Effect of natural antioxidants on physicochemical properties and lipid stability of pork liver pâté manufactured with healthy oils during refrigerated storage

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Revised: 15 May 2017 / Accepted: 27 September 2017 / Published online: 10 October 2017
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Abstract This study aimed to evaluate the effect of natural antioxidants in pork liver pâté manufactured with the combination of pork backfat, fish oil and olive oil. Phenolic composition of beer residue extract (BRE), chestnut leaves extract (CLE) and peanut skin extract (PSE) were identified and quantified. Four batches of pork liver pâté were produced: control, BRE, CLE and PSE. Pork liver pâté was evaluated for proximate composition, pH, instrumental colour, free fatty acid content, lipid-derived volatile compounds and lipid oxidation. The major compounds of BRE were benzoic acid and catechin (1.79 and 1.51 mg/L, respectively), in CLE were ellagic and gallic acid (10.26 and 2.70 mg/100 g fresh weight) and in PSE was catechin (20.66 mg/100 g dry weight). Proximate composition was similar for all batches. The pH values were not influenced by any natural antioxidant. Colour parameters were affected by storage time but slight differences were observed among batches. Lipid stability (TBARS and lipid-derived volatile compounds) was not remarkably affected by addition of natural extracts.

Keywords Flavonoids · Antioxidant activity · Lipid stability · Porcine liver pâté · Hexanal

Introduction

Although the development of meat products with partial substitution of animal saturated fat by unsaturated fatty acids is an important strategy to provide healthier meat products, the lipid stability can be impaired due to the sensibility of unsaturated fatty acids to pro-oxidant agents (de Ciriano et al. 2010). In a previous study, Lorenzo et al. (2016) observed that the incorporation of microencapsulated fish oil in konjac gel increased the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) content and decreased the $n - 6/n - 3$ PUFA ratio compared to the control group in Spanish salchichón. In addition, Domínguez et al. (2016) also noticed that lipid modification of pâté by substitution of pork backfat with fish oil has been shown to be a good strategy to improve their nutritional quality, specifically to increase long chain $n - 3$ fatty acids. In this type of product, the generation of free radicals and other oxidation products are also accelerated leading to reduction in shelf life. In addition to lipid oxidation, other undesirable changes such as loss of redness and development of off-flavour are commonly reported (Bernardi et al. 2016).

Phenolic compounds from natural sources have been receiving great attention due to the association between their consumption and reduced risk associated with development of severe diseases such as cancer and the increase of antioxidant status in human health (Rangel-Huerta et al. 2015). These antioxidant compounds are widely distributed in dietary sources although a significant content is lost in the large amount of wastes generated during food processing. Hence, the use of such residues as sources of phenolic compounds can reduce the loss of valuable materials and reduce economic and environmental impacts (Mirabella et al. 2014).

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Beer processing generates waste residues rich in phenolic compounds that are removed from beer to render a clear beverage and prevent the formation of haze. This residue is composed of catechin, epi-catechin, proanthocyanidins, caffeic, syringic acid and other compounds with antioxidant potential as cohumulone, humulone and lupulone (Munekata et al. 2016a). The extracts prepared from such residues were related to free radical scavenge and chain breaking activity (Barbosa-Pereira et al. 2014).

Chestnut leaves (*Castanea sativa*) are traditionally used to prepare hot beverage as tea and due to believed health effects are also used as folk medicine in people suffering from muscular pain (Díaz-Reinoso et al. 2011). Previous study revealed the presence of gallic and ellagic acid, rutin, quercetin, luteolin, epi-gallocatechin and kaempferol in the phenolic composition of chestnut leaves. Extracts obtained from chestnut leaves were evaluated by DPPH radical scavenge activity, reducing power and inhibition of lipid peroxidation revealing a high antioxidant potential (Barreira et al. 2008).

The residues from peanut processing can be exploited, particularly the skin from kernels due to the elevated phenolic content that are usually discarded or used as animal feed. The phenolic composition of peanut skin is largely composed by proanthocyanidins (polymeric structures formed by catechin and epicatechin) although other phenolic compound as resveratrol, ferulic acid and coumaric acid were also reported in literature (Ma et al. 2014). The antioxidant activity of peanut skin is also associated with scavenging of free radicals and reactive oxygen species (Wang et al. 2007).

Common alterations of muscle products during storage as lipid oxidation, colour changes, protein oxidation and development of off-flavour were noticed in dry-cured sausage chorizo (Lorenzo et al. 2013; Pateiro et al. 2015a), pork, chicken and sheep patties (Lorenzo et al. 2014a; Munekata et al. 2015, 2016b) and liver pâté (Pateiro et al. 2014, 2015a). Thus, the present study aimed to evaluate the phenolic composition and the effects of natural extracts from agro-industry residues (beer residue, chestnut leaves and peanut skin) on the instrumental colour, pH value, lipid oxidation, free fatty acids and volatile compounds of pork liver pâté manufactured with healthy oils during chilled storage.

Materials and methods

Extracts preparation

BRE was obtained according to Pateiro et al. (2015a). Briefly, phenolic compounds were separated from beer residue with a glass column filled with XAD-16 amberlite

that was previously equilibrated with distillate water, using ethanol as eluent. The extraction of chestnut leaves (CLE) was carried out according to Lorenzo et al. (2013) briefly, chestnut leaves were air-dried, grounded and extracted using acidified distilled water (HCl 0.1 M) in a proportion of 1:25 (m/v) for 90 min at 25 °C. The preparation of peanut skin extract (PSE) was performed according to Munekata et al. (2015) briefly, peanut skin was extracted with 80% ethanol at 60 °C for 50 min followed by sonication at room temperature for 15 min, centrifugation at 6000 rpm for 15 min.

Identification and quantification of phenolic compounds in natural extracts

Analyses were carried out using a Waters 2695 Separation Module equipped with a SunFire C18 Column (3.5 μ m, 3.0 $\times$ 150 mm) and a Waters 2996 Photodiode Array Detector, operating at 35 °C at a flow rate of 0.5 mL/min. The injection volume was 20 μ L. A nonlinear gradient of solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid) in the following proportions: 0 min, 85% A, 15% B; 1 min, 85% A, 15% B; 30 min, 40% A, 60% B; 40 min, 0% A, 100% B; 45 min, 0% A, 100% B; 47 min, 85% A, 15% B; 48 min 85% A, 15% B was used. Phenolic compounds were identified by comparison of the retention times and spectral features with those of authentic compounds. Quantification was performed at the corresponding wavelengths of maximum adsorption by calibration using the respective reference compound.

