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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/