Food Chemistry 371 (2022) 130995

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Improvements in the methodology for fatty acids analysis in meat products: One-stage transmethylation and fast-GC method

Trinidad Perez-Palacios *, Juan Carlos Solomando, Jorge Ruiz-Carrascal, Teresa Antequera

Research Institute of Meat and Meat Products (IProCar), University of Extremadura, Avda. de las Ciencias s/n, 10003 Cáceres, Spain

ARTICLE INFO

Keyword:
One-stage transmethylation
Fast chromatographic analysis
Fatty acid quantification
Meat products
Performance

Chemical compounds:
Chloroform (PubChem CID 6212)
Methanol (PubChem CID 887)
Sodium chloride (PubChem CID 5234)
Anhydrous sodium sulphate (PubChem CID 24436)
Sulphuric acid (PubChem CID 1118)
Sodium metal (PubChem CID 5360545)
Chlorotrimethylsilane (PubChem CID 6397)
Hydrochloric acid (PubChem CID 313)

ABSTRACT

The quantification of fatty acids (FA) in meat products is frequently carried out by two-stage methylation procedures followed by long gas chromatography (GC) runs. This work aimed to simplify this methodology by means of a one-stage transmethylation method and a fast GC run, evaluating the influence of sample preparation, reagents and type of heating on the amount of FA in different meat products and optimizing a fast GC-FID (flame ionization detector) run. This allowed to establish the optimum combination of parameters (methanol + chlorotrimethylsilane, lyophilized samples and oven heating) to achieve the quantification of the highest possible amount of FA and to reduce the time of GC run from 60 to 10 min. The quality evaluation of this method obtained satisfactory results. Thus, the quantification of FA in meat products was achieved in a straightforwardly and quickly way by using a one-stage transmethylation procedure followed by a fast GC-FID run.

1. Introduction

Fatty acid (FA) composition in meat products strongly influences their quality characteristics, such as firmness, oxidative stability, shelf life or flavour. In fact, in dry-cured hams, the FA profile has been used to predict some sensory traits (Pérez-Palacios, Ruiz, Ferreira, Petisca, & Antequera, 2012). The nutritional implications of dietary FA acid composition are also worth noting, since its relationship with promotion and prevention of different diseases, i.e. the consumption of low contents of saturated FA (SFA) and high of monounsaturated and polyunsaturated FA (MUFA and PUFA, respectively) seems to significantly reduce cardiovascular risk factors (Tindall et al., 2019).

Besides, in packaged products, the analysis of FA is required in the case of labelling with nutritional claims about the lipid profile, i.e. "LOW SATURATED FAT: *a claim that a food is low in saturated fat, and any claim likely to have the same meaning for the consumer, may only be made if the sum of saturated fatty acids and trans-fatty acids in the product does not exceed 1.5 g per100 g for solids or 0.75 g/100 ml for liquids and in either case the sum of saturated fatty acids and trans-fatty acids must not provide more than 10 % of energy*." or "HIGH MONOUNSATURATED FAT: *a claim that a food is high in monounsaturated fat, and any claim likely to have the same meaning for the consumer, may only be made where at least 45 % of the fatty acids present in the product derive from monounsaturated fat under the condition that monounsaturated fat provides more than 20 % of energy of the product*" (EU, 2010).

The methodology for the analysis of FA in meat products is not simple, requiring an exhaustive extraction of lipids from the sample, followed by the derivatization of the FA to obtain their volatile FA methyl esters (FAMEs) derivatives, and the analysis of such FAMEs, which is normally carried out by gas chromatography (GC). The usual process initiates with a solvent extraction of lipids followed by their transmethylation. Nowadays, this kind of two-stage procedures are

Abbreviations: FA, fatty acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; FAME, fatty acid methyl ester; GC, gas chromatography; FID, flame ionization detector; CTMS, chlorotrimethylsilane; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; RDS, relative standard deviation; R^2, coefficient of determination; ANOVA, one way analysis of variance.

* Corresponding author.
E-mail address: triny@unex.es (T. Perez-Palacios).

https://doi.org/10.1016/j.foodchem.2021.130995
Received 4 February 2021; Received in revised form 27 August 2021; Accepted 29 August 2021
Available online 1 September 2021

highly used in meat products (Ferrer-González, García-Martínez, & Totosaus, 2019; Holman et al., 2019; Rasinska, Rutkowska, Czarniecka-Skubina, & Tambor, 2019; Solomando, Antequera, & Perez-Palacios, 2020; Utama, Jeong, Kim, Barido, & Lee, 2019; Vasilev et al., 2020). In these recent studies, the preferred method for lipid extraction in meat samples is that described by (Folch, Lees, & Sloane Stanley, 1987), using a mixture of chloroform:methanol (2:1, v/v), and the transmethylation is carried out by using an acidic (boron trifluoride or sulfuric acid) or basic reagent (potassium hydroxide) with methanol (Pérez-Palacios & Estévez, 2020).

However, since these two-stage methods are time-consuming and laborious, research has been devoted to avoiding the solvent extraction step and set a one-stage procedure to simultaneously carry out the extraction of the lipids and the transmethylation of the FA. Thus, different one-stage transmethylation methods have been reported for the FA analysis of meat and meat products. These procedures mainly differ in the number of phases (one or two) of the transmethylation reaction and in the chemicals. The two phases transmethylation methods consist on firstly mixing the sample with sodium methoxide in anhydrous methanol and heating, and then adding acetyl chloride in anhydrous methanol and heating again (Agnew et al., 2019; Lee, Tweed, Kim, & Scollan, 2012; Schiavon et al., 2016). In the case of the transmethylation methods in one phase, different reagents have been proposed for being mixed with the sample and subsequently heated: hydrochloric acid in methanol (Carrapiso, Timón, Petrón, Tejeda, & García, 2000; Juárez et al., 2008), chlorotrimethylsilane (CTMS) in methanol (Tomàs et al., 2009) or sulfuric acid in methanol (Agnew et al., 2019). In this respect, Wang, Lim, Choi, Kang, and Lee (2013) demonstrated that the methylation performances of the reagents (hydrochloric acid in methanol, boron trifluoride in methanol and sulfuric acid in methanol) depend on the type of sample and these authors claimed that a judicious choice of the methylation reagent should be made. In all the reported methodologies, FAMEs are finally recovered after the addition of a salt solution and an organic solvent.