Liver pâté production and sampling procedures

For the present study, four different batches of liver pâté were manufactured: CON (control batch without antioxidant extract), BRE (1000 mg/kg), CLE (1000 mg/kg) and PSE (1000 mg/kg). An identical formula was used for all batches, except for the addition of the different antioxidants. The liver pâté formulation included pork liver (330 g kg⁻¹), pork lean (200 g kg⁻¹), pork backfat (150 g kg⁻¹), fish oil (75 g kg⁻¹), olive oil (75 g kg⁻¹), water (114.25 g kg⁻¹), NaCl (20 g kg⁻¹), powdered milk (20 g kg⁻¹), sodium caseinate (10 g kg⁻¹), potassium phosphate (5 g kg⁻¹), sodium nitrite (0.5 g kg⁻¹) and sodium ascorbate (0.25 g kg⁻¹).

Firstly, lean and liver was chopped (at 4 °C) in a bowl chopper (Sirman, mod C15VV, Marsango, Italy) and then mixed with NaCl, sodium nitrite and sodium ascorbate. This mix was maintained at 4 °C for 24 h. The day of the elaboration of pâté, backfat were chopped in small cubes and scalded at 65 °C for 15 min. Then, the mix (prepared the day before) and cooked fat were mixed with the

remaining ingredients and antioxidants (in the corresponding batch) until a homogeneous batter. Each batch was elaborated with 4.5 kg of meat batter. Finally, the meat batter were manually distributed into metal cans until completely full (100 g) and these were then hermetically closed prior to thermal treatment (80 °C during 30 min) in an autoclave (Ster PE 50–100 mini, ILPRA, Barcelona, Spain). The samples were cooled in a blast chiller (− 21 °C for 30 min) and then were stored in the dark at 4 °C for 160 days.

Five cans of pâté from each replicate and each batch were taken at 0, 45, 90, 125 and 160 days to determine pH, colour and thiobarbituric acid reactive substances (TBARS). The proximate composition and fatty acids profile of the manufactured pâté were analysed only at 0 days, while the volatile and the free fatty acids content was determined at the beginning and at the end of the storage period.

Proximate composition and physicochemical analysis

Moisture and protein were quantified according to the ISO recommended standards (ISO 1442: 1997 and ISO 937: 1978, respectively), while total fat was extracted according to the AOCS Official Procedure Am 5-04 (AOCS 2005). The pH of the samples was measured using a digital portable pH-meter (Hanna Instruments, Eibar, Spain) equipped with a penetration probe. Colour parameters were measured using a portable colorimeter (Konica Minolta CM-600d, Osaka, Japan) with pulsed xenon arc lamp filtered to illuminant D65 lighting condition. The colour was measured in five different points of each sample.

Lipid oxidation analysis

The TBARS assay was carried out according to the extraction method described by Vyncke (1975). TBARS values were calculated from a standard curve of malonaldehyde (MDA) and expressed as mg MDA/kg sample.

Fatty acids analysis

Total lipids were extracted from 12.5 g of sample, according to Bligh and Dyer (1959) procedure. Free fatty acids were separated from 20 mg of the extracted lipids using NH_2 -aminopropyl mini-columns (Sep-Pak Vac 3 cc, 500 mg, Waters, Milford, MA) as described by Kaluzny et al. (1985). The fatty acids were transesterified according to Domínguez et al. (2015) procedure. Separation and quantification of the fatty acid methyl esters (FAMES) was carried out using a gas chromatograph (GC-Agilent 6890 N; Agilent Technologies Spain, S.L., Madrid, Spain) equipped with a flame ionization

detector and an automatic sample injector HP 7683, and using a Supelco SPTM-2560 fused silica capillary column (100 m, 0.25 mm i.d., 0.2 μm film thickness). The chromatographic conditions were as described by Domínguez et al. (2015). Individual FAME peaks were identified by comparison of their retention times with those of the standards (Supelco 37 component FAME Mix, Supelco, USA). Quantification was performed using nonadecanoic acid (C19:0) at 0.3 mg/mL^{-1} as an internal standard. The results were expressed in grams per 100 g fat of detected FAMES and the analysis was carried out in triplicate.

Volatile compounds

The analysis of the volatile compounds was performed using HS-SPME-GC/MS method. One gram of each sample were weighed into a 24 mL headspace vial and sealed with a PTFE-faced silicone septum (Supelco, Bellefonte, PA, USA). A SPME device (Supelco, Bellefonte, PA, USA) containing a fused silica fibre (10 mm length) coated with a 50/30 μm layer of DVD/CAR/PDMS was used. Headspace SPME extraction and chromatographic conditions were carried out as described by Domínguez et al. (2014). The volatile content in pâté was calculated, based on the external standard technique, from a standard curve of peak total ion peak area vs. concentration. The results are expressed as pg/g of pâté.

Statistical analysis

A total of 200 liver pâté cans (five pâté cans for each batch x four batches x two replicates x five sampling points) were analysed for different parameters. The effect of different antioxidant extracts and the days of storage on physicochemical, proximate composition, lipid oxidation, fatty acids and volatile compounds content was examined using a mixed-model ANOVA, where these parameters was set as dependent variables, antioxidant extracts and the days of storage as fixed effect, and replicate as random effect. The pairwise differences between least-square means were evaluated by Duncan's method. Differences were considered significant if $P < 0.05$. The values were given in terms of mean values and standard deviation (SD). All statistical analysis was performed using IBM SPSS Statistics 19 software.

