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Stability of phenolic compounds in dry fermented sausages added with cocoa and grape seed extracts

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ABSTRACT

The level of eleven target phenolic compounds was evaluated in dry fermented sausages added with vegetable extracts. Grape seed (GSE1 and GSE2) and cocoa extracts, rich in phenolic compounds, were added in the formulation of dry fermented sausages ("salchichón" and "fuet"). Evolution of the major monomeric and oligomeric phenolic compounds of these extracts was evaluated during sausage shelf life by UHPLC-MS/MS. Kind of sausage did not affect significantly overall stability of the target compounds. At the end of the ageing process, catechin and epicatechin were at 54–61%, gallic acid and galloylated flavan-3-ols at 59–91%, oligomeric flavan-3-ols at 72–95% and glycosylated flavonols at 56–88% (in cocoa treatment) and 82–94% (in GSE treatment) of the contents that were added to the meat batter. All phenolic compounds levels did not decrease further significantly after ageing until the end of shelf life. Sensory analyses showed no important differences between control and cocoa added products, while grape seed addition gave these products abnormal sensory profiles. The 0.5% (w/w) addition of vegetal extracts was suitable to enrich dry fermented sausages with health-beneficial polyphenols.

1. Introduction

Unbalanced diets have been related to some chronic diseases, such as obesity, cancer and cardiovascular disease (WHO/FAO, 2003). Besides their fat content, meat products are an important source of highly bioavailable proteins, vitamins and minerals, so an improvement of their overall nutritional balance would be appealing from a nutritional point of view (Jiménez-Colmenero, Carballo, & Cofrades, 2001). Cured pork sausages, generally seasoned with spices, are very typical in the Spanish gastronomy. "Salchichón" is a large (35–90 mm Ø) acid fermented sausage while "fuet" is a thin (20–35 mm Ø) low acid fermented sausage.

Phenolic compounds constitute a group of plant secondary metabolites that includes flavonoids and phenolic acids (Crozier, Jaganath, & Clifford, 2009). Cocoa (*Theobroma cacao* L.) extracts are particularly rich in flavan-3-ols (catechin and epicatechin) and their oligomeric forms (procyanidins), and contain also glycosides of quercetin (Sánchez-Rabaneda et al., 2003). Grape (*Vitis vinifera* L.) seeds extracts are another important source of phenolic compounds, especially monomeric flavan-3-ols, procyanidins and several galloylated derivatives (Monagas, Garrido, Bartolomé, & Gómez-Cordovés, 2006).

Cocoa and grape seed phenolic compounds have exhibited positive health effects, such as anti-inflammatory activity and protection against free radicals, LDL peroxidation, carcinogenic metabolites and altered gene expression (Bagchi et al., 2000; Lamuela-Raventós, Romero-Pérez, Andrés-Lacueva, & Tornero, 2005; Singh, Tyagi, Dhanalakshmi, Agarwal, & Agarwal, 2004; Steinberg, Bearden, & Keen, 2003). Several works emphasized the potential of dietary phenolic compounds to prevent undesirable effects for consumers health related to the high consumption of fatty foods, such as the absorption of malondialdehyde (Gorelik, Ligumsky, Kohen, & Kanner, 2008) or some alterations of lipid metabolism (Quesada et al., 2009).

In recent years, many vegetal extracts rich in phenolic compounds have been put in the market as functional ingredients (Fernández-Ginés, Fernández-López, Sayas-Barberá, & Pérez-Alvarez, 2005). Also, the use of plant extracts rich in phenolic compounds has been suggested for their technological properties in meat products, such as to extend shelf life, improve quality and prevent rancidity (Ciriano et al., 2009; Coronado, Trout, Dunshea, & Shah, 2002; Gil, Bañón, Cayuela, Laencina, & Garrido, 2001; Hayes, Stepanyan, Allen, O'Grady, & Kerry, 2011). At the same time, health

Abbreviations: GSE, grape seed extract; MRM, multiple reaction monitoring.

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concerns about synthetic antioxidants stimulate the use of natural ingredients with antioxidant properties (Gil et al., 2001). Nevertheless, the use of vegetal extracts as functional ingredients in meat products should be supported by the evaluation of the phenolics content in these products and their evolution during shelf life. To the best of our knowledge, few data are available about the residual levels of phenolic compounds in dry fermented sausages added with vegetal extracts, namely hesperidin from orange fibre (Fernández-López et al., 2007).

Therefore, the aim of this work was to evaluate the concentration of main target phenolic compounds in typical Spanish dry fermented sausages added with three different vegetal extracts very rich in phenolic compounds, from their production to the end of the expected commercial shelf life. Two extracts from grape seed and one from cocoa were considered, and a suitable UHPLC–MS/MS protocol of analysis was developed to measure the concentration of the main target phenolic compounds in the specific matrices. Sensory analysis was carried out to sensory evaluate both extract-added and traditional products.

2. Material and methods

2.1. Chemicals

Ultrapure water was obtained with a Milli-Q Advantage system (Millipore Ibérica, Madrid, Spain). Acetone and methanol HPLC grade were from J.T. Baker (Deventer, The Netherlands). Acetonitrile LC-MS grade, CHAPS, formic acid, ascorbic acid, gallic acid and procyanidin B2 were obtained from Sigma–Aldrich (Madrid, Spain). Catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, procyanidin B1, quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside were purchased from Extrasynthèse (Genay, France). Procyanidin C1 was from PhytoLab (Vestenbergsgreuth, Germany) and quercetin-3-*O*-arabinoside from ChromaDex (Santa Ana, CA, USA).

Individual standard stock solutions at 1 g/l were prepared by dissolving the pure commercial standards in methanol. Fortified samples for calibration, accuracy and recovery assays were spiked with the appropriate amounts of a standard mix solution (50 mg/l in methanol). Standard solutions for MS infusion were prepared at 100 mg/l by diluting standard stock solutions with methanol. Chromatographic solutions of each standard were prepared by diluting stock solutions in mobile phase A.

