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GC/MS Analysis of Fatty Acids in Italian Dry Fermented Sausages

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Abstract: The present investigation reports a study about the evolution, during ripening, of the fatty acid profile and the fatty acid composition in acylglycerols of three different fermented sausages industrially produced in the Calabria region (Southern Italy). Statistical analysis (ANOVA) was applied to the results obtained for the profiles to check all the differences between samples. The study comprised also an evaluation of the lipid oxidation level. All kind of sausages showed a free fatty acids profile in which the monounsaturated fatty acids were predominant, followed by saturated and polyunsaturated fatty acids. In acylglycerols, a low content in linoleic acid (approximately 2% of total methyl esters) was displayed, while that found in the free fatty acids profile was higher (approximately 17% of total FFAs). In addition, the generation of aldehydes through secondary lipid oxidation was clearly confirmed after long ripening period by classical qualitative colorimetric method supported by ^{1}H NMR spectroscopy. The differences in fatty acids profiles observed in the free fatty acids and the acylglycerol fractions were certainly due to the high selectivity of lypase activity during the ripening.

Keywords: Acylglycerols, dry fermented sausages, fatty acids, gas chromatography-mass spectrometry, lipolysis.

1. INTRODUCTION

Dry fermented sausages are traditional meat pork products mainly manufactured in Countries of the Southern Europe like Italy [1]. In Calabria region (Southern Italy) several types of fermented sausages are still produced using traditional manufacturing processes but there is an increase in the demand for industrial production of these products. In the last decade numerous studies concerning technological, chemical, and sensory characterization of traditional dry fermented meat products have been performed [2]. The analysis of dry fermented sausages generally points out on some important parameters: breakdown products of lipolysis [3, 4] and proteolysis [5] i.e. peptides, amino acids [6], free fatty acids (FFAs) that contribute to the nutritional characteristics of fermented meat. Much attention is also paid to the determination of products of further degradation such as biogenic amines [7, 8] or compounds derived from oxidation of polyunsaturated fatty acids (PUFAs) [9, 10]. The analysis of fatty acids has become increasingly important in a modern society with dietary recommendations favoring a low intake of fats because more people are aware of their nutritional implication [11]. Furthermore, the growing interest in the control of the composition in acylglycerols and FFAs of sausages mainly is attributable to the necessity of producing "healthy" foods characterized by saturated/unsaturated fatty acid ratios all in favor of the latter ones. Epidemiological and biochemical studies have provided a great deal of evidence about the protective effect of ω-3 PUFAs against some common tumors, rheumatoid arthritis and cardiovascular diseases (CVD) [12-15]. The investigation of fatty acid composition in the lipid fraction of sausages could provide information about changes in the content in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) occurring during the ripening. This point should be of interest for the evaluation of the changes of nutritional characteristics of these products. In fact, modifications of the PUFA and MUFA content can have possible effects related to a low or high consumption of the fermented sausages. In this context, detailed information about the fatty acid profile provides researchers with the necessary knowledge in this field, as well as consumers about the shelf-life and acceptance of this kind of meat products.

In the present study the total lipid fraction extracted from three different Calabrian fermented sausages (known as “salsiccia”, “spianata” and “soppressata”) was analyzed by GC/MS. These sausages are typically produced in the South of Italy and represent a significant part of the culinary traditions of these regions. Moreover they are widely exported to many European Countries. The investigation

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covered the determination of fatty acid profiles in the respective acylglycerol portions as well as the composition of free fatty acids during a long time period of ripening (80 days).

2. MATERIAL AND METHODOLOGY

2.1. Sausages Formulation and Processing

The dry fermented sausages used in this study were prepared at an industrial establishment sited in the province of Cosenza (Calabria region, Southern Italy) following the current Italian industrial processing technology. Pork meat was ground and then mixed. Once out of the grinder and before mixing, sodium chloride (2.4% w/w) was added. Moreover, sodium nitrite (0.01% w/w), sodium nitrate (0.01% w/w), antioxidants (ascorbic acid, 0.02% w/w), sugars (dextrose and sucrose, 0.45% w/w), and spices (red hot pepper for "salsiccia" and "spianata", 0.6% w/w; black pepper and other spices for "soppressata" 0.6% w/w), and aromas (1% w/w) were added during mixing. Microbial starter formulations were also added as follows: i) SAGA T (0.01% w/w, 2 × 1010 ufc/g Pediococcus pentosaceus and Staphylococcus carnosus, Kerry Ingredients & Flavours Italia S.p.A., Mozzo, Bergamo, Italy) for "salsiccia" and "soppressata"; ii) LH30 (Lactobacillus helveticus fratelli Pagani, Milano, Italy) for "spianata". Mixtures were stuffed in casings and then spiked to allow entrapped air to escape. The sausages were stored in a warm room to dry under the following conditions: 24-26 °C at 75-80% relative humidity (RH) for 3 days, then 12-14 °C at 60-70% RH in the last days of the curing process. After 20 days, sausages were then stored under vacuum-packaging for the entire ripening period (80 days).