Results and discussion

Quantification of phenolic compounds and antioxidant activity in natural extracts

The quantification of phenolic compounds in BRE, CLE and PSE was performed with matching standards

(Table 1). The evaluation of BRE composition revealed the presence of nine compounds wherein the major compounds were catechin, (-)-epicatechin and benzoic acid (1.51, 0.94 and 0.79 mg/L, respectively). This result is in agreement with the study performed by Barbosa-Pereira et al. (2014) who reported catechin and epicatechin (41.30 and 50.59 mg/g extract, respectively) as major compounds in total phenolic composition (449 mg/g extract) of brewery waste stream. Phenolic acids as gallic acid, protocatechuic acid and 4-hydroxybenzoic acid (9.38, 16.69 and 3.15 mg/g, respectively) were also quantified but at lower concentration than flavonoids cited above. These authors also pointed out that composition and concentration of phenolic compounds in brewery residues can be influenced by many factors as plant species, system of cultivation, environmental conditions and processing technologies.

The evaluation of CLE revealed the presence of 10 compounds wherein gallic acid at 10.26 mg/100 g fresh weight (FW) was the major compound followed by catechin and protocatechuic acid (2.70 and 1.49 mg/100 g FW, respectively). Similarly, Dinis et al. (2012) reported gallic acid as major phenolic compound (in the range of 4.1–29.0 mg/g extract) in the phenolic composition of *Castanea sativa* Mill. cv. Judia in different ecotypes of Tras-os-Montes region. This study also pointed out that chestnut phenolic composition contains ellagic acid and flavonoids (in the ranges of 6.2–11.9 and 4.8–60.6 mg/g extract, respectively) which indicate that phenolic composition can be influenced by climatic conditions and other

environmental factors. Moreover, our results agree with the findings of other study, in which compounds such as catechin and ferulic acid were observed in chestnuts (*Castanea sativa* Mill.) (Nazzaro et al. 2011).

PSE was largely composed by catechin (20.66 mg/100 g dry matter). Other phenolic compounds as protocatechuic acid and rutin + isoquercetin were also present at the concentration of 3.79 and 2.57 mg/100 g dry matter. Similarly, Francisco and Resurreccion (2009) observed marked differences in phenolic content particularly for catechin that displayed concentrations of 74.35, 535.03 and 448.30 µg/g dry skin and for protocatechuic acid 7.62, 34.03 and 15.45 µg/g dry skin in Runner, Virginia and Spanish varieties, respectively.

The total phenolic content assay performed in previous studies (Pateiro et al. 2014; Munekata et al. 2015) revealed that BRE, CLE and PSE contained higher proportion of phenolic compounds. CLE showed the highest phenolic content (89 mg gallic acid equivalent (GAE)/g extract) compared to BRE and PSE (28.9 and 32.6 mg GAE/g extract, respectively). In this sense, the elevated proportion of gallic acid can be associated to the antioxidant potential of CLE (Dinis et al. 2012). In a similar way the elevated proportion of catechin can be related to the antioxidant activity of PSE (Francisco and Resurreccion, 2009) and particularly for BRE the combination of catechin, (-)-epicatechin and benzoic acid may explain the antioxidant activity of this natural extract (Barbosa-Pereira et al. 2014).

On the other side, the in vitro antioxidant evaluation indicated the potential application of BRE, CLE and PSE to prevent lipid oxidation (Lorenzo et al. 2013; Munekata et al. 2015; Pateiro et al. 2014, 2015a, 2015b). The results obtained from Trolox Equivalent Antioxidant Capacity assay indicated the higher antioxidant potential of CLE compared to BRE (0.27 and 0.09 g trolox/g extract). The radical scavenge activity measured by DPPH radical assay indicated similar relationship between CLE and BRE wherein DPPH values were 0.24 and 1.04 g equivalent BHT/g extract, respectively. This result may be explained by the higher concentration of gallic acid in CLE (Table 1).

Proximate composition, pH and colour parameters

The proximate composition revealed that the addition of natural antioxidants did not affect moisture (50.99–51.38 g/100 g), fat (51.64–57.78 g/100 g dry matter (DM)) and protein (29.25–30.04 g/100 g DM) content (Table 2). Since all batches were manufactured with the same formulation, this outcome was expected. In the same way, other authors reported no significant differences in the proximate composition for liver pâté manufactured with natural antioxidants compared to control treatments (Estévez et al. 2007; D'arrigo et al. 2004).

Table 1 Identification and quantification of specific phenolic compounds in BRE, CLE and PSE

Phenolic compound	BRE ^a	CLE ^b	PSE ^c
Benzoic acid	0.79	n.d.	n.d.
Caffeic acid	0.09	0.14	n.d.
<i>p</i> -Coumaric acid	0.75	0.54	n.d.
Ellagic acid	n.d.	0.54	n.d.
Ferulic acid	0.66	0.14	n.d.
Gallic acid	n.d.	10.26	n.d.
Protocatechuic acid	n.d.	1.49	3.79
Syringic acid	n.d.	0.27	n.d.
Vanillic acid	0.19	0.68	n.d.
Catechin	1.51	2.70	20.66
(-)-Epicatechin	0.94	n.d.	n.d.
Rutin + Isoquercitrin	0.09	n.d.	2.57
Vanillin	0.09	0.27	n.d.

BRE beer residue extract, CLE chestnut leave extract, PSE peanut skin extract, n.d. not detected

^amg/L

^bmg/100 g fresh weight

^cmg/100 g dry weight

A significant ($P < 0.001$) increase in pH values could be observed between 0 and 45 days followed by a reduction ($P < 0.001$) until day 160 of storage when pH value reached the 6.15 (Table 3). Changes in pH values during storage of pork liver pâté manufactured with natural extracts were also reported by other authors and may be considered normal for this type of product (Pateiro et al. 2014; Doolaege et al. 2012). On the other hand, statistical analysis did not indicate significant differences among batches elaborated with natural extracts, except in day 90. These findings suggest that properties of active compounds in the extracts (mainly phenolic acids) did not affect pH values of the batches. In this point of storage time, pâté elaborated with natural extracts (6.22, 6.21 and 6.20 in BRE, CLE and PSE batches, respectively) displayed significant ($P < 0.05$) lower pH value than control batch (6.27). A similar trend was reported by Lorenzo et al. (2013) who observed that pH values were not affected by the addition of antioxidants, although the highest pH values were found in the control group followed by BHT and chestnut batch. In addition, Jung, Shim, and Shin (2015) also did not find differences on final pH values between control and sausages manufactured with rosemary powder with mean pH values of 6.5. However, Pateiro et al. (2014) noticed the lowest pH values in liver pâté with chestnuts extract after 24 weeks of storage.

