pm 0.03	<0.001
Σ SFA	32.87 \pm 0.09	37.98 \pm 0.12	<0.001
C16:1 (n-7)	2.00 \pm 0.01	2.44 \pm 0.00	<0.001
C18:1 (n-9)	53.43 \pm 0.07	43.57 \pm 0.07	<0.001
C20:1 (n-9)	1.83 \pm 0.02	1.08 \pm 0.04	<0.001
Σ MUFA	57.52 \pm 0.06	47.58 \pm 0.10	<0.001
C18:2 (n-6)	7.71 \pm 0.11	12.23 \pm 0.04	<0.001
C18:3 (n-3)	0.49 \pm 0.01	0.61 \pm 0.01	<0.001
C20:2 (n-6)	0.57 \pm 0.02	0.45 \pm 0.23	0.296
C20:4 (n-6)	0.56 \pm 0.00	0.70 \pm 0.03	<0.001
Σ PUFA	9.63 \pm 0.29	14.40 \pm 0.37	<0.001

¹ Statistical significance in a student *t*-test for independent variables.

² Fatty acids expressed as percentages of total fatty acids analysed.

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

fatty acids and tocopherols on the activity of these substances.

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