In these one-stage transmethylation methods for meat products, the influence of some reaction parameters on obtained FAMEs amounts have been evaluated. Thus, the effect of temperature, reaction time, acid concentration, solvent volume, sample weight and sample moisture have been analysed (Agnew et al., 2019), with temperature and time showing the highest influence. Most reports indicate heating at high temperatures (60–80 °C) for long times (1–2 h) using a water bath (Agnew et al., 2019; Juárez et al., 2008; Lee et al., 2012; Schiavon et al., 2016). However, Tomàs et al. (2009) suggested to heat the samples by using microwave radiation, achieving an important time reduction for the transmethylation of meat samples to up to 30 s.

Regarding sample preparation in one-stage transmethylation methods in meat samples, the use of freeze-dried samples is required (Agnew et al., 2019; Juárez et al., 2008; Lee et al., 2012; Schiavon et al., 2016; Tomàs et al., 2009), which implies longer times and expensive equipment. This procedure may be also simplified by using thoroughly minced samples, but this issue has not been evaluated until now.

Once the FAMEs have been obtained, they are subsequently separated, preferably by using GC coupled to a flame ionization detector (FID), identified and quantified by using FAMEs standards and internal standards, such as undecanoic (C11:0) or tridecanoic (C13:0) acids (Pérez-Palacios & Estévez, 2020). This GC methods are usually time consuming, which is mainly related to the use of long capillary columns (more than 30 m) and extended oven temperature programs (around 1 h) (Agnew et al., 2019; Juárez et al., 2008; Lee et al., 2012; Wang et al., 2013). In this regard, the shortening of the GC run should be combined with the improvement of the one-stage transmethylation procedure for getting a rapid FA analysis.

Thus, this study aimed i) to evaluate the effect of sample preparation, reagents and heating mode of a one-stage transmethylation procedure on the FA acid composition of different meat products and ii) to set the GC parameters for a short run, with the final objective of simplifying and shortening the FA analysis in this kind of samples.

2. Material and methods

2.1. Samples

Different meat products (fresh pork loin, cooked sausages, dry-cured sausage and dry-cured ham) were purchased from a local supermarket. The products were minced or freeze-dried and refrigerated until analysis.

2.2. Reagents

Chloroform and methanol from Scharlau (Barcelona, Spain) were used for the lipid extraction; for this determination, butylated hydroxytoluene (BHT) and chloride and anhydrous sodium sulphate (Scharlau) were also needed. Sulphuric acid, sodium metal, CTMS, hydrochloric acid from Merck (Madrid, Spain) and chloroform and methanol were needed for carrying out different transmethylation procedures.

2.3. Experimental design

The first experiment of the present study dealt with the achievement of a one-stage transmethylation procedure for meat products. In order to address that, samples (four different meat products) were treated following twelve one-stage transmethylation procedures, varying in the sample preparation (minced by using a home grinder; freeze-dried by using the lyophilizer LABCONCO (Kansas City, USA)), the reagents (methanol + CTMS; sulphuric acid in methanol 1% + sodium metal in methanol; hydrochloric acid in methanol 3%) and the heating (by using oven or microwave). The obtained FAMEs were analysed following a GC-FID run of 60 min. The procedure yielding the highest quantity of FAMEs for most samples was selected.

The second experiment covered the setting of the chromatographic conditions to get a fast GC-FID method that accomplishes the analysis of major and minor FA of different meat products. For that, the chromatographic parameters (for inlet, oven, detector) of a GC-FID run of 10 min were established with a mixture of FAMEs standards.

Then, the improved procedure (one-stage transmethylation and fast GC run) was tested in the four products by comparing with the usual method of two-stage methylation and long-time GC-FID run. Finally, the quality of this new methodology for FA analysis of meat products was evaluated.

Determinations carried out for all procedures on each meat product were done in quintuplicate.

2.4. Two-stage transmethylation method

Firstly, total lipids were extracted following the method of (Folch et al., 1987) modified by (Pérez-Palacios, Ruiz, Martín, Muriel, & Antequera, 2008). Samples (5 g) were homogenized, in a Sorvall Omnimixer (OMNI international, US) at 12000 rpm for 2 min, with 100 ml of chloroform:methanol (2:1, v/v) with BHT (0.05%), centrifuged (10 min, 3000 rpm) and filtered. Then, the filtrate was mixed with 5 ml of distilled water, shaken and centrifuged (10 min, 3000 rpm), obtaining a byphasic system. The upper aqueous phase was eliminated, while the lower chloroformic phase was filtered through anhydrous sodium sulphate and collected. Chloroform was evaporated with a rotary evaporator under vacuum and nitrogen, to finally get the extracted lipids.

Secondly, FAMEs were prepared according to the method described by (Sandler & Karo, 1992). Extracted fat (10 mg) was added to 1 ml of chloroform containing 0.4 mg/ml tridecanoic acid (C13:0) (Merck), as an internal standard (IS). Then, chloroform was evaporated under nitrogen. Subsequently, 1 ml sodium metal in methanol (0.1 N) was added, vortexed and heated in an oven during 30 min at 80 °C. After heating, 1 ml of sulphuric acid (5%) in methanol was also added, and again