The evolution of colour parameters is displayed in Table 3. Generally, colour parameters were slight affected by natural extracts whereas significant changes during storage within each treatment were observed. The lightness (L^*) of all batches displayed gradual increase ($P < 0.001$) during refrigerated storage. The maximum L^* values were observed at day 90 wherein CLE batch (57.34) displayed the lowest luminosity among all batches (59.24, 58.57 and 59.38 for CON, BRE and PSE batches, respectively). The statistical evaluation of redness (a^* value) indicated significant differences among treatments in days 45, 90 and 125 wherein a^* values of CLE batch was lower than observed on CON, BRE and PSE batches (Table 3). Regarding the evolution of redness, this colour parameter displayed a common trend of increase and the same way as

L^* and b^* values, displayed maximum values in day 90. However, CON and BRE treatments displayed a significant decrease between days 90 and 160 but redness of CLE and PSE batches remained unchanged in the same period.

Similarly, the evolution of yellowness (b^* value) of all samples presented maximum values at day 90 in all treatments (b^* values around 21.22). Significant differences ($P < 0.01$) were observed by day 160 wherein BRE and CLE batches displayed the highest b^* values (20.57 and 20.79). These trends observed for colour parameters during storage are consistent with those reported by Estévez et al. (2007) in porcine liver pâté manufactured with rosemary and sage essential oils during 90 days of refrigerated storage. These authors also point out that redness stability was dependent on the type of pâté, since no significant differences were observed in control batch whereas antioxidants batches displayed significant reduction during storage. However, this result was not previously reported by Pateiro et al. (2014) who noticed different behaviours in each colour parameter of pork liver pâté manufactured with natural extracts. These authors observed that the increase in luminosity and reduction of redness were observed in pork liver pâté manufactured with green tea and chestnut extracts whereas no differences were observed for control group during refrigerated storage.

TBARS evolution

The lipid stability of pâté during refrigerated storage is shown in Table 4. Surprisingly, no differences were found between CON and antioxidant batches (BRE, CLE and PSE) for any day of refrigerated storage. In previous studies, we observed that the natural extracts presented elevated phenolic content (28.90, 89.01 and 32.60 mg GAE/g for BRE, CLE and PSE, respectively), in vitro antioxidant activity (TEAC: 0.09 and 0.27 g trolox/g extract for BRE and CLE, respectively) and potential to prevent lipid oxidation in meat products as pork patties (Lorenzo et al. 2014b), chicken patties (Munekata et al. 2015), chorizo (Lorenzo et al. 2013) and liver pâté (Pateiro et al. 2014). A possible explanation for these results may be

Table 2 Proximate composition of pig liver pâté elaborated with healthy oils and natural antioxidants (mean of five replicates)

(g/100 g)	Batches				Sig.
	CON	BRE	CLE	PSE	
Moisture	50.99 ± 0.94	51.12 ± 0.70	51.27 ± 0.7	51.38 ± 0.90	n.s.
Fat (dry matter)	52.67 ± 2.21	51.64 ± 2.27	57.78 ± 3.83	52.77 ± 2.44	n.s.
Protein (dry matter)	29.25 ± 0.75	30.04 ± 0.83	29.56 ± 0.76	29.40 ± 0.71	n.s.

Results are expressed as mean value ± standard deviation

Batches: CON control, BRE beer residue extract, CLE chestnut leave extract, PSE peanut skin extract

Sig significance, n.s. not significant

Table 3 The pH and colour parameters of pig liver pâté elaborated with healthy oils and natural antioxidants (mean of five replicates)

	Days	Batches				Sig.
		CON	BRE	CLE	PSE	
pH	0	6.23 ± 0.04 ^{βγ}	6.20 ± 0.05 ^β	6.21 ± 0.05 ^β	6.24 ± 0.06 ^β	n.s.
	45	6.26 ± 0.11 ^γ	6.32 ± 0.07 ^γ	6.29 ± 0.04 ^γ	6.32 ± 0.07 ^γ	n.s.
	90	6.27 ± 0.08 ^{βγ}	6.22 ± 6.20 ^{αβ}	6.21 ± 0.04 ^{αβ}	6.20 ± 0.04 ^{αβ}	*
	125	6.16 ± 0.03 ^{αβ}	6.17 ± 0.04 ^{αβ}	6.17 ± 0.02 ^α	6.17 ± 0.04 ^α	n.s.
	160	6.14 ± 0.05 ^α	6.15 ± 0.03 ^α	6.15 ± 0.03 ^α	6.16 ± 0.03 ^α	n.s.
	Sig.	***	***	***	***	
L*	0	49.80 ± 4.16 ^α	48.81 ± 6.25 ^α	49.52 ± 4.60 ^α	50.16 ± 6.40 ^α	n.s.
	45	52.95 ± 4.15 ^β	53.94 ± 3.09 ^{βγ}	51.86 ± 3.05 ^α	53.64 ± 3.21 ^{αβ}	n.s.
	90	59.24 ± 0.86 ^{βγ}	58.57 ± 0.81 ^{bd}	57.34 ± 0.91 ^{ab}	59.38 ± 0.92 ^{βγ}	***
	125	57.10 ± 2.21 ^γ	56.64 ± 2.63 ^{γδ}	55.21 ± 2.24 ^β	57.15 ± 1.67 ^{βγ}	n.s.
	160	49.20 ± 1.56 ^α	52.62 ± 3.65 ^β	50.42 ± 3.03 ^α	50.43 ± 3.90 ^α	n.s.
	Sig.	***	***	***	***	
a*	0	12.26 ± 0.82 ^α	12.16 ± 1.31 ^α	11.32 ± 1.30 ^α	12.08 ± 1.05 ^α	n.s.
	45	12.54 ± 0.80 ^{abα}	12.90 ± 0.45 ^{bb}	12.07 ± 0.55 ^{ab}	12.88 ± 0.58 ^{bb}	*
	90	13.13 ± 0.44 ^{bb}	13.29 ± 0.57 ^{bb}	12.73 ± 0.45 ^{ab}	13.24 ± 0.35 ^{bb}	*
	125	13.14 ± 0.48 ^{bb}	13.13 ± 0.62 ^{bb}	12.39 ± 0.73 ^{ab}	13.03 ± 0.57 ^{bb}	*
	160	12.62 ± 0.48 ^α	12.79 ± 0.56 ^{αβ}	12.54 ± 0.61 ^β	12.81 ± 0.65 ^β	n.s.
	Sig.	**	*	**	**	
b*	0	18.60 ± 1.59 ^α	18.36 ± 2.32 ^α	18.75 ± 2.19 ^α	18.66 ± 1.98 ^α	n.s.
	45	19.57 ± 1.45 ^β	20.16 ± 1.02 ^β	19.79 ± 1.15 ^{αβ}	20.37 ± 1.15 ^{βγ}	n.s.
	90	21.05 ± 0.46 ^γ	21.06 ± 0.64 ^γ	21.39 ± 0.52 ^γ	21.40 ± 0.53 ^γ	n.s.
	125	20.95 ± 0.66 ^γ	21.09 ± 0.85 ^β	21.01 ± 0.77 ^γ	21.21 ± 0.81 ^γ	n.s.
	160	19.11 ± 0.71 ^{αα}	20.57 ± 1.13 ^{bb}	20.79 ± 1.09 ^{bbγ}	20.09 ± 0.80 ^{abβ}	**
	Sig.	***	***	***	***	

