Title: "The use of linseed oil improves the nutritional quality of the lipid fraction of dry fermented sausages".

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ABSTRACT

Improvement of the nutritional quality of the lipid fraction of dry fermented sausages was achieved by a substitution of a quarter of the amount of pork backfat present in traditional formulations by an emulsion in which linseed oil was included. This improvement was particularly noticeable when 100 mg/Kg of butylhydroxytoluene and 100 mg/Kg of butylhydroxyanisol were added. P/S ratio increased from 0.4 in the control sausages to 0.6 in the batch with 3.3% linseed oil and to 0.7 in the batch with linseed (3.3%) and antioxidants. n-6/n-3 ratio decreased from 14.1 in control products to 1.7-2.1 in modified products as a consequence of the α-linolenic acid increment. No oxidation problems were detected during the ripening process, with TBA values always lower than 0.23ppm. Hexanal and nonanal showed the highest values in linseed oil containing products. Addition of antioxidants avoided the formation of decadienals and other aldehydes from lipid oxidation.

INTRODUCTION

The two main parameters currently used to assess the nutritional quality of the lipid fraction of food are P/S and n-6/n-3 ratios. P/S ratio is recommended nowadays to be above 0.4-0.5 (Enser, 2001; Wood et al., 2004) in order to prevent both an excess of saturated fatty acids with a negative effect on the LDL cholesterol plasmatic level, and an excess of polyunsaturated fatty acids, some of them being precursors of powerful clotting agents and also being involved in the aetiology of some cancers. n-6/n-3 ratio, which is estimated to be around 15-20 in the current Western diet should decrease below 5 or 4 (British Nutrition Foundation, 1992; Wood et al., 2004) to avoid the prothrombotic and proagregatory state induced by a high level of n-6 PUFA. Furthermore, a balanced n-6/n-3 ratio in the diet is essential for normal growth and development and should lead to decreases in cardiovascular disease and other chronic diseases and improve mental health (Simopoulos, 1999).

As meat and meat products are some of the most important sources of dietary fat, modification of the lipid profile of such products by enhancing n-3 polyunsaturated fatty acids can contribute to improve the nutritional quality of the occidental diet. Research has been done by feeding animals diets rich in polyunsaturated acids, basically n-3. Linseed has been widely used for this purpose, both as seeds (Romans, Johnson, Wulf, Libal, & Costello, 1995; Van Oeckel, Casteels, Warnants, N. & Boucque, 1997; Specht-Overholt et al., 1997; Matthews, Homer, Thies, & Calder, 2000) and as oil (Fontanillas, Barroeta, Baucells, & Guardiola, 1998; López-Ferrer, Baucells, Barroeta, & Grashorn, 2001; Rey, Kerry, Lynch, López-Bote, Buckley, & Morrisey, 2001; D´Arrigo, Hoz, López-Bote, Cambero, Pin, & Ordoñez, 2002a; D´Arrigo et al., 2002b; Hoz et al., 2003).

It has been observed that when animals are fed with n-3 enriched diets the oxidation rate of raw meats, including pork meat, increased (López-Bote, Rey, Sanz, Gray, & Buckey, 1997; Nurnberg, Kuchenmeister, Nurnberg, Ender, & Hackl, 1999). One of the strategies studied to avoid this problem has been the dietary addition of different amounts of α-tocopheryl acetate (D´Arrigo et al., 2002a; D´Arrigo et al., 2002b; Hoz et al., 2003), although in some cases also synthetic antioxidants such as BHA have been used in the animals´ feeding (Nam, Lee, Min, & Kang, 1997).

There are really few works dealing with the development of meat products enriched in n-3 fatty acids (Leskanich, Matthews, Warkup, Noble & Hazzledine, 1997; Specht-Overholt et al., 1997; Enser, Richardson, Wood, Gill & Sheard, 2000; Sheard, Enser, Wood, Nute, Gill & Richardson, 2000).

The modification of the ingredients used for the elaboration of dry fermented sausages instead of the use of dietary modified meat has been tested in different researches. Olive oil has been used as a source of MUFA obtaining technologically viable products without significant changes in PUFA and n-6/n-3 ratio (Bloukas, Paneras & Fournitzis, 1997; Muguerza, Fista, Ansorena, Astiasarán, & Bloukas, 2002). In order to modify the P/S ratio, soy oil had been also used in a previous work (Muguerza, Ansorena & Astiasarán, in press).