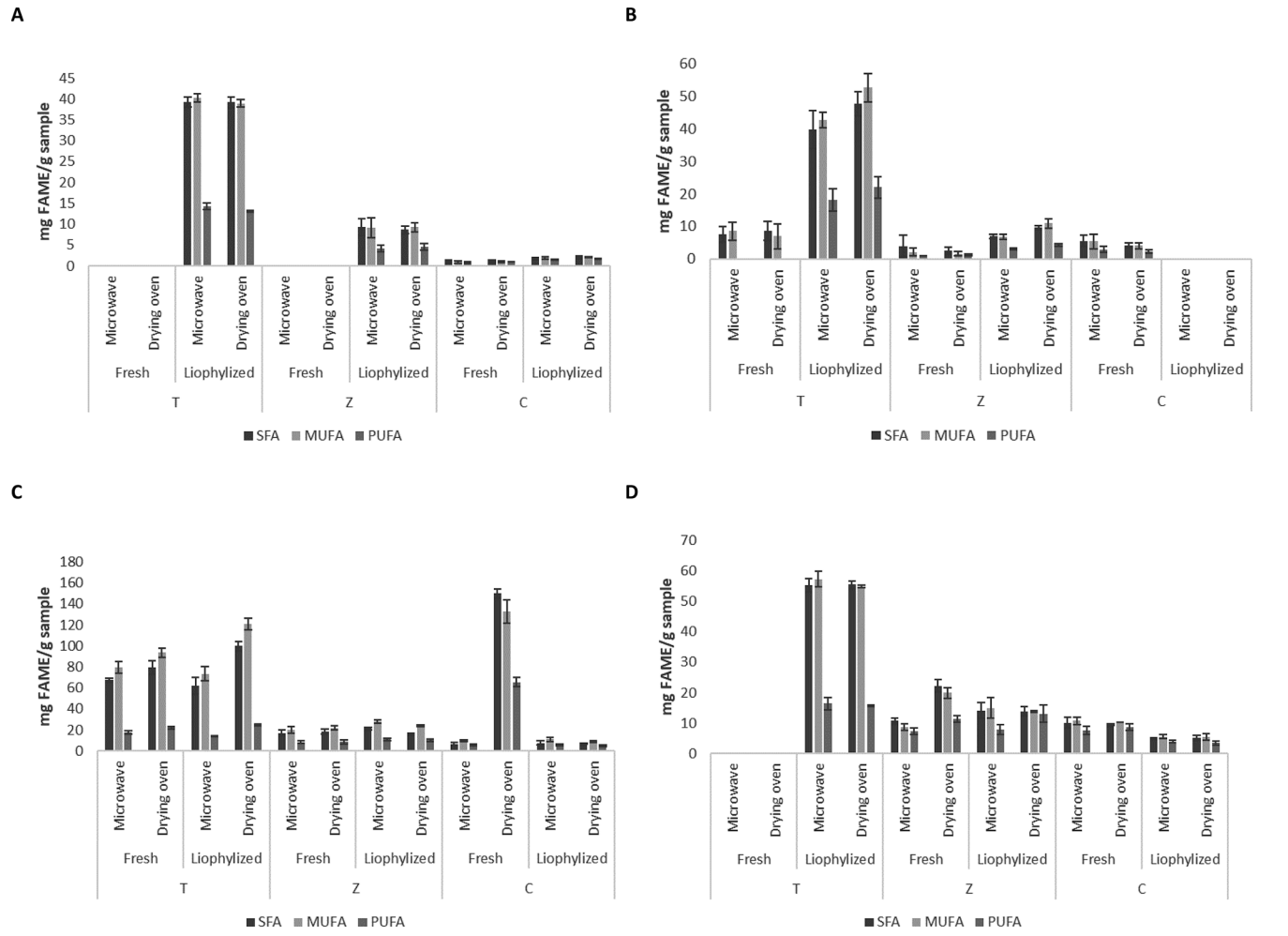

Fig. 1. Quantity (average values and standard deviation) of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid methyl esters (FAME) in different meat samples (A, pork loin; B, cooked sausage; C, dry-cured sausage; D, dry-cured ham) by means of one-stage transmethylation procedures differing in the reagents (T, Methanol + CTMS; Z, sulphuric acid in methanol + sodium metal; C, hydrochloric acid in methanol), sample preparation (minced or liophylized) and type of heating (by microwave or drying oven).

vortexed and heated in an oven during 30 min at 80 °C. After cooling, 1 ml of hexane and 1 ml of a supersaturated sodium chloride solution were added, followed by mixing and centrifuging for 15 min at 4500 rpm. The organic solvent top layer containing the FAMEs was pipetted into a 2 ml GC vial and evaporated under nitrogen. Finally, FAMEs were accurately dissolved in 1 ml of hexane.

2.5. One-stage transmethylation methods

Samples (50 mg dry matter), minced or freeze-dried, were added with the IS as explained above. Then, either methanol (1 ml) plus CTMS (0.5 ml) or 5% sulphuric acid (1 ml) in methanol plus 0.1 N sodium metal in methanol (0.5 ml) or hydrochloric acid in methanol 5 % (3 ml), were added, vortexed and heated. Heating was carried out by either using an oven (80 °C, 30 min) or a domestic microwave (800 W, 30 s). After cooling, the procedure followed as described above. Thus, twelve one-stage transmethylation procedures (2 sample preparation × 3 reagents × 2 heating) were evaluated in four meat products, giving a total of 48 samples.

2.6. Gas chromatographic conditions

FAMEs were analysed by GC using a Hewlett–Packard HP-5890A gas chromatograph, equipped with FID. Two chromatographic methods were tested. A time-consuming one (60 min per run) was carried out with an on-column injector and using a polyethylene glycol capillary column (Supelcowax-10, Supelco, Bellefonte, PA, USA) (60 m × 0.32 mm i.d. × 0.25 μm film thickness). The initial GC oven temperature was 180 °C that increased at 5 °C/min to 200 °C, being maintained 40 min at this temperature; thereafter, it increased at 5 °C/min to 250 °C, and then kept for an additional 21 min. The injector and detector temperatures were 250 °C. The carrier gas was helium at a flow rate of 0.8 ml/min.

For the faster chromatographic method (10 min per run), a cyanopropyl column (ZEBRON ZB-FAME, Phenomenex, California, USA) (20 m × 0.18 mm i.d. × 0.15 μm film thickness) with split injection (100:1) and Helium at a constant flow of 2.7 ml/min as the carrier gas was used. Injector and detector temperature were set at 250 °C. The temperature profile of the oven was 150 °C that increased at 10 °C/min to 180 °C. This was held for 1 min and increased again at 7 °C/min to 205 °C, which is maintained for 2 min.

Individual FAME peaks were identified by comparison of their retention times with those of standards (Supelco 37 component FAME mix, Merck).

2.7. Quantification of fatty acids

The external calibration curve method (usually applied) and the standard addition method were evaluated for the quantification of FA in the analysed meat samples. In the first case, five consecutive dilutions of the mix of standards (Supelco 37 component FAME mix, Merck) were

prepared and added with the corresponding amounts of methyl tridecanoate standard (Merck) to have the same concentration (0.4 mg/ml) of this FAME in the five dilutions. For the standard addition procedure, transmethylated meat samples (cooked and dry-cured sausages) were spiked with increased volumes (0, 25, 50, 100 and 200 μl) of the mix of standards. In both methods, the chromatographic analysis was carried out by applying the faster GC-FID conditions. For each FAME, calibration curve (FAME peak area/C13 peak area versus FAME amount/C13 amount) was constructed. In the standard addition method, the difference between control (0 μl) and added (25, 50, 100 or 200 μl) samples was used for calculating the FAME peak area/C13 peak area, and the FAME amounts in the different points of the curve. The results, expressed in mg FAME/g sample, were calculated using the exact weight of the sample.

2.8. Quality control of the fast GC-FID method

Quality control of the GC-FID analysis was performed through the routine analysis of procedural blanks and quality control standards and samples to ensure the absence of contaminants and the possible carry-over between samples and to assess the quality of the results. The method was evaluated by means of linearity, limit of detection (LOD) and quantification (LOQ), relative standard deviation (RSD) and percentage recovery.