2.2. Vegetal extracts

Two commercial grape seed extracts (*V. vinifera* L.), "Grape Seed Dry Extract" (Exxentia, Madrid, Spain) and "Leucocyanidins Grape Seed Extract" (Euromed, Barcelona, Spain), and a commercial cocoa extract (*T. cacao* L.) "Cocoanox Extract" (Natraceuticals, Valencia, Spain) were employed for the experiments.

2.3. Dry fermented sausages

Pork meat from shoulders and bellies was frozen at −20 °C for 3 days, thawed at 4 °C for 2 days and minced at −1 °C in a grinder (Tecmaq, Barcelona, Spain) with an adjustable plate set at diameter of 6 mm. For the production of "fuet", the formulation of ingredients per kilogram of ground meat (fresh weight basis) was as follows: 600 g pork shoulder meat, 400 g pork belly meat, 20 g sodium chloride, 0.15 g sodium nitrite, 0.15 g potassium nitrate, 0.5 sodium ascorbate, 2 g dextrose, 2.5 g black pepper, 15 g sodium lactate and 5 g of vegetal extracts (except control samples). The ingredients dosage in "salchichón" production per kg of ground meat (fresh weight basis) was as follows: 600 g pork shoulder meat, 400 g pork belly meat, 24 g sodium chloride, 0.15 g sodium nitrite, 0.15 g potassium nitrate, 0.50 g sodium ascorbate, 5 g dextrose, 2.5 g black pepper, 0.25 g lactic acid bacteria (starter) and 5 g of vegetal extracts (except control samples). About 13 kg and 40 kg of ground meat were employed for each treatment in "fuet" and "salchichón" respectively. Ground meat and other ingredients were mixed in a mixer (model 35P, Tecnotrip S.A., Terrassa, Spain).

Four different formulations for each kind of sausage were considered: *i*) Control (without any vegetal extract), *ii*) Cocoa (added with "Cocoanox Extract"), *iii*) GSE1 (added with "Grape Seed Dry Extract") and *iv*) GSE2 (added with "Leucocyanidins Grape Seed Extract").

"Fuet" samples were stuffed into 40 mm diameter natural casings and "salchichón" samples into 80 mm collagen casings (Fibran, Girona, Spain). Nine replicates for each formulation and for each production were considered, giving a total of $n = 36$ "fuet" and $n = 36$ "salchichón" samples. Fresh weight was about 350 g per piece for "fuet" and 1100 g per piece for "salchichón". "Fuet" sausages were dipped in a *Penicillium* spp. (Danisco, Copenhagen, Denmark) suspension to obtain the typical external appearance of this product, fermented at 18–20 °C and 80–85% of RH for 48 h to facilitate mould growth and then dried at 12–14 °C and 75–80% RH during 15 days. "Salchichón" samples were fermented at 20–22 °C with a relative humidity of 90–95%, until pH decreased to 5.0, and then ripened under controlled conditions (10–12 °C and 75–80% RH) for 30 days.

The weight loss during ageing was around 35% for "salchichón" and 45% for "fuet". Following the ageing process, both "fuet" and "salchichón" sausages were vacuum-packaged and stored at 4 °C during two months (expected commercial shelf life). Three samples of raw meat preparation (T0), three sausages at the end of the ageing process (T1) and three samples at the end of the commercial shelf life (T2) of "fuet" and "salchichón" from each treatment were vacuum packed and stored at −20 °C until the analyses were carried out. Three additional T1 samples were taken for both "fuet" and "salchichón" from each treatment for sensory evaluation. Fat and water content at T1 were measured by near infrared spectroscopy using FoodScan® (FOSS, Hillerød, Denmark).

2.5. UHPLC–MS/MS analysis

Different extractants were tested: *i)* methanol in water (50 ml/100 ml) with 0.1 g/100 ml ascorbic acid; *ii)* methanol in water (50 ml/100 ml) with 1 g/100 ml CHAPS and 0.1 g/100 ml ascorbic acid; *iii)* methanol in water (50 ml/100 ml) with 1 g/100 ml SDS and 0.1 g/100 ml ascorbic acid and *iv)* acetone in water (70 ml/100 ml) with 0.1 g/100 ml ascorbic acid.

Two grams of samples, added with 25 mg/kg of epigallocatechin from standard stock solution as internal standard, were homogenized with 15 ml of extracting solution, and then centrifuged in a J2-MC centrifuge (Beckman–Coulter, Fullerton, CA, USA). Clear phase was separated; pellet was mixed again with 5 ml of extracting solution, centrifuged as above and clear extracts reunified.

Two ml of supernatant were evaporated under nitrogen, redissolved in 1 ml mobile phase A, filtered through PTFE 0.2 μm porosity filters (Teknokroma, Sant Cugat, Spain) and injected to the UHPLC system. Chromatographic system consisted of an Acquity UPLC® (Waters, Milford, MA, USA), equipped with a diode array detector (DAD), an electrospray (ESI) as a source of ionization and a triple quadrupole mass spectrometer (TQD). The system was controlled by MassLynx 4.1 software (Waters, Milford, MA, USA). Chromatographic separation was carried out with a BEH C_{18} Shield column (150 × 1.0 mm id) with 1.7 μm particle size (Waters, Milford, MA, USA), kept at 35 °C. A linear gradient elution was carried out from 100% mobile phase A (5 ml/100 ml acetonitrile and 0.1 ml/

100 ml formic acid in water) and 0% B (60 ml/100 ml acetonitrile and 0.1 ml/100 ml formic acid in water) to 55% A in 15 min, with a flow rate of 0.130 ml/min. Sample vials on the injector were maintained at 4 °C and the injected volume was 5 μl.