2.2. Sampling

The sausages were sampled and analyzed in triplicate (three different sources) during three different periods of ripening (20 days: samples s**al20** and **spi20**; 40 days: samples **sal40**, **sop40** and **spi40**; 80 days: samples **sal80**, **sop80** and **spi80**). Samples were homogenized in a household kitchen-type blender before analysis.

2.3. Physicochemical Analyses

Total nitrogen (TN) and protein nitrogen (PN) were determined following the Kjeldahl method [16]. Moisture was determined according to the official methods of analysis [17]. Total lipids extracts were obtained by Soxhlet extraction according to the procedure reported in the literature [18].

2.4. Determination of Fatty Acid Composition

An aliquot of lipid extract (200 mg) was dissolved in 3 mL of *n*-hexane. A methanolic solution (5 mL) of sodium methoxide (10.1 mg/mL) was then added. A 20% excess sodium methoxide was used: this excess was calculated assuming that fat is constituted exclusively by triolein. The mixture was stirred at room temperature for 5 min. Afterwards, the hexane was separated from the methanolic phase. Then the methanolic phase was washed twice with hexane. The hexane was finally removed under reduced pressure conditions. The residue was dissolved in dichloromethane and analyzed by GC/MS. Fatty acid methyl esters (FAMEs) were identified by comparing the retention times of the chromatographic peaks with those obtained with the methyl esters from a mixture prepared with fatty acids from Sigma-Aldrich (Milano, Italy). The structural assignment was further confirmed by comparing their mass spectra with those of reference standards.

In a second time, the methanolic phase, containing the FFAs as carboxylate anions, was evaporated under reduced pressure. The residue was acidified with aqueous HCl 1N and then extracted with diethyl ether (5 x 3 mL). The combined ethereal extracts were washed once with a saturated sodium chloride aqueous solution, dried over anhydrous sodium sulfate (Na_2SO_4) and paper filtered and evaporated to dryness under vacuum.

2.5. FFAs Quantitative Analysis

Fatty acids were identified by comparison of their retention times to standards. Methyl pentadecanoate (C15:0) purchased from Sigma-Aldrich, normally not present in the lipid extracts, was used as the internal standard (20 mg/mL). This was added to aliquots of lipid extracts in order to determine the quantity of each fatty acid in the sample analyzed. FFAs were quantified as their corresponding FAMEs. To this aim, the residue obtained from transesterification was treated with a large excess of a 0.66N dichloromethane solution of diazomethane [19-21]. Until the solution exhibited a constant pale yellow color. After 30 min the organic solvent was removed under reduced pressure conditions, the residue was dissolved in 5 mL of dichloromethane and analyzed by GC/MS [7, 22].

FAMEs standard solutions: A mixture of standard free fatty acids (21.8 mg of myristic acid; 22.0 mg of palmitic acid; 26.9 mg of palmitoleic acid; 25.3 mg of linolenic acid; 23.8 mg of linoleic acid; 30.4 mg of oleic acid; 22.4 mg of stearic acid) was treated with a large excess of a 0.66 N dichloromethane solution of diazomethane using the procedure described above. The residue was dissolved in 10 mL of methylene chloride (solution A). Four stock solutions were then prepared starting from solution A: an aliquot of 0.1 mL of methyl pentadecanoate standard solution was added to 1, 2, 3 and 4 mL of solution A and each stock solution was diluted to 5 mL with methylene chloride to create standard solutions. 1µL of each FAMEs standard solution was analyzed by GC/MS.

2.6. GC/MS Analysis of FFAs and FAMEs

Analyses of FFAs and FAMEs were carried out using a 6890N Network GC System (Agilent Technologies Inc., Palo Alto, CA, U.S.A.) equipped with a HP- 5MS (30 m x 0.25 mm i.d., 0.25 µm film thickness) capillary column and with a 5973 Network MSD mass spectrometer, operated in electron-impact ionization mode (70 eV). GC-MS analyses were carried out in split mode (split ratio 1:50), using helium as the carrier gas (1 mL/min flow rate). The injector temperature was fixed at 250 °C. The sample volume injected was 1µl. Oven temperature was held at 150 °C for 2 min and then programmed at 5 °C/min to a final temperature of 280 °C, where it was maintained for 5 min.