The linearity was evaluated by the determination coefficient (R^2) of calibration curves of each FAME (FAME peak area/IS peak area versus FAME amount/IS amount). LOD and LOQ, based on a signal/noise ratio of 3:1 and 10:1, respectively, were determined using standard solutions ($n = 5$). For calculating the RSD run-to-run and day-to-day, five replicate analyses of samples were analysed in 1 day and in three different days, respectively. To calculate the percentage recovery, meat samples were spiked with appropriate amounts of FAMEs (0.25–6 μg) and were analysed using the established conditions and applying external calibration curves.

2.9. Statistical design

Influence of sample preparation (minced *vs*. freeze-dried), reagents (methanol + CTMS; sulphuric acid in methanol 1% + sodium metal in methanol; hydrochloric acid in methanol 3%) and heating (oven *vs*. microwave) on individual FAME quantities of each meat product was analysed by a multivariate linear regression model with interaction. When a significant effect ($p < 0.05$) was detected, paired comparisons between means were conducted using the Tukey's test. Differences on FAME quantities obtained from classical and improved procedures were evaluated by one-way analysis of variance (ANOVA). The statistics were run using the program IBM SPSS Statistics v.22 (IBM Co., New York, USA).

The slopes of the regression equations obtained for the external standard calibration curve and for the standard addition to sample curve were statistically compared by paired Student's *t*-test. Besides, the content of individual FAMEs in different meat samples obtained by these two methods were also analysed by ANOVA.

3. Results and discussion

3.1. One-stage transmethylation procedure: evaluation

Fig. 1 (A, B, C, D) shows the quantity of SFA, MUFA and PUFA in different meat samples (pork loin, cooked sausage, dry-cured sausage and dry-cured ham, respectively) by means of one-stage transmethylation procedures differing in the reagents (methanol + CTMS; sulphuric acid in methanol + sodium metal in methanol; hydrochloric acid in methanol), sample preparation (minced or lyophilized) and type of heating (microwave or oven). Besides, results on the statistical analysis (*p*-value) at evaluating these effects (individually or in combination) are shown in Table 1. As can be seen, reagents, sample preparation and the combined effects of reagents*sample preparation significantly influenced the quantities of SFA, MUFA and PUFA in the four types of meat samples. Overall, the use methanol + CTMS and lyophilized samples gave higher FA quantities than fresh samples treated with sulphuric acid in methanol or hydrochloric acid in methanol. These findings are quite in concordance with previous studies. The use of an in situ methylation method with hydrochloric acid in methanol had lower recoveries values for FAMEs in beef samples than usual two-stage methodology (Juárez et al., 2008), which the authors ascribed to both incomplete FAME extraction and methylation. In fact, hydrochloric acid in methanol seems to be mainly suitable for preparing FAMEs from short chain FA. (Carrapiso et al., 2000) obtained accurate results by using hydrochloric acid in methanol for the direct transesterification of FA from subcutaneous adipose tissue of pigs. Sulphuric acid in methanol is more appropriate for the FAME preparation of carboxylic acids and esters (Wang et al., 2013), while some studies have shown the effectiveness of CTMS for direct transmethylation of FA with quantification purposes in a high number of samples (Lee et al., 2012). In the comparative study of Wang et al. (2013) with samples of chicken, differences in the methylation performance were observed among reagents depending on the FA, for example, sulphuric acid in methanol achieved higher performance than hydrochloric acid in methanol for oleic (C18:1n-9) and linolenic (C18:3n-3) acids, while for stearic (C18:0) and arachidonic (C20:4n-6) acids the opposite behaviour was found. The significantly higher FA quantities found when using lyophilized than minced samples could be attributed to a more intense homogenization between the sample and the reagent (Lee et al., 2012) in the first case, which would eventually lead to the methylation of most FA.

The effect of the type of heating was only significant in dry-cured sausage (for SFA, MUFA and PUFA) and dry-cured ham (for SFA and MUFA), leading to higher FA quantities when using the oven. The same influence was observed for the rest of combined effects (reagents*heating, sample preparation*heating, reagents*heating*sample preparation). These results might be related to the lower control and homogeneity of temperature when using microwave, as previously pointed out by other authors (Tomàs et al., 2009). Nevertheless, some

Table 1
Results on the statistical analysis (*p*-value) at evaluating the influence of the reagents, the sample preparation and the type of heating (individually or in combination) of a new one-stage transmethylation procedure for quantifying saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in different meat samples.

		FRESH PORK LOIN	FRANKFURTER	DRY-CURED SAUSAGE	DRY-CURED HAM
Reagents	SFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	MUFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	PUFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Sample preparation	SFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	MUFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	PUFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Heating	SFA	0.832	0.432	**<0.001**	**0.003**
	MUFA	0.691	0.429	**<0.001**	**0.016**
	PUFA	0.535	0.399	**<0.001**	0.125
Reagents * sample preparation	SFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	MUFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
	PUFA	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Reagents * heating	SFA	0.804	0.522	**<0.001**	**<0.001**
	MUFA	0.643	0.689	**<0.001**	**<0.001**
	PUFA	0.088	0.621	**<0.001**	0.09
Sample preparation * heating	SFA	0.759	0.29	**<0.001**	**0.002**
	MUFA	0.631	0.206	**<0.001**	**<0.001**
	PUFA	0.398	0.334	**<0.001**	0.852
Reagents * sample preparation * heating	SFA	0.857	0.838	**<0.001**	**<0.001**
	MUFA	0.679	0.633	**<0.001**	**<0.001**
	PUFA	0.112	0.731	**<0.001**	0.841