Electrospray interface (ESI) was operated in the negative mode; the source temperature was fixed at 140 °C, the capillary voltage was set at −2.5 kV and the desolvation temperature was set at 350 °C. The cone gas (nitrogen) flow rate was 350 l/h and cone voltage was set at 30 V. MS experiments were carried out in "Scan" mode, in order to obtain *m/z* values of molecular ions. MS/MS experiments were also performed to obtain the fragmentation patterns of molecular ions, in "Daughter Ions" mode. The gas used in the collision cell was argon at a flow rate of 0.1 ml/min "Multiple Reaction Monitoring" (MRM) mode was used to quantify the phenolic compounds. MS/MS parameters were optimized with the AutoTune software by the infusion of standard solutions.

Target compounds were identified by comparing retention times, UV spectra, MS and MS/MS data with pure commercial standards. Standard addition calibration was created by spiking control samples with known amounts of standards, using epigallocatechin as internal standard. Quantitation was made by considering the areas obtained with a specific transition for each compound and epigallocatechin (*i.s.*) in the MRM mode. A qualification transition was also acquired to support identification. Limits of detection (LOD) were calculated on the basis of the calibration curves as the concentration corresponding to a signal to noise ratio (*s/n*) of 3. Absolute recoveries were estimated by comparing the response of three control samples, spiked before extraction with known amounts of pure standards, with the response of the same samples spiked after extraction at the same concentration. Accuracy was evaluated by analyzing 3 spiked control samples at different concentration levels. Repeatability of the method was estimated by analyzing three samples (at three different aging stages) per triplicate.

2.6. Sensory evaluation

Samples were unpacked and held at room temperature for 24 h before sensory evaluation. Eight selected and trained assessors (ASTM, 1981; ISO 8586-1, 1993; ISO 8586-2, 1994) undertook the sensory analysis on 2 mm slices of fermented sausages. The generation of the descriptors was carried out by open discussion in two previous sessions.

The descriptors retained were: cured colour intensity (intensity of cured colour of the lean), ability to peel-off the casing (difficulty of peeling), product adhered to casing (score of the amount of the fermented sausage adhered to the casing after to peel-off), odour intensity (intensity of typical odour of low-acid fermented product), ripened odour (pleasant odour developed by cured meat products), pungency (intensity of the pungent odour related with pepper), sweetness (basic taste sensation elicited by sugar), saltiness (basic taste sensation elicited by NaCl), acid taste (basic taste sensation elicited by an acid), bitterness (basic taste sensation elicited by caffeine and L-Tryptophan), piquantness (stinging sensation in the mouth and throat), ripened flavour (pleasant flavour characteristic of fermented sausages), hardness (force required to bite through the sample), gumminess (textural property that can be demonstrated using a rubber band), crumbliness (textural property characterised by ease with which a sample can be separated into smaller particles during chewing). Overall sensory quality was also evaluated by each individual panellist (scoring of the sensory quality of the sample by reference to the standard of quality for this product). A non-structured scoring scale (Amerine, Pangborn, & Roessler, 1965) was used, where 0 meant absence of the descriptor and 10 meant high intensity of the descriptor. Sensory evaluation was undertaken in 3 sessions per product ("fuet" and "salchichón") and a complete block design was used (Steel & Torrie, 1983), where each taster assessed all the treatments in each session. Samples were coded with three random numbers and were presented to the assessors balancing the first-order and the carry-over effects according to MacFie, Bratchell, Greenhoff, and Vallis (1989).

2.7. Statistical analysis

Statistical treatments of data were made using the JMP 8 software (SAS Institute Inc., Cary, NC, USA). A three-way ANOVA was carried out, to put in evidence the effect of the independent variables "Formulation" (Cocoa, GSE1 and GSE2), "Sampling time" (0, 1 and 3 months since production) and "Production set" ("fuet" and "salchichón") on the levels of target compounds. For sensory evaluation, data from the Quantitative Descriptive Analysis was performed over the mean score (8 panellists) for each "fuet"/"salchichón". Samples with grape seed extracts were not included in the model since they had appearance and flavour sensory characteristics too far from the typical products. The model included the batch (control and cocoa) and the tasting session as fixed effects. Interactions that were not statistically significant ($p \geq 0.05$) were dropped from the models. Differences between means were tested with the Tukey test ($\alpha = 0.05$).

3. Results and discussion

3.1. UHPLC–DAD–MS/MS analysis

Preliminary trials were carried out with different extracting solutions; better absolute recoveries were obtained by using

Table 1
UHPLC method validation parameters.

Compound	Calibration parameters[a]			Recovery (%) (mean ± SD)	% RSD	Accuracy (%) (mean ± SD)	Theoretical plates (*N*)	LOD[b] (μg/kg)
	b	*a*	r^2					
Gallic acid	2.567	$4.5 \cdot 10^{-2}$	0.998	96 ± 12	5.4	104 ± 13	1569	580
Catechin	0.852	$0.9 \cdot 10^{-2}$	0.998	89 ± 11	4.2	98 ± 13	31,408	470
Procyanidin B1	0.667	$-0.6 \cdot 10^{-2}$	0.999	62 ± 4	4.5	108 ± 3	33,856	300
Epicatechin	0.839	$0.5 \cdot 10^{-2}$	0.997	75 ± 5	6.8	94 ± 10	54,262	510
Procyanidin B2	0.682	$-0.5 \cdot 10^{-2}$	0.999	61 ± 5	7.1	97 ± 8	43,531	200
Epigallocatechin gallate	1.897	$-0.7 \cdot 10^{-2}$	0.997	69 ± 5	3.3	102 ± 13	57,712	100
Procyanidin C1	0.273	$-0.3 \cdot 10^{-2}$	0.999	56 ± 9	2.2	105 ± 11	67,981	290
Epicatechin gallate	1.229	$-0.6 \cdot 10^{-2}$	0.999	85 ± 7	2.3	110 ± 9	69,156	110
Quercetin-3-*O*-galactoside	3.270	$1.0 \cdot 10^{-2}$	0.993	88 ± 8	5.0	95 ± 12	112929	15
Quercetin-3-*O*-glucoside	3.473	$0.4 \cdot 10^{-2}$	0.996	89 ± 6	5.1	90 ± 4	115,936	20
Quercetin-3-*O*-arabinoside	1.335	$0.5 \cdot 10^{-2}$	0.997	95 ± 9	3.1	89 ± 3	124,872	20

[a] Regression equation: $Y = (b^*X) + a$, where *Y* is (Area compound/Area *i.s.*) and *X* is (mg kg^{-1} compound/mg kg^{-1} *i.s.*) obtained in MRM mode.
[b] LOD: limit of detection.