2.7. Kreis Test Methodology

In each experiment, a known quantity of lipid extract (2 g) was mixed with 2 mL of concentrated HCl in a test tube for 30 s. Then 2 mL of a 0.1% solution of phloroglucinol in diethyl ether were added and mixed thoroughly with the lipid-acid mixture [23]. A pink color formation indicated that the fat was slightly oxidized while a red color indicated that the fat was definitely oxidized.

2.8. NMR Analysis

For ^{1}H NMR analysis, aliquots of 200 mg of the lipid extracts were dissolved in 0.7 mL of deuterated chloroform ($CDCl_3$) in a 5 mm NMR tube. All spectra were recorded at 25 ± 0.1 °C under temperature control, using a Bruker Avance 300 Ultrashield spectrometer equipped with a 5-mm probe with Z-axis gradient coils. Spectra were obtained at the ^{1}H frequency of 300.132 MHz applying a standard zg90 pulse sequence, with the lock on the deuterium resonance of the solvent. Chemical shift values (δ) were reported in ppm by referencing them to tetramethylsilane (TMS) used as the internal standard.

2.9. Statistical Analysis

Mean and standard deviation of data are shown in all tables. The differences between samples obtained at the three periods of storage were tested for each type of sausage by applying the Kruskal-Wallis test and Mann-Whitney U test to both FAME and FFA concentration data. The Kruskal-Wallis test and Mann-Whitney U test are the analogous nonparametric methods of one-way between-groups of variance (analysis of variance, ANOVA) and t-test, respectively. Nonparametric techniques are also called "distribution free methods", since they are not dependent on a given distribution (such as in the case of ANOVA) but generally work for a broad range of different distributions. Since our statistical samples were not large enough to assume that the sampling distributions were normal, a nonparametric approach to treatment of data was necessary.

3. RESULTS AND DISCUSSION

The results for proximate composition of the three different types of dry sausages are reported in Table **1**. It can be observed from the data obtained that moisture in the "salsiccia" batches is low (27-30%): this fact is probably due to the small size and to the tight casing that allows a great water loss. Instead, the "soppressata" and the "spianata" samples that are prepared in larger casings are characterized by a moisture content that ranged between 37.5 and 41.2%. Table **1** gives also the protein percentages found for the dry sausages studied. These values ranged between 32.1% and 38.8%. The lipase activity influences the FFAs content during the drying and ripening steps [24, 25]. The total fat amount in the analysed sausages varied between 24.8% and 32.6%. Nevertheless, the fat obtained from all types of sausages by solvent extraction may also contain non-fat material. The analysis of total lipid extract often fails in accurately estimating nutritional values of biological materials. Evaluation of total fatty acids represents a better alternative for assessment of nutritional value than extractable lipids.

In dry sausages fatty acids are present both as esters linked to glycerol and phospholipids and as free (unesterified) fatty acids. The determination of fat composition in dry fermented sausages either in the acylglycerol or in the unesterified form was carried out by transesterification of the total lipid extract using a large excess of sodium methoxide.

The methods which utilize methanol in acidic medium for the transesterification of acylglycerols cannot be adopted because the results could be affected by the esterification of the FFAs present in the fat [26]. On the other hand, base-catalyzed transesterification of acylglycerols can suffer from the presence of FFAs that could neutralize the reaction environment thus leading to an incomplete transesterification [27]. The use of a large excess of sodium methoxide in dry environment enables the neutralization of the FFAs converting them rapidly into the corresponding sodium salts that are retained on the methanolic phase. At the same time,

Table 1. Moisture, protein and lipid content of the analyzed sausages from Southern Italy.