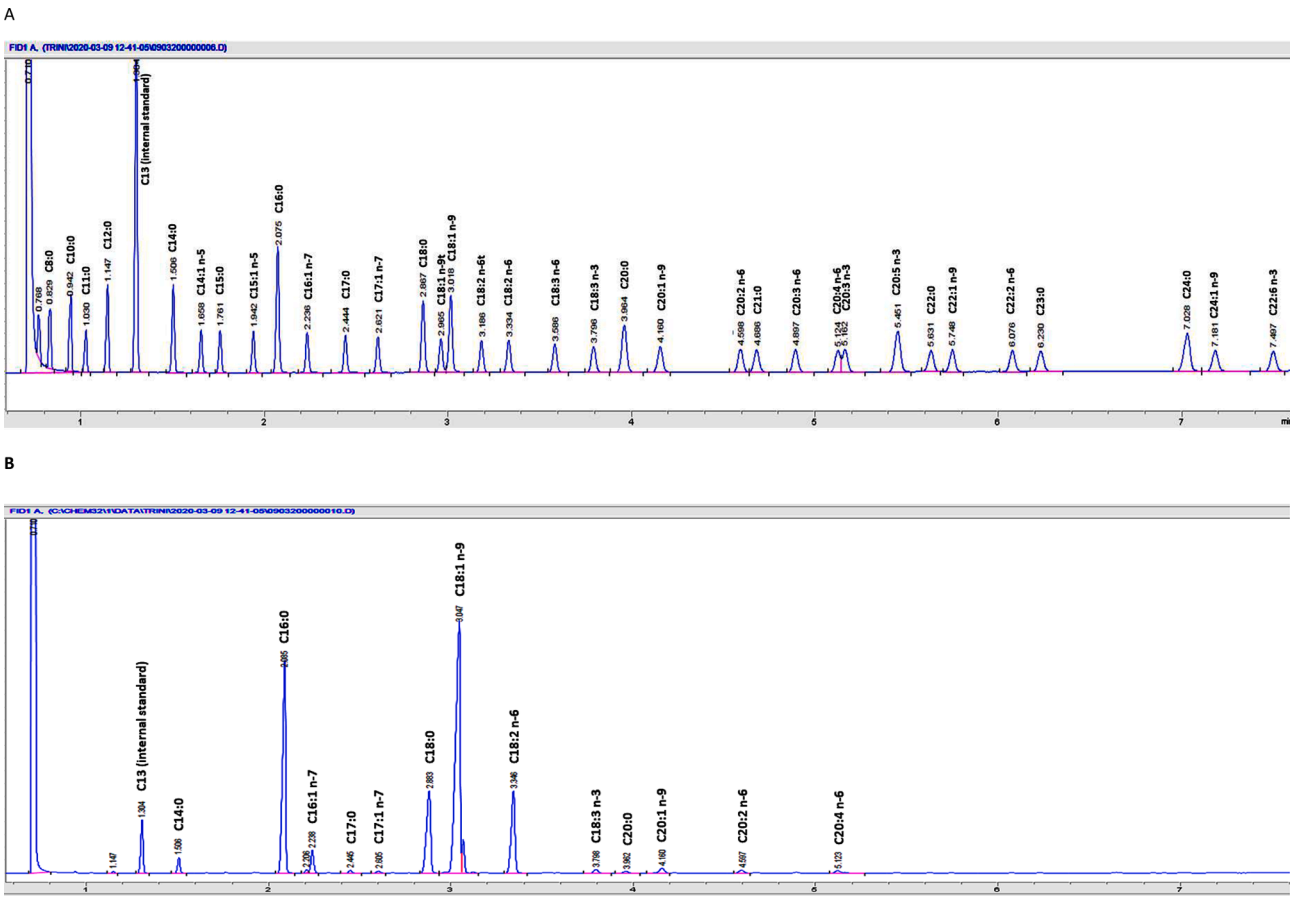

Fig. 2. Chromatograms of FAME peaks from a standard mix (A) and fresh pork loin (B) by using the settled fast GC-FID method.

previous studies have indicated the use of domestic microwaves for direct FA methylation without major issues (Armstrong, Metherel & Stark, 2008).

It is also noticeable that the influence of the type of heating is different depending on the type of meat product (Fig. 1). Similarly, Lee et al. (2012) also observed that the effect of the reagent on methylation performance varied with the sample, finding more significant differences in meat than in milk samples. Besides, the influence of the percentage of dry matter on the FA analysis in meat samples has been reported (Pérez-Palacios et al., 2012), being hindered as the content of dry matter increases, due to a major solvent absorption. This finding may explain the effect of the type of heating found in the present work.

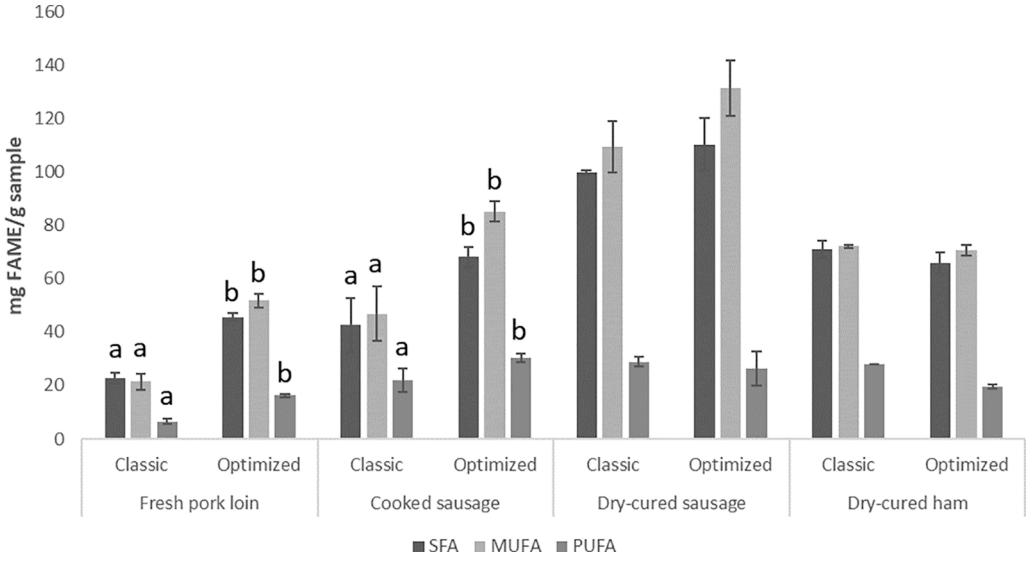

Fig. 3. Quantity (average values and standard deviation) of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid methyl esters (FAME) * determined by a classic (two-stage transmethylation + long-time gas chromatographic run) and optimized procedures (one-stage transmethylation + fast gas chromatographic run) in different meat samples.

Table 2
Quality parameters of each fatty acid detected under the optimised fast GC-FID method.