Table 2
Chromatographic, spectroscopic and spectrometric parameters used to identify and quantify the target phenolic compounds in samples added with cocoa and grape extracts.

Compound		t_R (mean ± SD) (min)	DAD signal	MS/MS parameters		
			λ_{max} (nm)	$[M-H]^-$ (m/z^-)	$MRM_{quantification}$ (m/z^-)	$MRM_{qualification}$ (m/z^-)
1	Gallic acid[a]	1.95 ± 0.01	271	169	169 > 125	169 > 79
	Epigallocatechin (*i.s.*)	5.51 ± 0.01	273	305	305 > 125	305 > 167
2	Catechin	5.95 ± 0.02	279	289	289 > 245	289 > 109
3	Procyanidin B1	6.19 ± 0.01	279	577	577 > 289	577 > 407
4	Epicatechin	7.39 ± 0.01	279	289	289 > 245	289 > 109
5	Procyanidin B2	7.90 ± 0.01	279	577	577 > 289	577 > 407
6	Epigallocatechin gallate[a]	9.59 ± 0.01	274	457	457 > 169	457 > 125
7	Procyanidin C1	10.00 ± 0.01	280	865	865 > 125	865 > 287
8	Epicatechin gallate	12.03 ± 0.02	278	441	441 > 169	441 > 125
9	Quercetin-3-*O*-galactoside[b]	12.78 ± 0.01	255, 355	463	463 > 301	463 > 271
10	Quercetin-3-*O*-glucoside	12.96 ± 0.01	256, 353	463	463 > 301	463 > 271
11	Quercetin-3-*O*-arabinoside[b]	14.09 ± 0.03	255, 353	433	433 > 301	433 > 271

[a] Only in samples added with grape seed extracts.
[b] Only in samples added with cocoa extract.

acetone in water (70 ml/100 ml) with 0.1 g/100 ml ascorbic acid. As a general rule, recoveries were higher than 75% for most compounds except for some oligomeric compounds, whose values were lower (Table 1). The higher molecular size of procyanidins and the well known interaction between such compounds and proteins (Manach, Scalbert, Morand, Rémésy, & Jimenez, 2004) could explain why procyanidin recoveries from a meat-based matrix were relatively lower.

The UHPLC conditions were chosen to obtain the chromatographic separation within 14 min. Under our conditions, the number of theoretical plates ranged from 1570 to 124,870, while LODs were comprised in the range between 1 and 60 μg/100 g (Table 1), being relatively high for flavan-3-ols and gallic acid, but still suitable for our scopes, taking into account the content of the target compounds in samples.

Table 2 shows the identification parameters for the analysis of the different compounds on the basis of chromatographic, spectroscopic and spectrometric properties of the pure standard solutions. Two specific MS/MS transitions and the DAD spectra were used to confirm the identity of the molecule.

Epigallocatechin was not detected in any treatment, pointing out the suitability of using it as internal standard. Fig. 1 shows a typical MRM profile of a "fuet" added with Cocoa extract, where nine phenolic compounds were identified. The profile of glycosylated flavonols, monomeric flavan-3-ols and their oligomers is in agreement with those reported for cocoa samples in other works (Sánchez-Rabaneda et al., 2003; Tomás-Barberán et al., 2007). In addition, a galloylated flavan-3-ol (epicatechin gallate) was quantified in samples added with Cocoa extract.

Fig. 2 shows the MRM profile of a "fuet" added with GSE2 extract. Detection of gallic acid, quercetin glucoside, monomeric, dimeric and trimeric flavan-3-ols and their galloylated derivatives as major compounds in grape seed ingredients is in accordance with previously reported data for this kind of extracts (Monagas et al., 2006).

3.2. Phenolic compounds stability in dry fermented sausages

ANOVA results showed that kind of sausage ("fuet" or "salchichón") did not affect significantly the phenolic compounds stability. On the contrary, the different source of the vegetable extracts significantly influenced, as expected, the phenolic profile of the samples. Significant effects on several target compounds were also found for the factor "sampling time" and the interaction "formulation × sampling time" (Table 3).