Results are expressed as mean value ± standard deviation

Batches: *CON* control, *BRE* beer residue extract, *CLE* chestnut leave extract, *PSE* peanut skin extract

^{a,b}Means in the same row (corresponding to same storage day) not followed by a common letter are significantly different ($P < 0.05$)

^{α,δ}Mean values in the same column (corresponding to the same batch) not followed by a common letter differ significantly ($P < 0.05$)

Sig significance: *** ($P < 0.001$), ** ($P < 0.01$) * ($P < 0.05$), n.s. (not significant)

Table 4 TBARs evolution of pig liver pâté elaborated with healthy oils and natural antioxidants (mean of five replicates)

	Days	Batches				Sig.
		CON	BRE	CLE	PSE	
TBARs (mg MDA/kg sample)	0	0.53 ± 0.08 ^{βγ}	0.51 ± 0.09 ^γ	0.52 ± 0.10 ^{βγ}	0.51 ± 0.07 ^{βγ}	n.s.
	45	0.62 ± 0.32 ^γ	0.52 ± 0.09 ^γ	0.57 ± 0.13 ^γ	0.53 ± 0.11 ^γ	n.s.
	90	0.48 ± 0.16 ^{βγ}	0.43 ± 0.04 ^{βγ}	0.44 ± 0.10 ^β	0.44 ± 0.08 ^β	n.s.
	125	0.29 ± 0.06 ^α	0.29 ± 0.05 ^α	0.31 ± 0.05 ^α	0.29 ± 0.07 ^α	n.s.
	160	0.38 ± 0.19 ^{αβ}	0.36 ± 0.15 ^{αβ}	0.44 ± 0.19 ^β	0.29 ± 0.12 ^α	n.s.
	Sig.	**	***	***	***	

Results are expressed as mean value ± standard deviation

Batches: *CON* control, *BRE* beer residue extract, *CLE* chestnut leave extract, *PSE* peanut skin extract

^{α,γ}Mean values in the same column (corresponding to the same batch) not followed by a common letter differ significantly ($P < 0.05$)

Sig: significance: *** ($P < 0.001$), ** ($P < 0.01$), n.s. (not significant)

related to the impact of thermal treatment during the manufacture of pâté. It is possible, therefore, that the temperature and time applied could induce changes in structure of compounds involved in antioxidant activity reducing its antioxidant potential (Su et al. 2003). This study confirms that thermal treatment can induce degradation of phenolic compounds in pure compounds or natural extracts and reduce their concentration (Volf et al. 2014).

On the other hand, the evolution of TBARS values varied significantly during storage. TBARS index gradually increased when the maximum values were observed in day 45 (values in the range of 0.52–0.62 mg de MDA/kg sample) followed by significant reduction until the end of storage. These findings support the idea of several studies (Lorenzo et al. 2014b; Delgado-Pando et al. 2012) where the increase in TBARS values can be associated with chopping and thermal treatment during pâté processing and presence of pro-oxidant agents as iron (released from myoglobin during heating) which leads to formation of oxidation products as MDA from polyunsaturated fatty acids. The further reduction in TBARS values during storage also accords with our earlier observations in this type of meat product (Lorenzo et al. 2014b) and it is possible related to stability of MDA molecule. Our TBARS values are in accordance with the results observed by Doolaege et al. (2012), who pointed out positive effects of rosemary extract on lipid stability of pork liver pâté.

Free fatty acids

The free fatty acid (FFA) content of pâtés is presented in Table 5. The total FFA content was similar among batches in days 0 and 160 indicating that natural extracts (BRE, CLE and PSE) did not remarkably affect the FFA content during processing and storage, respectively in agreement with data reported by Pateiro et al. (2015b) in chorizo (traditional Spanish dry-cured sausage). On the other hand, total FFA content increase ($P < 0.001$) from 15 mg/100 g fat to 47–60 mg/100 g fat in all treatments between day 0 and 160. These results were in accordance with other authors who observed a significant increase in total FFA content in meat products during refrigerated storage (Zhao et al. 2011).

The main fraction of FFA for all treatments at both 0 and 160 days was composed by monounsaturated fatty acids (MUFA) with values in the range of 5.61–6.55 and 18.34–25.57 mg/100 g fat, respectively. FFA was also composed by saturated fatty acids (SFA), which were also increased during storage ($P < 0.001$). At day 0, the SFA fraction on FFA for batches CON, BRE and PSE were 5.99, 6.07 and 6.10 mg/100 g fat, respectively, in a significant ($P < 0.05$) higher range than the observed for

samples elaborated with CLE (4.76 mg/100 g fat). Oleic acid (C18:1n9c) was the main FFA for all batches at day 0 with values between 4.64 and 5.37 mg/100 g fat. After 160 days, C18:1n9c remained as the major fatty acid (14.51–20.66 mg/100 g fat), although no significant differences were observed among treatments. Barros et al. (2011) reported C18:1n9c as major fatty acid in FFA composition of beef burgers manufactured with *Boletus edulis* extract, but these authors observed slight changes over 20 days of refrigerated storage.