	Linealidade (R^2)			LOD (mg/ml)	LOQ (mg/ml)	RSD (%)		Recovery (%)
	Standards	Cooked sausage	Dry-cured sausage			run-to-run	day-to-day	
C8:0	0.9742	0.9996	0.9992	0.0037	0.0124	3.17	10.68	95.56
C10:0	0.9923	0.9988	0.9989	0.0031	0.0102	1.56	10.79	94.16
C11:0	0.9897	0.9988	0.9987	0.0013	0.0044	6.11	10.48	94.99
C12:0	0.9920	0.9980	0.9999	0.0019	0.0063	6.27	11.14	94.15
C14:0	0.9729	0.9999	0.9986	0.0020	0.0067	9.02	11.81	96.76
C14:1n-5	0.9918	0.9976	0.9987	0.0012	0.0040	6.64	11.00	96.81
C15:0	0.9906	0.9979	0.9961	0.0012	0.0040	7.58	12.18	96.64
C15:1n-5	0.9922	0.9986	0.9999	0.0025	0.0085	6.10	10.65	96.37
C16:0	0.9973	0.9985	0.9998	0.0076	0.0252	2.57	6.70	95.16
C16:1n-7	0.9829	0.9980	0.9966	0.0034	0.0113	3.85	8.37	90.23
C17:0	0.9931	0.9996	0.9932	0.0060	0.0200	5.66	9.52	91.34
C17:1n-7	0.9907	0.9998	0.9981	0.0045	0.0150	6.21	10.94	94.89
C18:0	0.9965	0.9972	0.9978	0.0006	0.0022	4.58	7.14	95.10
C18:1n-9 t	0.9842	0.9997	0.9993	0.0012	0.0040	5.03	9.90	95.04
C18:1n-9	0.9993	0.9973	0.9954	0.0032	0.0107	1.72	1.85	96.32
C18:2n-6 t	0.9905	0.9996	0.9978	0.0013	0.0044	5.58	9.85	92.22
C18:2n-6	0.9861	0.9998	0.9986	0.0022	0.0073	6.45	9.12	92.12
C18:3n-6	0.9987	0.9999	0.9924	0.0010	0.0032	5.81	9.79	93.79
C18:3n-3	0.9860	0.9991	0.9948	0.0043	0.0144	6.69	8.79	95.48
C20:0	0.9987	0.9970	0.9914	0.0015	0.0050	6.81	10.75	91.87
C20:1n-9	0.9847	0.9947	0.9993	0.0046	0.0155	7.48	9.11	89.49
C20:2n-6	0.9909	0.9995	0.9968	0.0039	0.0131	5.59	9.43	88.27
C21:0	0.9888	0.9995	0.9999	0.0043	0.0142	5.67	9.40	90.34
C20:3n-6	0.9844	0.9985	0.9942	0.0062	0.0207	5.48	7.77	94.77
C20:4n-6	0.9875	0.9997	0.9929	0.0037	0.0125	4.25	9.10	91.19
C20:3n-3	0.9895	0.9915	0.9953	0.0041	0.0136	5.15	8.25	92.26
C20:5n-3	0.9933	0.9986	0.9950	0.0003	0.0010	6.11	9.89	89.90
C22:0	0.9811	0.9985	0.9970	0.0260	0.0866	5.73	9.18	90.43
C22:1n-9	0.9911	0.9994	0.9940	0.0058	0.0194	6.70	8.40	94.44
C22:2n-6	0.9941	0.9999	0.9945	0.0157	0.0522	3.72	8.90	92.98
C23:0	0.9933	0.9999	0.9989	0.0078	0.0259	6.51	9.52	95.40
C24:0	0.9905	0.9993	0.9968	0.0023	0.0077	5.77	10.00	91.14
C24:1n-9	0.9926	0.9904	0.9935	0.0068	0.0227	5.16	10.50	93.75
C22:6n-3	0.9920	0.9969	0.9981	0.0068	0.0228	4.81	10.00	91.18

Meat products affected by the heating (dry-cured sausage and dry-cured ham) show higher dry matter content (>45%) than non-affected samples (fresh pork loin, cooked sausage) (<65%) (Pérez-Palacios et al., 2012), which could lead to a less solvent volume to react with the FA in the formers. In addition, in these cases, the control and homogeneity of temperature is crucial and probably not totally achieved when heating by microwave (Tomàs et al., 2009), which should affect the lipid extraction and/or FA transesterification. This may also explain the different effect of the type of heating depending on the meat sample.

Considering these findings, the use of lyophilized samples, methanol + CTMS and oven for the one-stage transmethylation method of FA in meat samples seems to be the most efficient method for obtaining FA methyl esters.

3.2. Fast GC-FID run for the FA quantification in meat samples

Once established the conditions for the one-stage transmethylation procedure, the following stage focused on setting up the chromatographic conditions to get a fast GC-FID run, which are detailed in the material and methods section. Fig. 2 shows the chromatograms obtained by applying the settled conditions to analyse the 37 component FAME mix standards and a meat sample (fresh pork loin). 35 in 37 FAME standards have been separated and identified, butyric (C4:0) and caproic (C6:0) acids methyl esters remaining undetected. This is probably due to the initial temperature of the oven, which is higher (150 °C) than that recommended for detection of short chain FA (80 °C to 160 °C at 40 °C/min) (Phenomenex, 2017). In fact, the initial temperature was increased purposely to save time, since these short chain FA are not of interest in meat samples. For samples containing these short chain FA, such as milk and derived products, initial temperature should be decreased. In the fresh pork loin samples, the identified FAMEs were: myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1n-9), margaric (C17:0), heptadecenoic (C17:1n-10), stearic (C18:0), oleic (C18:1n-9), linoleic (C18:2n-6), linolenic (C18:3n-3), arachidic (C20:0), eicosenoic (C20:1n-9), behenic (C22:0) and arachidonic (C20:4n-6) acids, which are according to the FA profile described in the scientific literature for this product (Martin, Antequera, Muriel, Perez-Palacios, & Ruiz, 2008; Pérez-Palacios et al., 2012).

Besides, the quantities of SFA, MUFA and PUFA in different meat samples obtained when using the optimized procedure (one-stage transmethylation and fast GC run) were compared with the classic method of two-stage methylation and long-time GC-FID run (Fig. 3). In the case of fresh pork loin and cooked sausage samples, the optimized methodology achieved significantly higher SFA, MUFA and PUFA quantities than the classic one, while no significant differences were found in dry-cured sausage and dry-cured ham. Accordingly, results in previous works at evaluating the use of CTMS (Tomàs et al., 2009) showed higher FA quantities than the method with boron trifluoride in methanol, and no differences between freeze-dried and ground samples have also been reported (Lee et al., 2012). Nevertheless, these referenced methods have not improved the chromatographic analysis. Furthermore, it is worth noting the notable time saving with the fast GC-FID run (<10 min) in comparison to the usual method (60 min).