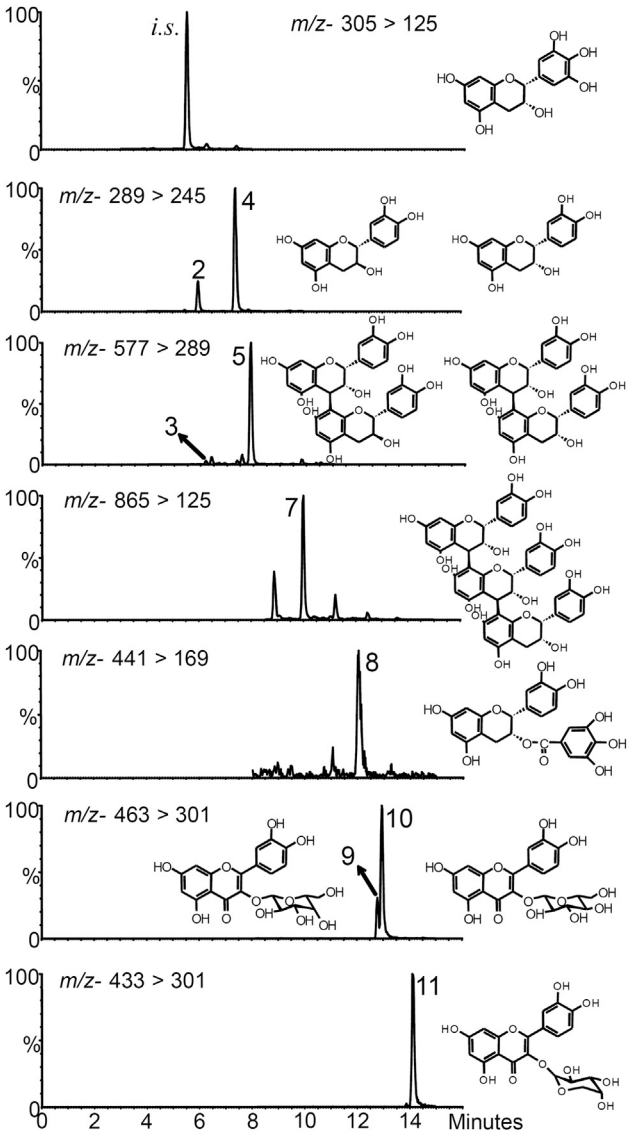

Fig. 1. Multiple Reaction Monitoring (MRM) profile of "fuet" with Cocoa extract. Peaks: ***i.s.***, epigallocatechin (internal standard); **2**, catechin; **3**, procyanidin B1; **4**, epicatechin; **5**, procyanidin B2; **7**, procyanidin C1; **8**, epicatechin gallate; **9**, quercetin-3-*O*-galactoside; **10**, quercetin-3-*O*-glucoside; **11**, quercetin-3-*O*-arabinoside.

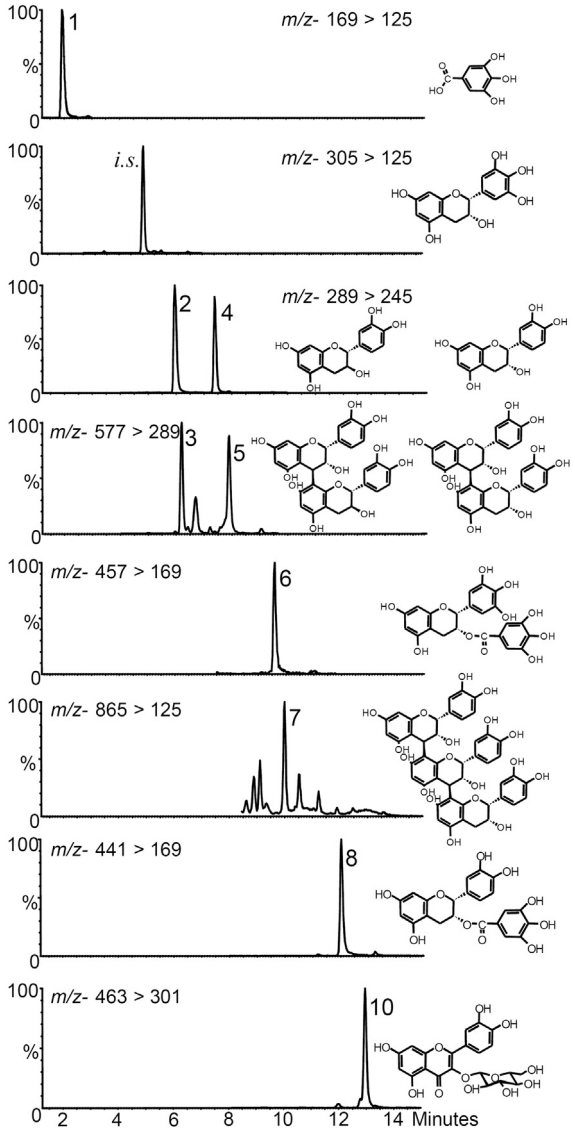

Fig. 2. Multiple Reaction Monitoring (MRM) profile of "fuet" with grape seed extract (GSE2). Peaks: **1**, gallic acid; ***i.s.***, epigallocatechin (internal standard); **2**, catechin; **3**, procyanidin B1; **4**, epicatechin; **5**, procyanidin B2; **6**, epigallocatechin gallate; **7**, procyanidin C1; **8**, epicatechin gallate; **10**, quercetin-3-*O*-glucoside.

Figs. 3–4 show the mean values of the target compounds for the combinations of factors "formulation" and "sampling time" to highlight the level and the behaviour of the phenolic compounds in fermented sausages during their production and storage. Phenolic profile of cocoa samples was characterized by high levels of epicatechin, procyanidins B2 and C1, and glycosylated flavonols. Samples added with the two grape seed extracts showed distinct phenolic profiles, being GSE1 samples more rich in catechin and epicatechin (GSE1), while GSE2 samples had higher content of procyanidins, gallic acid and galloylated flavan-3-ols.

As a general rule, phenolic compounds showed an initial reduction at the end of the ageing process (T1, Figs. 3–4). Anyway, levels of bioactive polyphenols did not decrease further significantly, at least until the end of commercial shelf life (T2). These results seem in concordance with the work of Fernández-López et al. (2007), who found that hesperidin, a phenolic compound from orange by-products, was very stable in dry fermented sausages over time.

At the end of ageing, catechin and epicatechin were at 54–61% of their content at the beginning of production (Fig. 3). Oligomeric flavan-3-ols (procyanidins) generally showed higher residual levels than their corresponding monomeric compounds (72–95% residual). During this study no significant changes of the procyanidins concentration was observed in the samples added with grape seed extract GSE1; in a similar way, catechin and procyanidin B1 in the samples added with the cocoa extract, and procyanidin C1 in the samples added with grape seed extract GSE2 did not vary significantly over time (Fig. 3).

Epigallocatechin gallate and gallic acid were found only in samples added with extracts from grape seeds, and once again, the relative content of these compounds was significantly different depending on the extracts. Gallic acid showed a high residual content at T1 in GSE2 samples (81% of the initial content), and did not significantly decrease in samples added with GSE1 extract (Fig. 4). Epigallocatechin gallate was at 59–68% of its initial content at T1. Epicatechin gallate showed a similar behaviour in GSE2 samples (61%), while its stability was significantly better in GSE1 and Cocoa samples (Fig. 4).