The objective of this work was to evaluate the lipid modifications undergone in dry fermented sausages during the ripening process when preemulsified linseed oil was used in the formulation, mainly focussing attention on the changes in the P/S and n-6/n-3 ratios. Furthermore, the effect of the incorporation of antioxidants was tested in order to prevent a detrimental oxidation process.

MATERIAL AND METHODS

Sausage preparation

Chorizo de Pamplona, a type of traditional Spanish dry fermented sausage, was elaborated according to the procedure described by Muguerza, Gimeno, Ansorena, Bloukas and Astiasarán (2001). Three batches of fermented sausages, about 5 Kg each, were prepared. The control sausage was based on a traditional formulation of 75% pork meat and 25% pork backfat. In the other two batches a 25 % of the total pork backfat was substituted by preemulsified linseed oil with soy protein. The emulsion was made according to the procedure described by Hoogenkamp (1989). Eight parts of hot water were mixed for 2min with one part of isolated soy protein, and the mixture was emulsified with 10 parts of olive oil for 3min. 100 mg/Kg of butylhydroxytoluene (BHT) and 100 mg/Kg of butylhydroxyanisole (BHA) were added as antioxidants in one of the batches that included linseed oil in the formulation. The percentages of meat and fat sources are detailed in table 1. The fatty acid profile of the linseed oil, expressed in mg/100g fatty acids was as follows: Myristic (0.11), Palmitic (6.28), Palmitelaidic (0.18), Palmitoleic (0.18), Stearic (3.91), Oleic (24.54), Linoleic (46.30), Linolenic (46.30), Eicosapentaenoic (0.11), Docosahexaenoic (0.07).

Analysis of parameters was carried out in the initial mixture (day 0) and after 3, 15 and 30 days of ripening. Three replications of the experiment were carried out.

Chemical analysis

Extraction of lipids was carried out using a chloroform/methanol mixture (Folch, Lees and Stanley, 1957). Fatty acids were determined in the lipid extracted by gas chromatography. Boron trifluoride/methanol was used for the preparation of fatty acid methyl esters (AOAC, 2002a). A Perkin-Elmer Autosystem XL gas chromatograph fitted with a capillary column SP^{TM}-2560 (100 m x 0.25 mm x 0.2 μm) and flame

ionization detection was used. The temperature of both the injection port and detector was 220ºC. the oven temperature was programmed to increase from 170 to 200ºC at a rate of 0.2ºC/min. The carrier gas was hydrogen, 20.5 psi. The sample size was 0.5 μl. The identification of the fatty acid methyl esters was done by comparison of the retention times of the peaks in the sample with those of standard pure compounds (Sigma, St. Louis, MO, USA) and by spiking the sample with those compounds. The quantification of individual fatty acids was based on the internal standard method, using heptadecanoic acid methyl ester (Sigma, St. Louis, MO, USA).

Thiobarbituric acid (TBA) value was determined according to the method used by Tarladgis, Watts, Younathan and Dugan (1960) with modifications of Tarladgis, Pearson and Dugan (1964) and Zipser and Wats (1962). Peroxides were determined according to the AOAC method (2002b).

Determination of lipid oxidation compounds

Likens-Nickerson Extraction. 25 g of frozen sausage were ground and placed in a 250 ml flask with 100 ml of water. A second flask with 5 ml of dichloromethane and 150 μg of dodecane (internal standard) was also attached to a modified Likens-Nickerson apparatus. 5 ml of dichloromethane were also added to fill the apparatus solvent return loop. Both solvent and sample mixture were heated to 70ºC and boiling temperature, respectively, maintaining these conditions for 2 h. After cooling to ambient temperature, the extract of dichloromethane was collected and dried over anhydrous Na_2SO_4. Two distillations per batch of sausage were carried out.