3.3. Performance of the fast GC-FID method

Linearity of external calibration curve method and standard addition method (in samples from both cooked and dry-cured sausages) was good (higher than 0.9 for most FA detected) (Table 2). Besides, there was not significant differences between the slopes of the regression lines obtained for the external standard calibration curve and for the standard addition to sample curves, with Student's *t*-test values higher than the

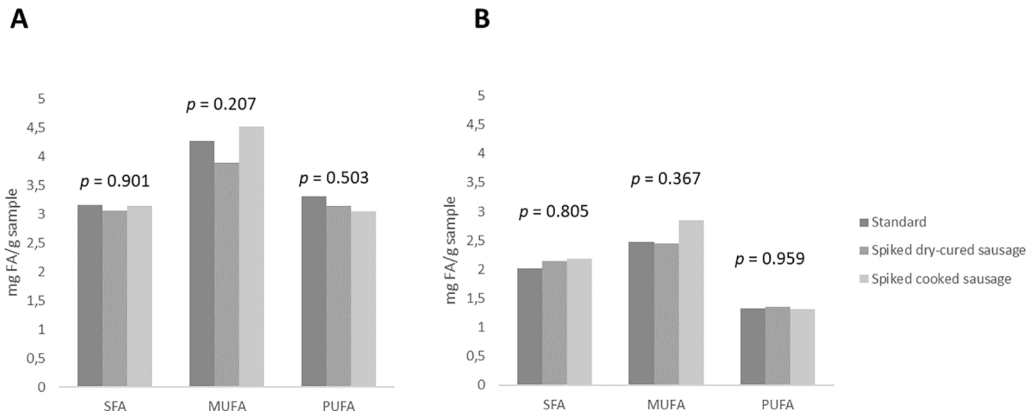

Fig. 4. Results on the quantity of the sum of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) of cooked (A) and dry-cured sausages (B) when applying calibration curves from standard solution of FAMEs and spiked meat samples.

critical level for v = 6 and 95% probability of success ($t = 2.447$). This finding has been corroborated by comparing the quantities of FAMEs in cooked and dry-cured sausages by means of an external calibration curve method and standard addition method in both samples (Fig. 4), without any significant differences in the sum of SFA, MUFA or PUFA among curves in any of the two products. This evidences no matrix effects and indicates the equivalence between the compared data (external standard calibration curve, standard addition to cooked sausage curve, standard addition to dry-cured sausage curve), which points out to the use of external calibration curve method as the preferred method, since it is simpler than the standard addition one.

To evaluate the performance of this suggested methodology, quality parameters for each individual FA were determined using standard solutions (Table 2). LOD and LOQ were around 10×10^{-3}-52×10^{-3} and 32×10^{-3}-0.12 mg FAME/ml. These values are lower than those reported in previous works with longer GC-FID runs (Araújo, Barbosa, Malafaia, & Napoleão, 2018; Juárez et al., 2008). For run-to-run and day-to-day assays, the RSD ranged between 1.56 and 7.58% and 1.85–11.81%, respectively. A percentage of variation lower than 20% must be achieved to consider that a method is precise (Ribani, Bottoli, Collins, Jardim, & Melo, 2004). Therefore, the optimized GC-FID method showed a good precision. The recovery of the FA ranged between 88.27 and 96.81 %. According to (Ribani et al., 2004), the rate of recovery may vary between 70 and 120%, percentages accomplished by the method proposed in the present study.

The quantity of major FA of cooked and dry-cured sausages by means of the improved methodology was also analysed (Supplementary material). As expected, the highest quantities were found for oleic acid (139.23 ± 7.29 and 78.16 ± 7.11 mg FAME/g sample), followed by palmitic (79.03 ± 4.21 and 48.51 ± 4.88 mg FAME/g sample), stearic (41.08 ± 4.39 and 18.85 ± 3.41 mg FAME/g sample) and linolenic acids (26.66 ± 1.78 and 27.69 ± 2.16 mg FAME/g sample), respectively for cooked and dry-cured sausages, while the quantities of myristic, palmitoleic, heptadecanoic, heptadecenoic, eicosapentenoic, α-linolenic and arachidonic acids were lower than 10 mg/g sample. These findings are quite in accordance with previous studies with similar meat products (Asuming-Bediako et al., 2014).

4. Conclusions

This study successfully achieves a simplification in the FA analysis in meat products by means of a one-stage transmethylation procedure followed by a fast GC-FID run of 10 min. Reagents and sample preparation are significant parameters of the procedure for one-stage FAME preparation in meat samples, while the type of heating is less influencing. The use of methanol + CTMS, lyophilized samples and heating in oven for the transmethylation of FA of meat samples in one-stage appears as the most appropriate procedure. A fast GC-FID method that allows appropriate separation, identification and quantification of FA (from C8:0 to C22:6n-3) in meat samples has been set up, with excellent quality parameters that ensure its suitability. For very short chain FA (C4:0, C6:0), GC condition (oven initial temperature) should be adjusted.

CRediT authorship contribution statement

Trinidad Perez-Palacios: Conceptualization, Methodology, Validation, Formal analysis, Resources, Supervision, Funding acquisition. **Juan Carlos Solomando:** Validation, Investigation. **Jorge Ruiz-Carrascal:** Investigation. **Teresa Antequera:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Authors acknowledge to Juan González for his help in the laboratory.

Funding

Agencia Estatal de Investigación (AEI) and the Fondo Europeo de Desarrollo Regional (FEDER), which supported the project AGL2016-73260-JIN (AEI/FEDER/UE).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130995.

References

Agnew, M. P., Craigie, C. R., Weralupitiya, G., Reis, M. M., Johnson, P. L., & Reis, M. G. (2019). Comprehensive evaluation of parameters affecting one-step method for quantitative analysis of fatty acids in meat. *Metabolites, 9*(9), 189. https://doi.org/10.3390/metabo9090189.

Araújo, K. S., Barbosa, M. O., Malafaia, C. B., & Napoleão, D. C. (2018). Validation and application of a chromatographic method for evaluation of commercial vegetable oils possibly adulterated. *Revista Eletrônica Em Gestão, Educação e Tecnologia Ambiental, 22*, 13. https://doi.org/10.5902/2236117035071.

Asuming-Bediako, N., Jaspal, M. H., Hallett, K., Bayntun, J., Baker, A., & Sheard, P. R. (2014). Effects of replacing pork backfat with emulsified vegetable oil on fatty acid composition and quality of UK-style sausages. *Meat Science, 96*(1), 187–194. https://doi.org/10.1016/j.meatsci.2013.06.031.