Significantly higher amounts of glycosylated flavonols (quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside and quercetin-3-*O*-arabinoside) were found in samples added with cocoa extract (Fig. 4). At T1, flavonols from cocoa extract were found in dry fermented sausages at a concentration comprised between 56% and 88% of their initial content, while the concentration of quercetin-3-*O*-glucoside in GSE1 and GSE2 samples was not significantly modified over time (Fig. 4).

Table 3
Three-way ANOVA results, with main effects of independent variables and their interactions.

Dependent variables	Independent variables			Interactions			
	Type of sausage (*a*)	Formulation (*b*)	Sampling time (*c*)	*a* × *b*	*a* × *c*	*b* × *c*	*a* × *b* × *c*
Gallic acid	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Catechin	*n.s.*	***	***	*n.s.*	*n.s.*	*	*n.s.*
Procyanidin B1	*n.s.*	***	**	*n.s.*	*n.s.*	**	*n.s.*
Epicatechin	*n.s.*	***	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Procyanidin B2	*n.s.*	***	***	*n.s.*	*n.s.*	*	*n.s.*
Epigallocatechin gallate	*n.s.*	***	***	*n.s.*	*n.s.*	***	*n.s.*
Procyanidin C1	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Epicatechin gallate	*n.s.*	***	***	*n.s.*	*n.s.*	***	*n.s.*
Quercetin-3-*O*-galactoside	*n.s.*	***	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Quercetin-3-*O*-glucoside	*n.s.*	***	***	*n.s.*	*n.s.*	***	*n.s.*
Quercetin-3-*O*-arabinoside	*n.s.*	***	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*

n.s. : not significant ($p \geq 0.05$).
*: $p < 0.05$.
**: $p < 0.01$.
***: $p < 0.001$.

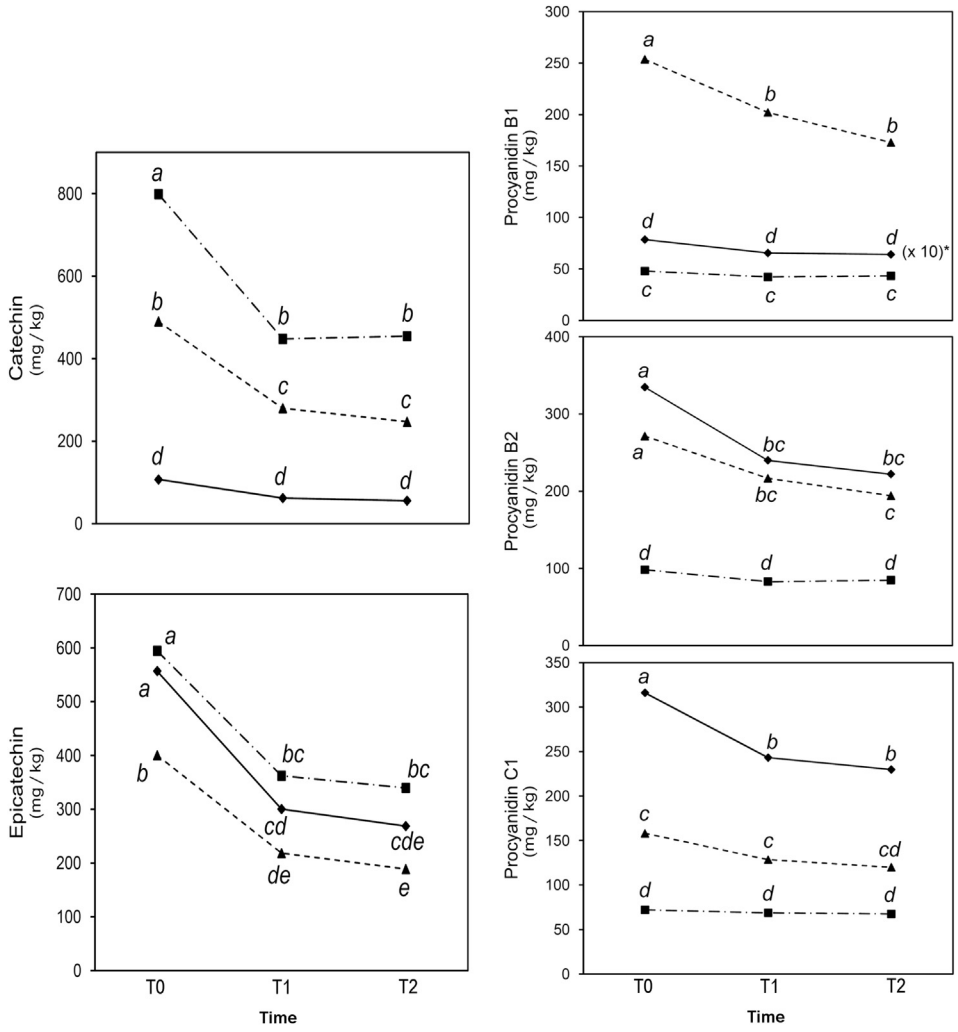

Fig. 3. Levels of catechin, epicatechin and oligomeric flavan-3-ols ($n = 6$, mg/kg dry weight) in dry fermented sausages added with cocoa extract (solid line), GSE1 extract (dash dotted line) and GSE2 extract (dashed line). Levels of phenolic compounds in "control" formulation were always under the limits of detection. T0 = raw meat preparation; T1 = at the end of ageing; T2 = at the end of shelf life (two months after the end of ageing). Small letters indicate significant differences ($p < 0.05$) between means. *Values expressed as (mg/kg) × 10.

Phenolics have been pointed out to have a clear protective action against lipid oxidation in meat products, especially in sausages with high lipid content and for large storage periods (Coronado et al., 2002; Hayes et al., 2011). Under our conditions, the satisfactory stability of the phenolic compounds in dry fermented sausages was probably related with the use of a traditional formulation, including antioxidant agents (nitrites, nitrates and ascorbic acid), as well as with a not very high fat content (18.7 ± 0.5 g/100 g and 15.4 ± 0.1 g/100 g for "fuet" and "salchichón" respectively, at T1) which increased protection against oxidation. Results are in concordance with the work of Fernández-López et al. (2007), who found that hesperidin, a phenolic compound from orange by-products, was very stable in dry fermented sausages over time.