Sausages	Samples	Moisture (Mean ± SD)	Protein(Mean ± SD)	Fat (Mean ± SD)
"Salsiccia"	Sal20	27.9 ± 0.7	37.5 ± 0.7	32.6 ± 0.6
	Sal40	27.4 ± 1.1	37.8 ±1.1	31.8 ± 0.8
	Sal80	29.9 ± 1.1	38.8 ± 0.6	29.5± 0.9
"Soppressata"	Sop40	41.2 ± 0.7	32.1 ± 1.1	24.7 ± 0.9
	Sop80	38.7 ± 1.1	33.1 ± 1.3	26.3 ± 0.9
"Spianata"	Spi20	37.6 ± 0.5	33.3 ± 0.9	27.2± 0.7
	Spi40	37.5± 1.2	32.2 ± 1.1	28.3 ± 0.7
	Spi80	39.3 ± 1.3	33.1 ± 1.1	29.4 ± 0.5

Data are expressed as mean value ± SD in percentage, (n=3). Measures were performed in triplicate.

the use of an excess sodium methoxide ensures, through the complete transesterification of acylglycerols, the formation of fatty acid methyl esters (FAMEs) that are soluble in hexane. GC/MS analysis of the hexane layers showed the presence of FAMEs (Fig. **1**). Their identification was achieved by comparing their individual peak retention times and mass spectra with those of reference standards.

FFAs were recovered after acidification of the methanolic solution and were then subjected to the derivatization procedure and measurement. In fact, fatty acids need to be converted into FAMEs in order to improve their volatility and thus ensuring better gas chromatographic peak shape. Derivatization was performed with diazomethane according to the method of Schlenlg and Gellerman (1960) [28] that enables the conversion of the FFAs into the corresponding methyl esters. Table **2** reports the data obtained for FFAs in all products considered. The three "salsiccia" batches showed a lower content of FFAs (about 6% of total fat) than those recovered in the samples from "soppressata" and "spianata" (7.5-10% ca of total fat).

The percentage of FFAs in the total fat was calculated as the sum of individual fatty acids. The main identified fatty acids in the three different types of sausages were: oleic, palmitic, linoleic, stearic, palmitoleic and myristic acid. This profile coincides basically with that found by other authors in pork fat [29].

All the examined samples (Table **2**) showed a higher content of MUFAs and PUFAs than SFAs. In particular, the saturated/unsaturated free fatty acid ratio (S/U) achieves values around 0.22 and 0.36. Oleic acid (C18:1 cis-9) was predominant among MUFAs, being also the most prevalent individual FFA in all kinds of sausages, with values around 58-68%. Linoleic acid (C18:2 cis-9, cis-12) was the dominating PUFA with values around 9-18%. SFAs were also determined, with palmitic acid (C16:0) contributing for the highest amounts (12-18%); stearic acid (C18:0) being

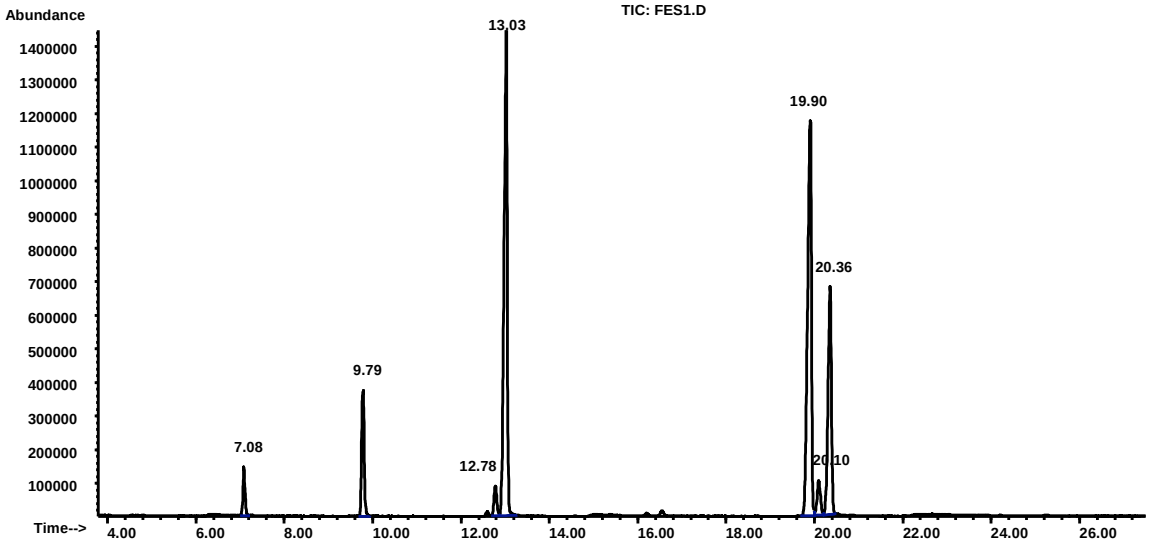

Fig. (1). GC/MS analysis of FAMEs obtained from the transesterification of sample sal20 (r.t. 7.08 myristic acid methyl ester; r.t. 9.79 pentadecanoic acid methyl ester (reference standard); r.t. 12.78 palmitoleic acid methyl ester; r.t. 13.03 palmitic acid methyl ester; r.t. 19.90 oleic acid methyl ester; r.t. 20.10 linoleic acid methyl ester; r.t. 20.36 stearic acid methyl ester).