FFA amount in all batches with the exception of CLE batch where SFA was the main group, showed the following relationship of FA groups: MUFA > SFA > PUFA. This suggested a preferential release of MUFA than other FAs. Liberation of FA from molecules as phospholipids and triglycerides is a phenomenon influenced by several factors as endogenous lipases, raw materials, processing conditions additives and ingredients. In this sense, prior studies have noted the importance of endogenous lipases (Lorenzo et al. 2014a) as central players in the lipolysis process.

Interestingly, lower content ($P < 0.05$) of free n-3 fatty acids: α -linolenic acid (C18:3n3), *cis*-5,8,11,14,17-eicosapentaenoic acid (C20:5n3), docosapentaenoic acid (C22:5n3) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6n3) were present in FFA fraction of CLE batch compared to CON, BRE and PSE after 160 days of storage. Total free n – 3 fatty acid content of CLE treatment was also lower ($P < 0.05$) than observed for CON, BRE and PSE (1.71 vs 3.55, 4.08 and 3.52 mg/100 g fat for CON, BRE and PSE batches, respectively). Such outcome for CLE batch may be explained by the development of lipid oxidation and formation of derived volatile compounds, particularly for 3-methyl-butanal content (Table 6).

Volatile compounds of pork liver pâté with mix of oils and natural antioxidants

The four major lipid-derived volatile compounds between the first and last day of storage are shown in Table 6. Regarding the hexanal content, statistical analysis on the first day of storage indicated that CLE batch had the lowest concentration (51.97 pg/g) followed by intermediate concentrations of BRE and PSE treatments (71.16 and 72.18 pg/g, respectively) and the highest concentration in CON batch (82.85 pg/g). Such outcome can be associated with total phenolic content and antioxidant potential evaluated in previous studies where CLE had higher phenolic content than BRE and PSE (Pateiro et al. 2014; Munekata et al. 2015). However, antioxidant potential must be interpreted with caution because hexanal content was similar among batches at the end of storage period. In this sense our results were in disagreement with those reported

Table 5 Free fatty acid of pig liver pâté elaborated with healthy oils and natural antioxidants (mean of five replicates)

(mg/100 g fat)	Days	Batches				Sig.
		CON	BRE	CLE	PSE	
C12:0	0	0.01 ± 0.01 ^α	0.01 ± 0.01 ^α	0.01 ± 0.01	0.01 ± 0.01	n.s.
	160	0.06 ± 0.01 ^β	0.06 ± 0.01 ^β	0.47 ± 1.06	0.18 ± 0.39	n.s.
	Sig.	***	***	n.s.	n.s.	
C14:0	0	0.23 ± 0.05 ^{abz}	0.22 ± 0.05 ^{abz}	0.18 ± 0.03 ^{αα}	0.26 ± 0.05 ^{bx}	*
	160	0.74 ± 0.27 ^β	0.85 ± 0.25 ^β	0.62 ± 0.09 ^β	0.72 ± 0.31 ^β	n.s.
	Sig.	***	***	***	***	
C15:0	0	0.03 ± 0.01 ^α	0.04 ± 0.01	0.03 ± 0.01 ^α	0.04 ± 0.01	n.s.
	160	0.10 ± 0.06 ^β	0.06 ± 0.04	0.07 ± 0.04 ^β	0.06 ± 0.04	n.s.
	Sig.	**	n.s.	*	n.s.	
C16:0	0	3.69 ± 0.65 ^α	3.72 ± 0.62 ^α	2.83 ± 1.22 ^α	3.75 ± 0.64 ^α	n.s.
	160	12.25 ± 1.53 ^β	12.96 ± 2.94 ^β	11.39 ± 2.56 ^β	11.86 ± 4.45 ^β	n.s.
	Sig.	***	***	***	***	
C16:1n7	0	0.40 ± 0.05 ^{bx}	0.40 ± 0.05 ^{abz}	0.35 ± 0.05 ^{αα}	0.43 ± 0.05 ^{bx}	*
	160	1.45 ± 0.28 ^β	1.70 ± 0.56 ^β	1.21 ± 0.46 ^β	1.49 ± 0.46 ^β	n.s.
	Sig.	***	***	***	***	
C17:0	0	0.08 ± 0.02 ^{abz}	0.06 ± 0.03 ^{αα}	0.07 ± 0.02 ^{αα}	0.09 ± 0.02 ^{bx}	*
	160	0.23 ± 0.05 ^β	0.22 ± 0.11 ^β	0.18 ± 0.07 ^β	0.26 ± 0.05 ^β	n.s.
	Sig.	***	***	***	***	
C17:1n7	0	0.05 ± 0.01 ^α	0.05 ± 0.01	0.04 ± 0.01 ^α	0.04 ± 0.02 ^α	n.s.
	160	0.12 ± 0.09 ^β	0.13 ± 0.10	0.10 ± 0.04 ^β	0.17 ± 0.04 ^β	n.s.
	Sig.	*	n.s.	0.002	***	
C18:0	0	1.87 ± 0.35 ^α	1.94 ± 0.28 ^α	1.56 ± 0.22 ^α	1.85 ± 0.42 ^α	n.s.
	160	6.46 ± 1.07 ^β	6.48 ± 1.17 ^β	6.16 ± 1.29 ^β	6.56 ± 1.15 ^β	n.s.
	Sig.	***	***	***	***	
C18:1n9c	0	5.37 ± 1.12 ^α	5.27 ± 0.94 ^α	4.64 ± 0.84 ^α	5.23 ± 1.11 ^α	n.s.
	160	19.90 ± 4.41 ^β	20.66 ± 8.03 ^β	14.51 ± 5.01 ^β	19.48 ± 6.03 ^β	n.s.
	Sig.	***	***	***	***	
C18:1n7c	0	0.32 ± 0.04 ^α	0.33 ± 0.06 ^α	0.29 ± 0.04 ^α	0.35 ± 0.06 ^α	n.s.
	160	1.38 ± 0.27 ^β	1.49 ± 0.47 ^β	1.18 ± 0.26 ^β	1.43 ± 0.32 ^β	n.s.
	Sig.	***	***	***	***	
C18:2n6c	0	1.72 ± 0.30 ^α	1.87 ± 0.32 ^α	1.81 ± 0.61 ^α	1.82 ± 0.26 ^α	n.s.
	160	7.37 ± 1.50 ^β	7.73 ± 2.49 ^β	5.91 ± 1.98 ^β	7.93 ± 2.02 ^β	n.s.
	Sig.	***	***	***	***	
C20:0	0	0.03 ± 0.01 ^α	0.03 ± 0.01 ^α	0.03 ± 0.01 ^α	0.04 ± 0.01 ^α	n.s.
	160	0.17 ± 0.08 ^β	0.24 ± 0.16 ^β	0.14 ± 0.07 ^β	0.40 ± 0.33 ^β	n.s.
	Sig.	***	***	***	**	
C18:3n6	0	0.02 ± 0.01 ^α	0.02 ± 0.01 ^α	0.01 ± 0.01 ^α	0.01 ± 0.01	n.s.
	160	0.17 ± 0.15 ^β	0.12 ± 0.08 ^β	0.14 ± 0.09 ^β	0.65 ± 1.40	n.s.
	Sig.	***	***	***	n.s.	
C20:1n9	0	0.14 ± 0.04 ^{αα}	0.14 ± 0.09 ^{αα}	0.13 ± 0.02 ^{αα}	0.23 ± 0.10 ^{bx}	**
	160	0.86 ± 0.23 ^{bb}	0.87 ± 0.38 ^{bb}	0.45 ± 0.18 ^{ab}	0.75 ± 0.33 ^{bb}	*
	Sig.	***	***	***	***	
C18:3n3	0	0.14 ± 0.04 ^α	0.17 ± 0.06 ^α	0.16 ± 0.03 ^α	0.18 ± 0.07 ^α	n.s.
	160	0.69 ± 0.09 ^{bb}	0.67 ± 0.18 ^{bb}	0.44 ± 0.15 ^{ab}	0.69 ± 0.20 ^{bb}	**
	Sig.	***	***	***	***	
9c,11t-CLA	0	0.05 ± 0.01 ^α	0.04 ± 0.01 ^α	0.04 ± 0.01 ^α	0.05 ± 0.01 ^α	n.s.
	160	0.17 ± 0.10 ^β	0.15 ± 0.02 ^β	0.18 ± 0.03 ^β	0.16 ± 0.02 ^β	n.s.
	Sig.	***	***	***	***	
C20:2n6	0	0.05 ± 0.02 ^α	0.06 ± 0.01 ^α	0.06 ± 0.01 ^α	0.07 ± 0.02 ^α	n.s.
	160	0.24 ± 0.10 ^β	0.28 ± 0.10 ^β	0.18 ± 0.06 ^β	0.24 ± 0.10 ^β	n.s.
	Sig.	***	***	***	***	