Analysis. The volatile compounds were analyzed in a HP 6890 GC system (Hewlett-Packard, Palo Alto, USA) coupled to a 5973 mass selective detector (Hewlett-Packard). A total of 1 μl of the extract was injected into the GC, equipped with a capillary column

(30 m X 250 μm X 0.25 μm nominal HP-5MS). The carrier gas was He (1ml/min), and the chromatographic conditions were as follows: initial oven temperature was maintained during 10 min at 40ºC and subsequently programmed from 40 to 120ºC at a rate of 3ºC/min and at a rate of 10ºC/min from 120 to 250ºC, at which it was held for another 5 min; injector temperature, 250ºC; transfer line temperature, 280ºC; ion source temperature, 230ºC; scan speed, 4.49 scan/sec; mass range, 33-350 amu (atomic mass units); solvent delay, 3 min; electron impact at 70 eV. Identification of the peaks was based on the comparison of their mass spectra with the spectra of the Wiley library (HPCHEM Wiley 275 6th Ed.) and, in some cases, a comparison of their retention time with those of standard compounds was also carried out. The Kovats indices were also calculated according to the method of Tranchant (1982) and were compared with available literature data (Kondoyan & Berdagué, 1996). Only compounds related to lipid oxidation are shown. Area of peaks was measured by integration of the total ion current of the spectra or by calculation of the total area based on integration of a single ion. Semiquantitative determination of the volatile compounds was based on the ratio of their peak to that of dodecane (i.s.), and the results were expressed as nanograms of dodecane per gram of dry matter.

Data Analysis.

For each replication of the experiment, three samples were analyzed from each type of sausage and period of analysis. Each parameter was determined four times in each sample. In tables, mean values are shown. An ANOVA test was carried out in order to determine significant differences among sausages depending on the type of formulation. Data analysis was carried out with an SPSS 9.0 program (© 1998, SPSS inc. Chicago, version 9.0. Illinois).

RESULTS AND DISCUSSION

The analysis of the total lipid content at the end of the ripening process showed values of 33.7% for the control sausage, 31.8% for the linseed oil containing batch and 29.7% for the sausages with linseed oil and antioxidants. The fatty acid profiles of the products, expressed as g/100g fatty acids, are presented in table 2. It can be seen that the values obtained along the ripening process for every type of sausage did not show noticeable changes among the different days of analysis. Consequently, the desiccation process did no affect the total fatty acid profile. Differences shown in this table among batches are due to the differences in the raw matters used in control and modified products. The greatest difference was observed for α-linolenic acid that, at the end of the ripening, increased from 0.92g/100g fatty acids in control to 7.99/100g fatty acids in modified products without antioxidants. Enser et al. (2000) analyzing the fatty acid composition of different tissues and meat products from pork fed linseed obtained increments of α-linolenic of 1.36 fold in relation to sausages elaborated from control raw materials. Those authors found also some significant increases in EPA. In linseed containing products eicosapentaenoic acid (EPA) showed slight amounts during the first steps of maturation, but this acid disappeared at the end of the ripening. In a previous paper, soy oil was used in the same concentration (Muguerza et al., 2003), leading to increments for myristic, palmitic, palmitoleic, oleic acids and specially linoleic acid. No differences were found for EPA and DHA. Nam et al. (1997) found that arachidonic acid (C20:4, n-6) content decreased significantly when increasing feeding of linseed oil in poultry meat. In our work arachidonic acid was not detected in any sample. Those authors also found that MUFA fraction was not affected by the diet.

Analyzing the effect of the addition of antioxidants, higher values for linoleic and α-linolenic acids were detected in antioxidant containing sausages than in linseed sausages

from the 15th day of ripening. These data pointed out that the presence of antioxidants could reduce the oxidation of these fatty acids, which are the most susceptible to suffer oxidation. Different compounds with antioxidant properties have been used to avoid the oxidation of PUFAs. Van Ruth, Shaker and Morrise (2001) showed the efficiency of methanolic extracts of soybean seeds to inhibit linseed oxidation. Lauridsen, Nielsen, Henckel and Sorensen (1999) observed a slight decrease of n-6 PUFA in fats when pigs were fed supplemented α-tocopheryl acetate and rapeseed oil. However Monahan et al. (1992) and López-Bote et al. (1997) did not find any effect of α-tocopheryl acetate supplementation on the fatty acid composition in muscle. Rey et al. (2001) explained the differences found for the effect of vitamin E by the fatty acid composition in the use of different fat sources in animal feeding for the different experiments. It is demonstrated that phospholipids in muscle cell membranes are the main lipids susceptible of oxidation in meat (Pikul, Leszczynski, & Kummerow, 1984; Buckley et al., 1989). In our case no problems have been found in relation to the oxidation process during ripening, maybe because the enrichment of n-3 PUFA was made basically through the addition of triglycerides, whereas the enrichment by dietary treatment modified both triglycerides and phospholipids of meat.

