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Alpha-tocopherol stereoisomer analysis as discriminant method for distinguishing Iberian pig feed intake during the fattening phase

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ABSTRACT

The use of stereoisomers of α-tocopherol to correctly classify Iberian pig fat samples according to their feeding system was investigated. Samples were obtained over two different seasons in controlled farms from the four categories of pigs described in the Industry Quality Policy (FREE-RANGE: pigs fed exclusively under free-range conditions; FREE-FEED: pigs fed free-range and supplemented with feed; FEED-OUT: pigs fed outdoors with access to grass and a mixed diet; and FEED: pigs fed exclusively a mixed diet). A higher presence of RRR-stereoisomer indicated a greater consumption of the natural form of tocopherol provided by acorns or grass, whereas a higher proportion of S forms were related to a higher mixed diet intake. Validation results showed 90% success in fat sample classification. Analysis of the RRR-stereoisomer together with γ- and α-tocopherol determination can be considered as a potent tool for distinguishing fat from pigs fed under free-range conditions or exclusively with acorns and grass from those receiving a supplemented diet at any time of their fattening phase.

1. Introduction

Free-range production systems based on natural resources have been received more interest in recent years. Consumers demand products that are healthy, of high quality and from animals fed under traditional conditions, and are willing to pay higher prices for them, so increasing their economic importance (López-Bote, 1998). One example of this production type is the Iberian pig, which is traditionally reared in the evergreen oak forest located in the south-west of Spain. Feeding pigs mainly on acorns and grass provides monounsaturated fatty acids, antioxidants (Rey, Daza, Lopez-Carrasco, & Lopez-Bote, 2006a; Rey, Isabel, Cava, & López-Bote, 1998) and other phenolic compounds (Cantos, Espín, López-Bote, Ordóñez, & Tomás-Barberán, 2003), and generates products of the highest quality and commercial value (García et al., 1996; López-Bote, 1998). There are other rearing regimens for Iberian pigs according to the Industry Quality Policy (BOE, 2007). Hence, another possibility is an additional mixed diet supplement if the availability of acorns and grass is insufficient. It is also possible to rear Iberian pigs in outdoors conditions with grass availability and a feed supplement or exclusively with a formulated diet in intensive conditions. In these three cases, products are of reduced quality and price (López-Bote, 1998).

For distinguishing between feeding groups as a quality control practice and to avoid alterations or fake practices, fatty acid analysis by gas chromatography has been used (BOE, 2001) for many years. However, the use of oleic acid-enriched diets has increased the number of incorrect classifications between pigs fed under intensive and those fed under free-range conditions (Ventanas, Ventanas, Tovar, García, & Estevez, 2007). Consequently, new methods have been developed over the last few years to identify the feed type of Iberian pigs. Hence, some compounds present in their particular feed such as tocopherols (gamma- and alpha-) have been determined in Iberian pig tissues to categorise the feeding regimen. It has been reported that Iberian pigs fed under free-range conditions have higher concentrations of γ-tocopherol (mainly present in acorns) in muscle and fat than those fed a mixed diet and this antioxidant accumulation is related to the time of free-range feeding and weight gained outdoors (Rey, Daza, López-Carrasco, & López-Bote, 2006b). However, the concentration of α-tocopherol in tissues depends not only on grass availability as the principal source and acorn consumption but also on α-tocopherol supplementation in feeds (Rey et al., 2006a). Analysis of α-tocopherol stereoisomeric forms might provide additional information and improve the classification method.

Tocopherols occur in four forms (α, β, γ, δ) determined by the number and position of methyl groups on the chromanol ring. Alpha-tocopherol is the form preferentially absorbed and the most widely studied (Brigelius-Flohé & Traber, 1999). It is a chiral molecule that presents eight stereoisomer forms differing in group orientation at the 2′, 4′ and 8′ positions of the phytyl tail. The

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predominant stereoisomer derived from plant sources is *RRR*-α-tocopherol (Brigelius-Flohé & Traber, 1999). However, synthetic α-tocopherol (all-rac-α-tocopheryl acetate), which is the commercially available form of vitamin E for feed supplementation, is composed of an equimolar mixture of 8 stereoisomers and only one of these is identical to the *RRR*-α-tocopherol stereoisomer. The biodiscrimination between the eight α-tocopherol stereoisomers is specially affected by the presence of the 2R-configuration (Kiyose, Muramatsu, Kameyama, Ueda, & Igarashi, 1997), because of their higher affinity for the α-tocopherol transfer protein (Hosomi et al., 1997). Some authors (Röhrle et al., 2011) have previously investigated the potential application of stereoisomer analysis of α-tocopherol to discriminate between cattle raised at pasture or fed concentrates containing synthetic vitamin E. Moreover, in Iberian pigs Rey, López-Bote, Daza, and Lauridsen (2010) reported that acorn feeding provided ham with higher proportion of RRR-α-tocopherol stereoisomer than when pigs were fed a mixed diet and suggested that this could be used to discriminate between the feeding backgrounds of the animals. However, in this study only two feeding types of those described in the Industry Quality Policy (BOE, 2007) (free-range with acorns and feeding with a mixed diet) were compared.

Until recently there were no studies investigating differences in α-stereoisomers affected by Iberian pig feed type in commercial conditions. Taking into account that outdoors-based systems are characterised by a heterogeneous production (López-Bote, 1998; Pugliese & Sirtori, 2012), more research is necessary in order to use α-tocopherol stereoisomer analysis as a tool for discriminating feeding groups. In addition, although the feasibility of α and γ-tocopherol quantification for distinguishing Iberian pig feeding systems has been recently studied in practical situations (Rey, Amazan, & García-Casco, 2013), the α-tocopherol stereoisomer proportion was not considered.