Table 5 continued

(mg/100 g fat)	Days	Batches				Sig.
		CON	BRE	CLE	PSE	
C22:0	0	0.01 ± 0.01 ^α	0.01 ± 0.01 ^α	0.01 ± 0.01 ^α	0.01 ± 0.01 ^α	n.s.
	160	0.09 ± 0.02 ^β	0.09 ± 0.02 ^β	0.09 ± 0.02 ^β	0.10 ± 0.02 ^β	n.s.
	Sig.	***	***	***	***	
C20:3n6	0	0.03 ± 0.01 ^α	0.04 ± 0.01 ^α	0.03 ± 0.01 ^α	0.03 ± 0.01 ^α	n.s.
	160	0.12 ± 0.03 ^β	0.17 ± 0.06 ^β	0.17 ± 0.09 ^β	0.24 ± 0.23 ^β	n.s.
	Sig.	***	***	***	*	
C20:4n6	0	0.28 ± 0.04 ^α	0.31 ± 0.05 ^α	0.31 ± 0.06 ^α	0.31 ± 0.08 ^α	n.s.
	160	1.25 ± 0.27 ^β	1.35 ± 0.41 ^β	1.01 ± 0.46 ^β	1.20 ± 0.31 ^β	n.s.
	Sig.	***	***	***	***	
C20:5n3	0	0.11 ± 0.02 ^α	0.14 ± 0.05 ^α	0.17 ± 0.08 ^α	0.16 ± 0.05 ^α	n.s.
	160	0.68 ± 0.17 ^{bβ}	0.76 ± 0.31 ^{bβ}	0.33 ± 0.16 ^{aβ}	0.65 ± 0.26 ^{bβ}	**
	Sig.	***	***	*	***	
C22:5n3	0	0.07 ± 0.01 ^α	0.08 ± 0.02 ^α	0.08 ± 0.01 ^α	0.10 ± 0.03 ^α	n.s.
	160	0.40 ± 0.10 ^{bβ}	0.47 ± 0.19 ^{bβ}	0.24 ± 0.11 ^{aβ}	0.39 ± 0.15 ^{bβ}	*
	Sig.	***	***	***	***	
C22:6n3	0	0.25 ± 0.05 ^{aα}	0.29 ± 0.17 ^{aα}	0.39 ± 0.16 ^{ab}	0.50 ± 0.18 ^{abα}	**
	160	1.89 ± 0.63 ^{bβ}	1.96 ± 1.04 ^{bβ}	0.59 ± 0.40 ^a	1.58 ± 0.94 ^{bβ}	**
	Sig.	***	***	n.s.	**	
SFA	0	5.99 ± 1.00 ^{bα}	6.07 ± 0.91 ^{bα}	4.76 ± 1.31 ^{aα}	6.10 ± 1.09 ^{abα}	*
	160	21.20 ± 5.05 ^β	21.08 ± 4.43 ^β	19.49 ± 3.92 ^β	20.41 ± 5.49 ^β	n.s.
	Sig.	***	***	***	***	
MUFA	0	6.51 ± 1.21 ^α	6.38 ± 1.02 ^α	5.61 ± 0.86 ^α	6.55 ± 1.25 ^a	n.s.
	160	24.44 ± 5.34 ^β	25.57 ± 9.66 ^β	18.34 ± 5.90 ^β	24.16 ± 7.11 ^β	n.s.
	Sig.	***	***	***	***	
PUFA	0	2.74 ± 0.34 ^α	3.05 ± 0.48 ^α	3.07 ± 0.65 ^α	3.25 ± 0.51 ^a	n.s.
	160	12.13 ± 4.34 ^β	13.95 ± 4.66 ^β	9.39 ± 3.11 ^β	13.99 ± 3.32 ^β	n.s.
	Sig.	***	***	***	***	
n3	0	0.59 ± 0.10 ^{aα}	0.70 ± 0.26 ^{aα}	0.81 ± 0.24 ^{abα}	0.95 ± 0.32 ^{bα}	*
	160	3.55 ± 1.22 ^{bβ}	4.08 ± 1.70 ^{bβ}	1.71 ± 0.85 ^{aβ}	3.52 ± 1.59 ^{bβ}	*
	Sig.	***	***	**	***	
n6	0	2.11 ± 0.33 ^α	2.31 ± 0.36 ^α	2.22 ± 0.62 ^α	2.25 ± 0.32 ^α	n.s.
	160	8.35 ± 3.51 ^β	9.71 ± 3.09 ^β	7.50 ± 2.44 ^β	10.33 ± 2.08 ^β	n.s.
	Sig.	***	***	***	***	
Total	0	15.25 ± 2.29 ^α	15.50 ± 2.30 ^α	13.44 ± 2.47 ^α	15.90 ± 2.41 ^α	n.s.
	160	57.80 ± 8.86 ^β	60.62 ± 18.36 ^β	47.49 ± 10.95 ^β	58.68 ± 14.07 ^β	n.s.
	Sig.	***	***	***	***	