The mentioned fatty acid modifications gave rise to some differences in the total fatty acids fractions, SFA, MUFA and PUFA, supplied by the product (table 3). Decreases in Σ SFA and Σ MUFA are observed in both modified products, reaching the lowest amounts in those with antioxidants. Comparing these sums with those shown by sausages with 25% substitution of pork back fat with soy oil (Muguerza et al., 2003) it could be observed that, referred to the respective control, the decreases for both fractions were lower when linseed oil was used. Also, the increase of PUFA fraction was lower for linseed oil added products. However, sausages with linseed oil plus

antioxidants showed the highest decrease in SFA and the highest increase of PUFA. P/S ratio raised significantly in modified sausages with linseed oil, being in the case of sausages with antioxidants nearly 2 fold the value found for the control. It has to be taken into account that the increase in this ratio in relation to control is due basically to the increase in α-linolenic acid. Effectively, n-6/n-3 ratio decreased from 13.49 in control products to 1.80-1.93 in modified products as a consequence of the α-linolenic acid increment. It is demonstrated that the dietary n-6/n-3 ratio can influence the pig muscle n-6/n-3 ratio of polar and neutral lipids (Hogberg, Pickova, Andersson & Lundstrom, 2003). When modifications of this ratio are obtained through feeding pigs with diets with linseed, n-6/n-3 ratios of meats are very diverse. Some authors got values around 3.5 - 4.5 in muscle (Leskanich et al., 1997; Rey et al., 2001), 1.34 in subcutaneous fat (D´Arrigo et al., 2002) and 4.9-4.6 in sausages elaborated from raw modified meats (Leskanich et al., 1997; Enser at al., 2000).

The aroma of dry fermented sausages is greatly influenced by volatile compounds resulted by the oxidation process affecting the lipid fraction during the ripening (Mateo & Zumalacárregui, 1996; Edwards, Ordóñez, Dainty, Hierro & Hoz, 1999; Ansorena, Astiasarán & Bello, 2000). Lipid oxidation parameters and some of the most common volatile lipid derived compounds are gathered in table 4. TBA and peroxides showed very low values in the three analyzed products. Modified sausages without antioxidants showed a significantly higher value for TBA than for control. Also hexanal (from oxidation of linoleic) and nonanal (from oxidation of oleic acid) showed significantly higher amounts for modified sausages without antioxidants, being these results in agreement with TBA values. Different results have been found in relation to the effect of enrichment of meats with n-3 PUFA over the oxidation lipid intensity and the development of volatile compounds. No effects have been found on flavour and in

general on sensorial meat quality in some research works (Enser et al., 2000; Sheard, Enser, Wood, Nute, Gill & Richardson, 2000; Leskanich et al., 1997; Melton, 1990). On the contrary, Elmore, Mottram, Enser and Wood (1999, 2000) studying the effects of α-linolenic acid enrichment on the volatile compounds of cooked beef and lamb concluded that there were significant increases in some of the lipid oxidation products. These authors concluded that autoxidation appeared to be prompted by increased levels of PUFAs. Octenal an tt2,4-decadienal did not show significant differences between control and modified products without antioxidants. The addition of antioxidants kept low values for all compounds, and especially avoided the formation of dienals, characterized by their off-flavour. It has to be pointed out, however, that the concentrations of these compounds in control and linseed containing sausages was not considered high, being within the range of commercial sausages (Ansorena et al., 2001). Although no sensorial evaluation was carried out by a trained panel in the analyzed products, no differences were observed in appearance and odour among the three batches.

In conclusion, the results obtained in this work indicated that the addition of linseed oil to the formulation of dry fermented sausages has a relevant influence on the nutritional quality of the products, without modifying substantially the flavour and oxidation status of the ready to eat products. However, more research is needed to conclude about the evolution of the lipid oxidation process during the self life of linseed containing sausages.

Table 1. Percentages of meat and fat sources and presence of antioxidants in the three batches of dry fermented sausages elaborated.

	Control	**Linseed**	**Linseed + Antioxidants**
Lean Meat	75%	75%	75%
Pork Backfat	25%	18.75%	18.75%
Linseed oil	-	3.3%	3.3%
BHT + BHA	-	-	100 mg/Kg + 100 mg/Kg

Table 2. Total fatty acids along ripening process for the three types of sausages (g/100g fatty acids).