Phenolic compounds concentrations at T1 are shown in Table 4. Their values in "fuet" were higher than in "salchichón" due to their different weigh loss (33.6 ± 2.1 g/100 g and 49.1 ± 1.5 g/100 g of mean water content in "fuet" and "salchichón" respectively, these values are typical for this kind of meat products). A balanced diet requires a certain intake of natural antioxidants, which can be improved by supplementing foods with vegetable extracts rich in phenolic compounds. A portion of 25 g of T1 "fuet" or T1 "salchichón" added with vegetable extracts provides an intake of 11–19 mg of monomeric and oligomeric phenolic compounds, which is a relevant contribution to the dietary intake of these compounds. As an example, a recent EFSA scientific opinion states that a daily consumption of 200 mg of cocoa flavan-3-ols helps to maintain endothelium-dependent vasodilation, which contributes to normal blood flow (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2012). A portion (25 g) of T1 cocoa-added dry fermented sausage contains 14.17 ± 2.03 mg ("fuet") or 11.00 ± 1.13 mg ("salchichón") of monomeric and oligomeric flavan-3-ols.

This paper demonstrates that the stability of phenolic compounds added in dry fermented sausages enables the use of vegetable extracts, aiming to produce a final product with significant levels of natural antioxidants. However, the fat content is still a factor that hampers the healthy properties of traditional dry fermented sausages. An interesting approach is to replace the fat content with vegetable polysaccharides such as short-chain fructooligosaccharides or inulin. This strategy has been implemented successfully in dry fermented sausages in previous works (Muguerza, Gimeno, Ansorena, & Astiasarán, 2004; Salazar, García, & Selgas, 2009).

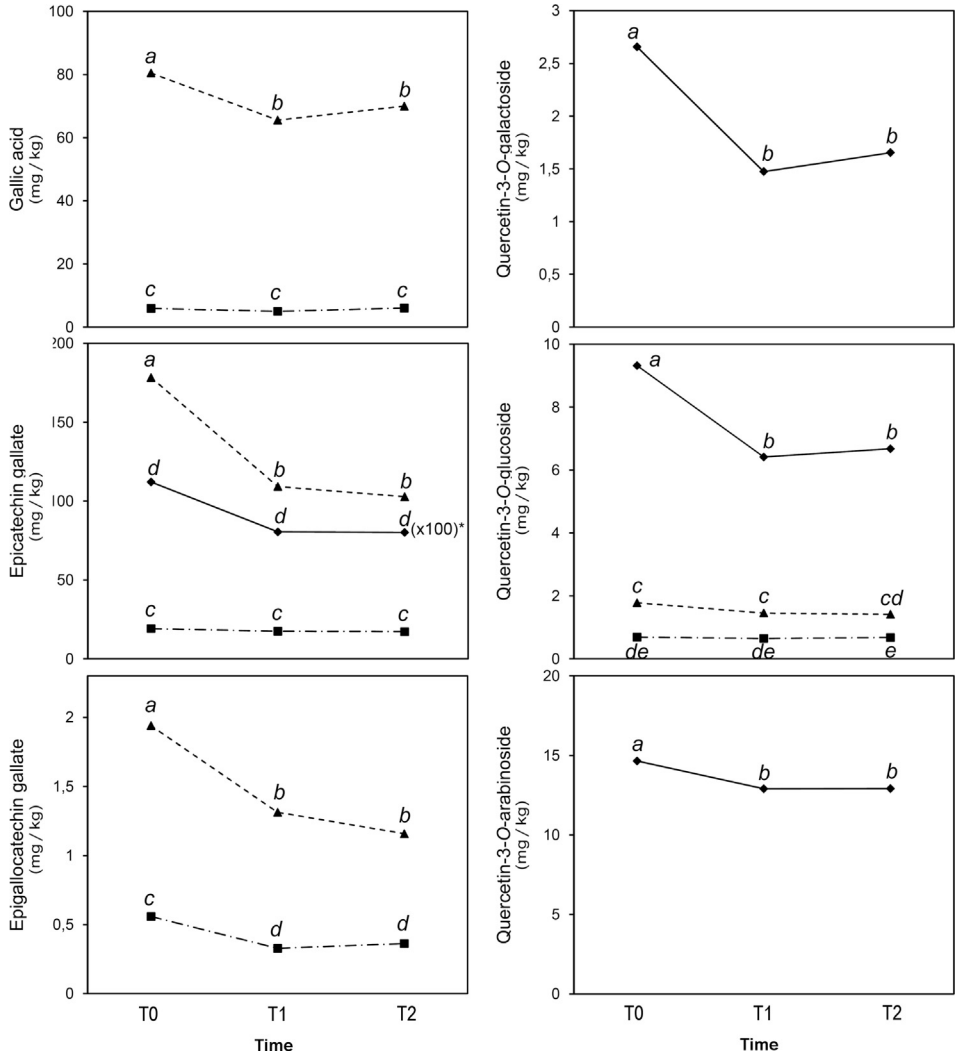

Fig. 4. Levels of gallic acid, galloylated flavan-3-ols and glycosylated flavonols ($n = 6$, mg/kg dry weight) in dry fermented sausages added with cocoa extract (solid line), GSE1 extract (dash dotted line) and GSE2 extract (dashed line). Levels of phenolic compounds in "control" formulation were always under the limits of detection; levels of gallic acid for "Cocoa" samples and levels of quercetin-3-*O*-galactoside and quercetin-3-*O*-arabinoside for GSE1 and GSE2 samples were under the limit of quantification (LOQ = 3 × LOD). T0 = raw meat preparation; T1 = at the end of ageing; T2 = at the end of shelf life (two months after the end of ageing). Small letters indicate significant differences ($p < 0.05$) between means. *Values expressed as (mg/kg) × 100.