Table 2. FFAs profile of the analyzed sausages form Southern Italy, determined by GC/MS.

	"Salsiccia"			**"Soppressata"**		**"Spianata"**		
	Sal20	**Sal40**	**Sal80**	**Sop40**	**Sop80**	**Spi20**	**Spi40**	**Spi80**
FFAs in total fat	5.70 ± 0.12	6.12 ± 0.11	6.73 ± 0.20	9.92 ± 0.18	8.66 ± 0.16	7.48 ± 0.27	7.54 ± 0.19	8.33 ± 0.26
Myristic acid ($C_{14:0}$)	1.95 ± 0.05	1.22 ± 0.04	1.37 ± 0.05	0.98 ± 0.02	1.11 ± 0.04	1.14 ± 0.03	1.46 ± 0.04	1.37 ± 0.04
Palmitic acid ($C_{16:0}$)	17.09 ± 0.06	12.53 ± 0.03	14.46±0.03	12.15±0.05	17.72±0.08	14.00±0.09	11.96±0.04	16.18 ± 0.02
Stearic acid ($C_{18:0}$)	6.92 ± 0.13	6.23 ± 0.07	7.05 ± 0.01	5.24 ± 0.14	7.72 ± 0.10	5.88 ± 0.06	6.99 ± 0.11	6.37 ± 0.12
Palmitoleic acid ($C_{16:1}$)	4.60 ± 0.02	3.25 ± 0.05	3.36 ± 0.02	3.78 ± 0.11	2.94 ± 0.03	3.45 ± 0.03	3.62 ± 0.04	3.32 ± 0.04
Oleic acid ($C_{18:1}$)	58.56 ± 0.01	58.84 ± 0.05	62.16±0.05	68.72±0.20	58.61±0.07	58.56±0.16	60.95±0.04	62.50 ± 0.12
Linoleic acid ($C_{18:2}$)	10.87 ± 0.02	17.93 ± 0.03	11.59±0.05	9.13 ± 0.03	11.91±0.02	16.93±0.02	14.96±0.04	10.28 ± 0.07
S/U	0.35	0.25	0.29	0.22	0.36	0.26	0.26	0.31

Data are expressed as mean value ± SD in percentage, (n=3). Measures were performed in triplicate. S/U: saturated/unsaturated free fatty acid ratio.

below 8% and the myristic acid (C14:0) concentration being less than 2%.

To this aim the FAMEs recovered in the hexane phase obtained from the transesterification were subjected to quantitative analysis. The individual FAMEs measurement allowed to understand the composition of the fatty acids in the acylglycerols fraction of the examined sausages (Table **3**).

It is worth noting that the content of unsaturated fatty acids bound to glycerol was still significantly high with values around 60%; nevertheless, an increase in the content of SFAs was observed. Palmitic and stearic acids were the prevailing SFAs being a percentage of total fatty acids about 25 and 12%, respectively. A content of oleic acid lower than that in FFAs was found in acylglycerols (between 50 % and 57%). However, the most interesting result was the drastic decrease of linoleic acid content with values above 2%. As a consequence, the S/U ratio of the fatty acids constitutive of acylglycerols in the sausages increases reaching values between 0.58 and 0.77.

These results show a different profile of fatty acids contained in acylglycerols respect to the FFAs. The Kruskal-Wallis test (for "salsiccia" and "spianata") and Mann-Whitney U test (for "soppressata") were applied to both FAME and FFA concentration data grouped in three categories for each type of sausage corresponding to the three ageing periods in order to find possible statistical differences between groups and, therefore, to study the influence of ageing period on FAME and FFA contents. As shown in Table **4**, many more differences were found in the FFA amounts than in the FAME ones. In particular, the concentration values of all the FFAs analyzed in the three types of sausages, except for stearic acid in salsiccia, were significantly affected by ageing process.

The decrease in the content of ω-6 fatty acids mostly represented by linoleic acid is consistent with the fact that there is a significant lipase activity. Linoleic acid represents together with oleic acid one of the preferred substrates for the hydrolytic activity of the lipases [30, 31]. Therefore, lipases act selectively releasing MUFAs and PUFAs with 18 carbon atoms. This enzymatic selectivity can explain the FFAs profile found in the examined sausages.