	Control				Linseed				Linseed + Antioxidants			
Days	**0**	**3**	**15**	**30**	**0**	**3**	**15**	**30**	**0**	**3**	**15**	**30**
SFA												
Lauric	0.13a	0.13a	0.13b	0.13a	0.12a	0.11a	0.11a	0.12a	0.17b	0.16a	0.12a	0.13a
Myristic	1.58c	1.57c	1.55b	1.55b	1.24a	1.24a	1.25a	1.33a	1.41b	1.41b	1.31a	1.35a
Palmitic	25.23b	25.14b	24.93b	24.82b	21.84a	21.94b	21.90a	22.14a	21.19a	21.12a	20.59a	21.50a
Stearic	14.53c	14.49c	14.18c	14.27c	12.99b	12.91b	12.48b	12.65b	11.85a	11.62a	11.47a	11.38a
Arachidic	0.21a	0.22a	0.15a	0.15a	0.21a	0.21a	0.22b	0.24b	0.19a	0.19a	0.20ab	0.17a
Behenic	0	0a	0.42a	0.31a	0	0a	0.28a	0.27a	0	0.35b	0.24a	0.25a
MUFA												
Palmitoleic	2.75b	2.72b	2.64b	2.66b	2.32a	2.31a	2.37a	2.37a	2.40a	2.39a	2.44ab	2.42a
Oleic	39.56c	39.75b	39.83a	39.99b	37.36b	38.36ab	37.68a	37.41a	37.11a	37.05a	37.25a	36.74a
PUFA												
Linoleic	14.24a	14.19a	14.22a	14.24a	14.15a	14.21a	14.42a	14.66a	16.38b	16.36b	16.81b	16.69b
α-Linolenic	0.88a	0.88a	0.88a	0.93a	7.95b	7.81b	7.87b	8.03b	7.91b	8.30b	8.87c	8.57c
Arachidonic	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a
EPA	0a	0a	0a	0a	0.7b	0.07b	0.05b	0a	0a	0a	0a	0a
DHA	0.13b	0.13a	0.13a	0.13b	0.11b	0.10a	0.28a	0.10a	0.04a	0.34a	0.09a	0.07a
TRANS												
t-Palmitoleic	0.46c	0.45a	0.45c	0.44c	0.35a	0.34a	0.33a	0.33a	0.40b	0.24a	0.39b	0.40b
Elaidic	0.18a	0.19a	0.31a	0.22a	0.26b	0.27b	0.23a	0.20a	0.33b	0.33b	0.22a	0.16a
t-Linoleic	0.10a	0.09a	0.14a	0.15a	0.10a	0.10a	0.12a	0.12a	0.12a	0.12a	0.09a	0.16a
Brassidic	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a	0a

For each parameter and time of analysis, different letters denote significant differences among types of sausages. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

Table 3. Lipid fractions (g/100g fatty acids) and ratios of nutritional interest obtained for the dry fermented sausages ripened for 30days.

	Control	**Linseed**	**Linseed + Antioxidants**
Σ SFA	41.68	36.41	34.80
Σ MUFA	42.31	40.68	39.51
Σ PUFA	15.25	22.27	24.33
P/S	0.4	0.6	0.7
n-6/n-3	14.1	1.7	2.1

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
P/S: Polyunsaturated fatty acids / Saturated fatty acids.

Table 4. Lipid oxidation parameters of the control and modified sausages at the end of the ripening process.

	Control	**Linseed**	**Linseed + Antioxidants**
TBA (mg malonaldehyde/Kg sample)	0.08a	0.23b	0.08a
Peroxides (meq O_2/Kg fat)	2a	0.83a	0a
Hexanal (ng dodecane/g dm)	226.5a	308.5b	218.5a
Octenal (ng dodecane/g dm)	60b	66.5b	33.5a
Nonanal (ng dodecane/g dm)	291.5a	591.5b	314a
Tt2,4-decadienal (ng dodecane/g dm)	25.5b	22.5b	0a
2,4-decadienal (ng dodecane/g dm)	104.5c	91.5b	0a

Different letters in the same row denote significant differences among types of sausages. dm: dry matter.

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