Carrapiso, A. I., Timón, L. M., Petrón, J. M., Tejeda, J. F., & García, C. (2000). In situ transesterification of fatty acids from Iberian pig subcutaneous adipose tissue. *Meat Science, 56*(2), 159–164. https://doi.org/10.1016/S0309-1740(00)00035-8.

Commission Regulation (EU) (2010) No 116/2010. Amending regulation (EC) No 1924/2006 of the European Parliment and of the Council with regard to thelist of nutricion claims. Official Journal of the European Union, 37, 16–18.

Ferrer-González, B. M., García-Martínez, I., & Totosaus, A. (2019). Textural properties, sensory acceptance and fatty acid profile of cooked meat batters employing pumpkin seed paste or soybean oil oleogel as fat replacers. *Grasas y Aceites, 70*(3), 1–11. https://doi.org/10.3989/gya.1055182.

Folch, J., Lees, M., & Sloane Stanley, G. . (1987). a Simple, 55(5), 999–1033.

Holman, B. W. B., Ponnampalam, E. N., Kilgannon, A. K., Collins, D., Plozza, T., & Hopkins, D. L. (2019). Moisture content, fatty acid profile and oxidative traits of aged beef subjected to different temperature-time combinations. *Meat Science, 157* (April), Article 107876. https://doi.org/10.1016/j.meatsci.2019.107876.

Juárez, M., Polvillo, O., Contò, M., Ficco, A., Ballico, S., & Failla, S. (2008). Comparison of four extraction/methylation analytical methods to measure fatty acid composition by gas chromatography in meat. *Journal of Chromatography A, 1190*(1–2), 327–332. https://doi.org/10.1016/j.chroma.2008.03.004.

Lee, M. R. F., Tweed, J. K. S., Kim, E. J., & Scollan, N. D. (2012). Beef, chicken and lamb fatty acid analysis - A simplified direct bimethylation procedure using freeze-dried material. *Meat Science, 92*(4), 863–866. https://doi.org/10.1016/j.meatsci.2012.06.013.

Martin, D., Antequera, T., Muriel, E., Perez-Palacios, T., & Ruiz, J. (2008). Effect of dietary conjugated linoleic acid in combination with monounsaturated fatty acids on the meat composition and quality traits of dry-cured loin. *Meat Science, 80*(4), 1309–1319. https://doi.org/10.1016/j.meatsci.2008.06.006.

Pérez-Palacios, T., & Estévez, M. (2020). Analysis of lipids and lipid oxidation products. *Meat Quality Analysis, 217–239*. https://doi.org/10.1016/B978-0-12-819233-7.00013-6.

Pérez-Palacios, T., Ruiz, J., Ferreira, I. M. P. L. V. O., Petisca, C., & Antequera, T. (2012). Effect of solvent to sample ratio on total lipid extracted and fatty acid composition in meat products within different fat content. Meat Science, 91(3), 369–373. doi:10.1016/j.meatsci.2012.02.021.

Pérez-Palacios, T., Ruiz, J., Martín, D., Muriel, E., & Antequera, T. (2008). Comparison of different methods for total lipid quantification in meat and meat products. *Food Chemistry, 110*(4), 1025–1029. https://doi.org/10.1016/j.foodchem.2008.03.026.

Phenomenex (2017). ZB-FAME The Fast FAME GC Column. Available at https://www.phenomenex.com.

Rasinska, E., Rutkowska, J., Czarniecka-Skubina, E., & Tambor, K. (2019). Effects of cooking methods on changes in fatty acids contents, lipid oxidation and volatile compounds of rabbit meat. *Lwt, 110*(March), 64–70. https://doi.org/10.1016/j.lwt.2019.04.067.

Ribani, M., Bottoli, C. B. G., Collins, C. H., Jardim, I. C. S. F., & Melo, L. F. C. (2004). Validação em métodos cromatográficos e eletroforéticos. *Química Nova, 27*, 771–780. https://doi.org/10.1590/S0100-40422004000500017.

Sandler, S. R., & Karo, W. (1992). *Sourcebook of advanced organic laboratory preparations*. Academic Press.

Schiavon, S., Pellattiero, E., Cecchinato, A., Tagliapietra, F., Dannenberger, D., Nuernberg, K., … Bittante, G. (2016). The influence of different sample preparation procedures on the determination of fatty acid profiles of beef subcutaneous fat, liver and muscle by gas chromatography. *Journal of Food Composition and Analysis, 50*, 10–18. https://doi.org/10.1016/j.jfca.2016.05.001.

Solomando, J. C., Antequera, T., & Perez-Palacios, T. (2020). Evaluating the use of fish oil microcapsules as omega-3 vehicle in cooked and dry-cured sausages as affected by their processing, storage and cooking. Meat Science, 162(December 2019), 108031. doi:10.1016/j.meatsci.2019.108031.

Tindall, A. M., Petersen, K. S., Skulas-Ray, A. C., Richter, C. K., Proctor, D. N., & Kris-Etherton, P. M. (2019). Replacing saturated fat with walnuts or vegetable oils improves central blood pressure and serum lipids in adults at risk for cardiovascular disease: A randomized controlled-feeding trial. *Journal of the American Heart Association, 8*(9), 9–12. https://doi.org/10.1161/JAHA.118.011512.

Tomàs, A., Tor, M., Villorbina, G., Canela, R., Balcells, M., & Eras, J. (2009). A rapid and reliable direct method for quantifying meat acylglycerides with monomode microwave irradiation. *Journal of Chromatography A, 1216*(15), 3290–3295. https://doi.org/10.1016/j.chroma.2009.01.055.

Utama, D. T., Jeong, H. S., Kim, J., Barido, F. H., & Lee, S. K. (2019). Fatty acid composition and quality properties of chicken sausage formulated with pre-emulsified perilla-canola oil as an animal fat replacer. *Poultry Science, 98*(7), 3059–3066. https://doi.org/10.3382/ps/pez105.

Vasilev, D., Dimovska, N., Hajrulai-Musliu, Z., Teodorović, V., Nikolić, A., Karabasil, N., … Mirilović, M. (2020). Fatty acid profile as a discriminatory tool for the origin of lamb muscle and adipose tissue from different pastoral grazing areas in North Macedonia – A short communication. Meat Science, 162(October 2018), 108020. doi:10.1016/j.meatsci.2019.108020.

Wang, T., Lim, J. N., Choi, S. H., Kang, H. S., & Lee, H. G. (2013). Comparative studies on derivatization methods of single or mixed fatty acids. *Food Science and Biotechnology, 22*(6), 1573–1579. https://doi.org/10.1007/s10068-013-0253-z.