The dry fermented sausages cases of study are known as MUFAs and PUFAs rich foods. PUFAs are responsible for the oxidative phenomena. Oxidative stress and thermally induced conditions provoke lipid peroxidation of PUFAs in appreciable extents, with particular regard for linoleic acid, since its molecular structure displays a series of double carbon-carbon bonds which can undergo oxidative disruption.

In order to assess the extent of oxidation processes the Kreis assay (Table **5**) was performed on the three different type of sausages.

This classical assay has a qualitative validity in establishing the presence of aldehydes in the lipid portions extracted from meat products. In particular, the oxidative processes that occur in sausages are responsible for the formation of alkanals and alkenals by oxidation of the double bonds of linoleic acids present in the sausages [32, 33].

One sample for each kind of sausages gave positive test (samples **sal80**, **sop80**, **spi80**) indicating the presence of aldehydes in the sausages. However, the purity of reagents used for the test are sometimes responsible for false determination of the presence of aldehydes, especially when the concentration of these compounds in the sample is very moderate.

We decided, at this point, to subject one of the lipid extract positive to the Kreis test to further investigation using other more sensitive instrumental techniques [34]. As an example of analysis, we subjected to the nuclear magnetic resonance investigation the sample **sal80**. It has been shown that high resolution ^{1}H NMR is an appropriate tool for the evaluation in fats and edible oils of primary oxidation compounds, as well as of secondary oxidation products [34, 35]. The lipid extracts were analyzed directly after their obtainment, with no work-up, to make the procedure as short in time as possible and to zeroing any further undesired lipid

Table 3. FFAs profile in acylglycerols of the analyzed sausages form Southern Italy, determined as FAMEs by GC/MS.

	"Salsiccia"			**"Soppressata"**		**"Spianata"**		
	Sal20	**Sal40**	**Sal80**	**Sop40**	**Sop80**	**Spi20**	**Spi40**	**Spi80**
FAMEs	89.29 ± 1.94	88.72 ± 1.68	88.37± 1.25	87.20±1.51	90.08± 1.61	90.34 ± 1.15	87.18 ± 1.37	87.14 ± 1.16
Myristic acid ($C_{14:0}$)	1.72 ± 0.06	1.74 ± 0.06	1.74 ± 0.05	1.53 ± 0.05	1.54 ± 0.04	1.63 ± 0.04	1.49 ± 0.03	1.48 ± 0.04
Palmitic acid ($C_{16:0}$)	28.49 ± 0.31	26.94 ± 0.13	25.04 ±0.08	23.71±0.15	25.55±0.28	25.71±0.24	23.85±0.25	23.42 ± 0.43
Stearic acid ($C_{18:0}$)	13.17 ± 0.48	12.83 ± 0.17	12.28 ±0.25	12.39±0.29	13.49±0.18	10.46±0.22	11.61±0.42	11.99 ± 0.18
Palmitoleic acid ($C_{16:1}$)	4.10 ± 0.06	4.37 ± 0.07	3.77 ± 0.03	4.70 ± 0.12	3.25 ± 0.07	3.58 ± 0.11	3.39 ± 0.10	3.66 ± 0.08
Oleic acid ($C_{18:1}$)	50.33 ± 0.64	51.71 ± 0.35	54.64 ±0.44	55.19±0.21	54.07±0.36	55.93±0.04	57.07±0.55	56.75 ± 0.24
Linoleic acid ($C_{18:2}$)	2.19 ± 0.07	2.41 ± 0.06	2.53 ± 0.04	2.49 ± 0.04	2.42 ± 0.03	2.35 ± 0.03	2.58 ± 0.10	2.71 ± 0.10
S/U	0.77	0.71	0.64	0.60	0.68	0.61	0.59	0.58

Data are expressed as mean value ± SD in percentage, (n=3). Measures were performed in triplicate. S/U: saturated/unsaturated free fatty acid ratio.

Table 4. Significant differences between the three different ageing periods (20 days (1), 40 days (2), 80 days (3)) obtained by applying the Kruskal-Wallis test (for Salsiccia and Spianata) and Mann-Whitney U test (for Soppressata) to both FAME and FFA data.