The objectives of the present study were firstly to quantify isomers (α- and γ-tocopherol) and α-stereoisomers in subcutaneous fat from Iberian pigs fed in different commercial feeding situations; and secondly, to study the suitability of γ- and α-tocopherol concentrations together with the α-stereoisomer proportion for discriminating groups from different feeding backgrounds.

2. Material and methods

All the experimental procedures used in this study were in compliance with the Spanish guidelines for the care and use of animals in research (BOE, 2005).

2.1. Samples

Samples (subcutaneous fat) were obtained from Iberian pigs raised under controlled feeding conditions over two different seasons: 2008–2009 and 2009–2010. During the 2008–2009 season, fat ($n = 208$) was collected from the following feeding groups: FREE-RANGE: pigs raised in free-range conditions and exclusively fed acorns and grass (they put on at least 46 kg) in the ever-green oak forest in different geographical areas (Free-Range-1 ($n = 29$) in Ciudad Rodrigo, Salamanca; and Free-Range-2 ($n = 32$) in Cabeza de Vaca, Badajoz, Spain); FREE-FEED: pigs that put on at least 29 kg in free-range conditions during a different period of time and also received a mixed diet (Free-Feed-1 ($n = 28$) staying 83 days in free-range; and Free-Feed-2 ($n = 12$) stayed 60 days); FEED-OUT: pigs fed a conventional diet and with either access to grass ($n = 23$) (Feed-Out-1) or limited access to acorns ($n = 13$) (Feed-Out-2) or pigs fed a fat-enriched diet and access to grass ($n = 39$) (Feed-Out-3); and FEED: pigs fattened with a standard commercial pig diet ($n = 32$).

During the 2009–2010 season fat samples ($n = 200$) were obtained according to the following feeding systems: FREE-RANGE: Free-Range-3 ($n = 25$) (fed in Fuente Obejuna, Córdoba); Free-Range-4 (fed in Valdesequera, Badajoz); FREE-FEED: pigs raised in free-range conditions that received feed either at the end of the fattening phase ($n = 25$) (Free-Feed-3) or during all the fattening period ($n = 25$) (Free-Feed-4); FEED-OUT: Feed-Out-4: pigs fed a fat-enriched diet with limited access to grass ($n = 25$); Feed-Out-5: pigs fed a conventional diet and with access to grass ($n = 25$); Feed-Out-6: pigs fed a fat-enriched diet and access to grass ($n = 25$); FEED: $n = 25$.

Pigs from these two seasons were raised in controlled farms located at different locations in Spain (FuenteOvejuna, Córdoba; Olivenza, Badajoz; Alburquerque, Badajoz; Torrecampo, Córdoba; Ciudad Rodrigo, Salamanca; Cabeza de Vaca, Badajoz) or experimental institutions (Valdesequera, Badajoz), which had implemented a traceability system and a periodic inspection program.

Animals were stunned and slaughtered at a live weight of approximately 158.5 (±3.5 kg) and samples of subcutaneous fat were obtained from the tail insertion area in the coxal region and kept frozen at −20 °C until analysis.

For the α-tocopherol stereoisomer determination, samples ($n = 54$) of different feeding groups ($n = 6$ per treatment) from both seasons were selected at random, in order to reduce the cost of analysis. From season 2008–2009 the chosen feeding groups were: Free-range-1 and Feed-Out-2. From season 2009–2010: Free-range-3, Free-Feed-3, Free-Feed-4, Feed-Out-4, Feed-Out-5, Feed-Out-6; Feed. The feeding groups from both seasons were selected in order to have a high variability of origin.

2.2. Laboratory analysis

2.2.1. Tocopherol quantification

Concentrations of α- and γ-tocopherol in subcutaneous fat were quantified as described by Rey et al. (2006b), in which samples (0.05 g) were saponified in the presence of KCl (1.15%) and KOH (50%). Tocopherols were extracted with hexane and the upper layer containing tocopherol was evaporated to dryness and dissolved in 200 μl ethanol prior to analysis by reverse-phase HPLC (Agilent 1100, provided with diode array and fluorescence detectors; Agilent Technologies, Waldbronn, Germany). Separation was carried out on an Agilent Technologies Lichrospher RP-C18 column (250 mm × 4 mm i.d., 5 μm particle size); the mobile phase was methanol/water (97:3 v/v) at a flow rate of 2 ml/min and peaks were recorded at 292 nm. Peaks were detected by fluorescence detector (Agilent Technologies, Series 1200) set at λ-excitation: 295 nm and λ-emission: 330 nm. Identification and quantification of both tocopherols was carried out by means of a standard curve ($r^2 = 0.9999$) developed with the pure compounds (Sigma, Alcobendas, Madrid). Results were expressed as μg of α-tocopherol or γ-tocopherol per g. Samples were assayed in duplicate. Recovery of γ-tocopherol and α-tocopherol were not lower than 80% and 95% respectively. An internal standard (α-tocopheryl acetate) was added.

2.2.2. Alpha-tocopherol stereoisomers in Fat

Distribution of the stereoisomers of α-tocopherol in fat samples was determined by HPLC according to Lauridsen and Jensen (2005). Briefly, the heptane extract containing 1–2 μg of α-tocopherol in 9 mL was evaporated to dryness under a nitrogen stream. Then the α-tocopherol extract was derivatised to its methyl ester following the method described by Drotleff and Ternes (2001). The methyl ether derivative was extracted with 1.50 ml heptane, of which 100 μl were injected into the HPLC. Chromatographic separation was achieved on a Chiralcel OD-H column (25 × 0.46 cm; 5-μm particle size, cellulose tris(3,5-dimethylphenylcarbamate);