3.3. Sensory evaluation of dry fermented sausages with vegetal extracts

Panelists discarded dry fermented products elaborated with extracts of grape seeds because they were abnormal from the typical products, in terms of appearance and flavour sensory characteristics. The results of sensory analysis of fermented sausages elaborated with cocoa extract are shown in Table 5. No important differences were found between the control (traditional) and cocoa-supplemented "fuet" and "salchichón", namely in colour intensity (higher in cocoa-supplemented samples).

Table 4
Concentration of phenolic compounds (Means ± SD, mg/100 g fresh weight, $n = 3$) in "fuet" and "salchichón" added with vegetal extracts at the end of the ageing process (T1).

Compounds	"Fuet"			"Salchichón"		
	Cocoa	GSE1	GSE2	Cocoa	GSE1	GSE2
Σ Monomeric flavan-3-ols[a]	21.8 ± 4.3	56.7 ± 9.8	30.9 ± 7.2	20.3 ± 3.1	39.4 ± 6.1	27.1 ± 1.9
Σ Gallic acid and galloylated flavan-3-ols[b]	0.07 ± 0.05	1.75 ± 0.03	11.95 ± 1.87	0.05 ± 0.02	0.96 ± 0.43	8.73 ± 0.43
Σ Oligomeric flavan-3-ols[c]	34.6 ± 5.8	13.1 ± 3.2	33.4 ± 3.4	23.6 ± 1.4	9.7 ± 1.1	30.2 ± 3.2
Σ Glycosylated flavonols[d]	1.53 ± 0.17	0.05 ± 0.01	0.10 ± 0.01	0.96 ± 0.16	0.03 ± 0.01	0.07 ± 0.01
Total	58.0 ± 10.4	71.6 ± 13.0	76.4 ± 12.5	45.0 ± 4.6	50.1 ± 7.6	66.1 ± 5.5

[a] Sum of catechin and epicatechin.
[b] Sum of gallic acid, epicatechin gallate and epigallocatechin gallate.
[c] Sum of procyanidin B1, procyanidin B2 and procyanidin C1.
[d] Sum of quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-arabinoside.

Table 5
Least squares means ($n = 8$, ±SD) for each sensory descriptor and for each product ("fuet" and "salchichón") obtained from the sensory analysis.

Attributes	"Fuet"		"Salchichón"	
	Control	Cocoa	Control	Cocoa
Appearance				
Colour intensity	4.9 ± 0.7b	5.7 ± 0.7a	4.0 ± 1.3b	4.9 ± 1.3a
Ability to peel-off the casing	3.1 ± 1.6	2.6 ± 1.2	0.8 ± 0.6	0.9 ± 0.8
Product adhered to the casing	2.3 ± 1.6	1.4 ± 0.9	0.7 ± 0.6	0.8 ± 0.7
Odour				
Intensity	4.7 ± 1.4	5.1 ± 1.2	4.4 ± 1.8	4.7 ± 1.7
Ripened	3.2 ± 1.8	3.8 ± 1.6	2.5 ± 1.8	2.7 ± 1.6
Pungency	3.0 ± 1.3	3.6 ± 1.6	3.0 ± 1.6	3.2 ± 1.5
Flavour				
Sweetness	3.3 ± 2.2	2.9 ± 2.2	1.4 ± 1.6	1.5 ± 1.6
Saltiness	3.5 ± 1.4	3.5 ± 1.7	3.9 ± 1.4	4.1 ± 1.4
Acid taste	n. a.	n. a.	4.5 ± 2.0	4.4 ± 1.5
Bitterness	0.7 ± 1.0	1.1 ± 1.5	0.4 ± 0.6	0.7 ± 0.7
Piquantness	3.9 ± 1.7	4.7 ± 1.4	3.7 ± 1.4	3.8 ± 1.6
Ripened	n. a.	n. a.	2.8 ± 1.9	2.9 ± 1.8
Texture				
Hardness	3.2 ± 1.2	3.9 ± 1.1	2.8 ± 1.1	3.1 ± 1.0
Gumminess	2.3 ± 1.7	2.6 ± 1.7	1.0 ± 1.2	1.1 ± 1.3
Crumbliness	4.8 ± 1.6	4.7 ± 1.2	4.0 ± 1.4	4.0 ± 1.5
Overall sensory quality	5.7 ± 1.9	6.2 ± 1.0	4.0 ± 1.5	4.3 ± 1.4

Within a row, least squares means with different superscripts differ significantly ($p < 0.05$).
n.a. (no evaluated).
Sensory attributes were scored by a non-structured scoring scale where 0 meant absence of the descriptor and 10 meant high intensity of the descriptor.

4. Conclusions

For the first time, the concentration of eleven phenolic compounds in "fuet" and "salchichón" dry fermented sausages added with cocoa and grape seed extracts was monitored from their production to the end of shelf life. A rapid sample extraction and a suitable UHPLC-DAD-MS/MS method were employed to quantify the phenolic compounds which are characteristic of the vegetal extracts of this study, but these conditions could be easily adapted to assess the behaviour of different vegetal extracts in other works on dry fermented sausages.

In both kinds of dry fermented sausages, the main phenolic compounds showed an initial reduction at the end of the ageing process, and their level did not further decrease significantly, at least until the end of the commercial shelf life. At the end of the ageing process (*i.e.*, when product is ready to be consumed), catechin and epicatechin were at 54–61% of their initial concentration, while higher residual percentages were found for gallic acid, galloylated flavan-3-ols, oligomeric procyanidins and glycosylated flavonols.

To develop balanced meat products is a challenge for food technologists and represents an appealing research line for novel foods. This paper demonstrates that is possible to produce dry fermented sausages with natural antioxidants from vegetable extracts without affecting their sensory properties. These results establish a good basis for further studies, such as the use of fat substitutes or vegetable fats, to develop balanced dry fermented sausages with higher nutritional value.

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