FAME		
Variable	**Sausage**	**Periods[a]**
Linoleic acid	Salsiccia	1-3
Palmitoleic acid	Soppressata	1-2
Stearic acid	Soppressata	1-2
FFA		
Variable	**Sausage**	**Periods[a]**
Total amount	Salsiccia Soppressata	1-3 1-2
Miristic acid	Salsiccia Soppressata Spianata	1-2 1-2 1-2
Palmitoleic acid	Salsiccia Soppressata Spianata	1-2 1-2 2-3
Palmitic acid	Salsiccia Soppressata Spianata	1-2 1-2 2-3
Oleic acid	Salsiccia Soppressata Spianata	1-3 1-2 1-3
Linoleic acid	Salsiccia Soppressata Spianata	1-2 1-2 1-3
Stearic acid	Soppressata Spianata	1-2 1-2

[a] Periods between which the significant difference was observed

oxidation. The proton NMR spectrum of the total lipid extract obtained from the sausage case of study displayed the expected set of signals attributable to the content of acyl chains, which were present in the sausage fat either as components of acylglycerols and as FFAs (Fig. **2A**).

All these signals were unambiguously assigned to the corresponding spin systems of the different types of protons belonging to the various acyl chains, according to the literature values [36, 37]. No other signals were visible in the recorded spectrum, at least upon normally scaled graphic conditions. Afterward, we undertook the analysis of the sub-spectrum: the downfield region between 9.00 and 10.00 ppm showed some interesting peculiarities, principally due to the presence of a series of well-distinct signals (Fig. **2B**). All these resonances were attributed to the formyl protons of aldehydes generated from the lipid secondary oxidation pathway. In the absence of specimens of these aldehydes, the data already published [38] supported us in establishing by analogy the nature of compounds generating the above set of signals. Therefore, the signal appearing at 9.75 ppm was attributed to the series of overlapped triplets generated by the aldehydic proton in n-alkanals. The two lines at 9.56 and 9.59 ppm were indicative of the presence of 4-hydroxy-trans-2-alkenals in the products of oxidation of the mature sausage

Table 5. Kreis test of sausages

Sample	Kreis test[a]
Sal20	Negative
Sal40	Negative
Sal80	Positive ++
Sop40	Negative
Sop80	Positive ++
Spi20	Negative
Spi40	Negative
Spi80	Positive +

[a] + = pink; ++ = deep pink.