Results are expressed as mean value ± standard deviation

^{a,b}Mean values in the same row (corresponding to the same storage time) not followed by a common letter differ significantly ($P < 0.05$)

^{α,γ}Mean values in the same column (corresponding to the same batch) for each fatty acid or sum of fatty acid not followed by a common letter differ significantly ($P < 0.05$)

Batches: CON control, BRE beer residue extract, CLE chestnut leave extract, PSE peanut skin extract

Sig: significance: *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$), n.s. (not significant)

by Pateiro et al. (2014) who observed that hexanal content extracted from liver pate was lower in batches elaborated with tea and grape seed extract with respect to control group.

After 160 days of storage, the hexanal content was lower than observed in the first day of storage ($P < 0.001$) for CON, BRE and PSE whereas no significant differences

were observed for CLE batch. Such reduction is in agreement with results obtained in TBARS assay that indicated a slight reduction in lipid oxidation between the first and last day of storage (Table 4). Ansorena and Astiasarán (2004) reported no significant differences in hexanal concentration of dry fermented sausages manufactured with olive oil between 2 and 5 months of storage (vacuum package).

Table 6 Volatile compounds of pig liver pâté elaborated with healthy oils and natural antioxidants (mean of five replicates)

pg/g sample	Days	Batches				Sig.
		CON	BRE	CLE	PSE	
Hexanal	0	82.85 ± 19.64 ^{bβ}	71.16 ± 10.68 ^{abβ}	51.97 ± 2.23 ^{αα}	72.18 ± 16.58 ^{abβ}	*
	160	44.84 ± 5.19 ^{αα}	40.67 ± 7.85 ^{αα}	41.30 ± 8.78 ^{αα}	39.95 ± 6.27 ^{αα}	n.s.
	Sig.	***	***	n.s.	***	
Heptanal	0	2.36 ± 0.62	2.33 ± 0.49	1.91 ± 0.24 ^α	2.54 ± 0.54	n.s.
	160	2.62 ± 0.52	2.98 ± 1.22	3.28 ± 0.96 ^β	2.54 ± 0.33	n.s.
	Sig.	n.s.	n.s.	*	n.s.	
Pentanal	0	0.00 ± 0.00 ^α	0.00 ± 0.00 ^α	0.00 ± 0.00 ^α	0.00 ± 0.00 ^α	n.s.
	160	133.65 ± 29.44 ^β	139.01 ± 26.14 ^β	156.23 ± 22.17 ^β	140.27 ± 17.97 ^β	n.s.
	Sig.	***	***	***	***	
3-Methyl-butanol	0	119.16 ± 38.10 ^β	123.61 ± 8.07 ^β	145.70 ± 23.15	109.22 ± 25.12 ^β	n.s.
	160	78.68 ± 10.11 ^{αα}	79.30 ± 17.14 ^{αα}	132.99 ± 25.58 ^b	60.09 ± 9.89 ^{αα}	***
	Sig.	*	**	n.s.	**	

Results are expressed as mean value ± standard deviation

Batches: *CON* control, *BRE* beer residue extract, *CLE* chestnut leave extract, *PSE* peanut skin extract

^{a,b}Mean values in the same row (corresponding to the same storage time) not followed by a common letter differ significantly ($P < 0.05$)

^{α,γ}Mean values in the same column (corresponding to the same batch) for each fatty acid or sum of fatty acid not followed by a common letter differ significantly ($P < 0.05$)

Sig: significance: *** ($P < 0.001$), ** ($P < 0.01$), * ($P < 0.05$), n.s. (not significant)

Differently than observed in the present study, Estévez et al. (2007) reported a significantly lower hexanal content in liver pâté produced with sage and rosemary extracts compared to control treatment after 90 days of chilled storage.

Conclusion

The addition of natural extracts (sources of phenolic compounds) did not promote remarkable changes in oxidative stability of pork liver pâté manufactured with mix healthy oils during 160 days of refrigerated storage. Natural extract reduced the hexanal content in the first day of storage but no further significant effects were observed. The proximate composition and other parameters as instrumental colour, pH, TBARS index, FFA and lipid-derived volatile compounds were not affected by natural antioxidants during storage. Therefore, the natural extracts applied did not promote additional protection for pork liver pâté healthy oils during refrigerated storage.

Acknowledgements This work was supported by the Xunta de Galicia (Grant Number FEADER 2013/34). The authors would like to thank National Council for Scientific and Technological Development (CNPq n.º 248705/2013-0) and Biomega Natural Nutrients S.L. for the fish oil.

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