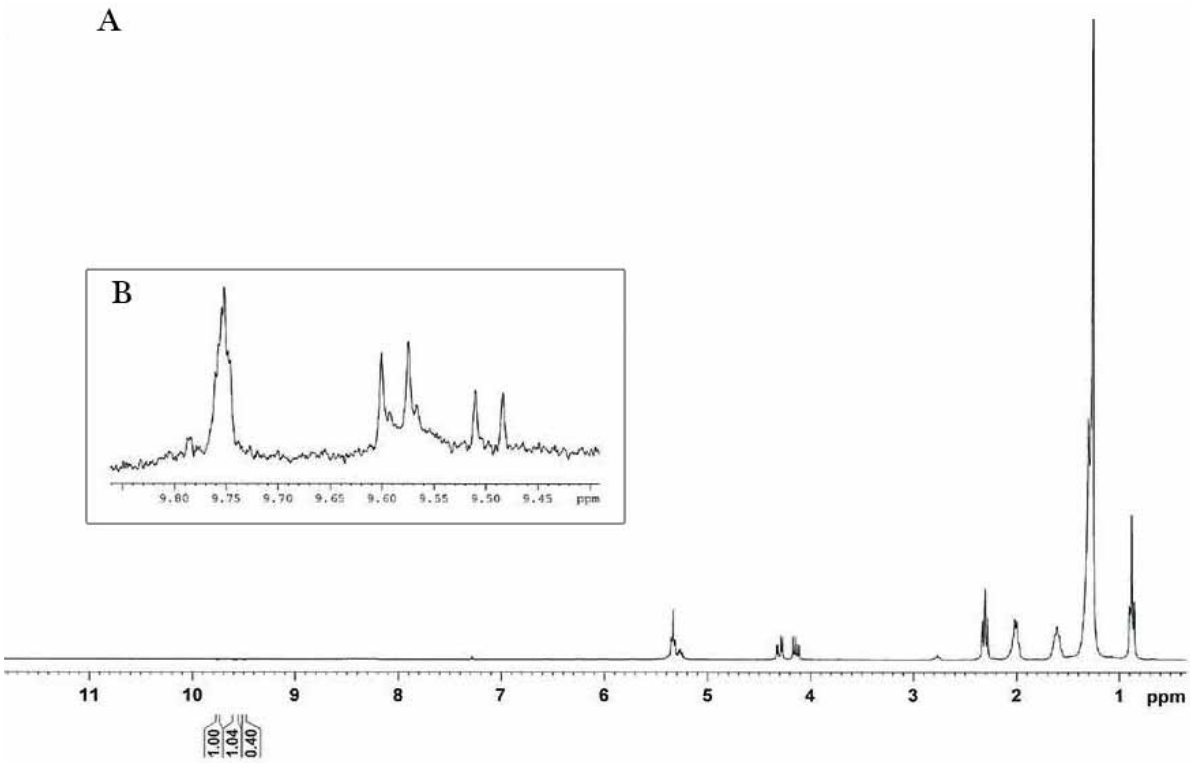

Fig. (2). High resolution ^{1}H NMR spectrum of a sample of total lipid extract obtained from sample **sal80** (**A**), and region of the aldehydic protons (**B**).

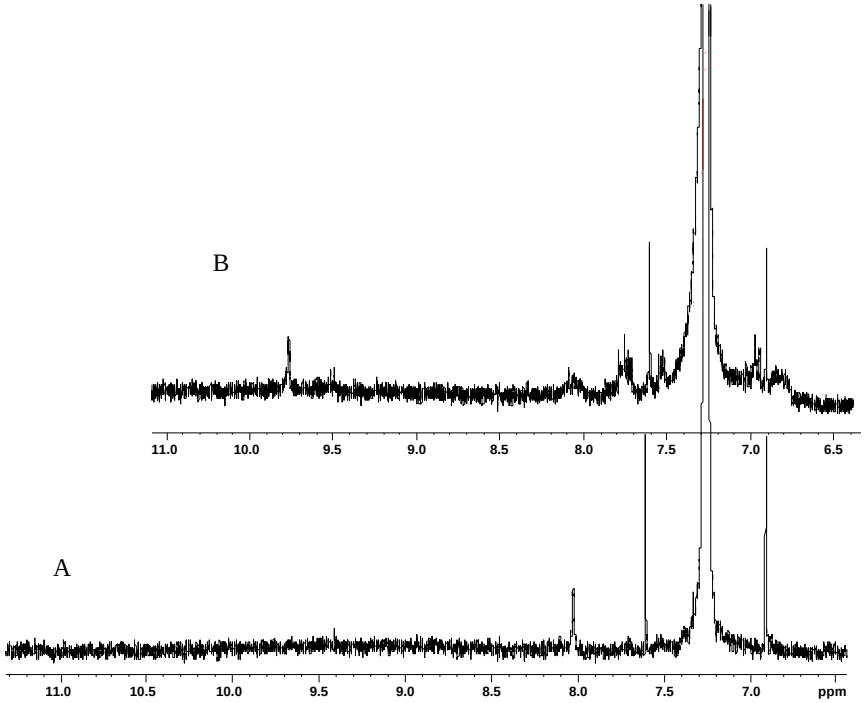

Fig. (3). Aldehydic proton windows of the high resolution ^{1}H NMR spectra recorded on samples **spi40** (spectrum **A**) and **spi80** (spectrum **B**).

fat. For this family of compounds the values of 9.560 and 9.586 ppm are reported, demonstrating the perfect matching between our analysis and the literature data. The spectrum displayed two further doublets: the first, with lines at 9.57 and 9.60 ppm, was attributable to the aldehydic protons of 4-hydroperoxy-trans-2-alkenals; the second, generated by the aldehydic group of trans-2-alkenals, showed its lines at 9.48 and 9.51 ppm. The appearance of the discussed signals in the spectrum, together with the absence of the triplet typically recognized for the methyl group of linolenic acid at 0.97 ppm indicated that PUFAs underwent oxidation generating the observed series of aldehydes.

^{1}H NMR analysis was also performed on samples **spi40** and **spi80**. The respective aldehydic proton windows are displayed in Fig. (**3**). As it can be observed from spectrum A the analysis of **spi40**, sample negative to Kreis test, did not show signals attributable to secondary lipid oxidation products. Aldehydic proton resonances of weak intensity were instead observed in the case of the slightly oxidized sample **spi80** (spectrum B).

CONCLUSION

In conclusion, GC/MS analysis was used for determining the FFA content and the fatty acid profiles in acylglycerols of three kinds of dry fermented sausages from Calabria during their ripening. The data obtained indicates that lipolysis plays an essential role in developing FFAs during ripening. A preferential hydrolysis of linoleic and oleic acid was observed. The generation of aldehydic products by the oxidative degradation of unsaturated fatty acid chains was also studied by ^{1}H NMR. The spectroscopic investigation confirmed the data obtained by Kreis test.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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