**Identification and quantification of dimethyl acetals from plasmalogenic lipids in
lamb intramuscular fat under different derivatization procedures**

P. Gómez-Cortés^a, V. Rodríguez-Pino^a, A.L. Martínez Marín^b, M.A. de la Fuente^{a*}

^a Instituto de Investigación en Ciencias de la Alimentación (CSIC-UAM), Nicolás
Cabrera, 9., 28049 Madrid, Spain

^b Departamento de Producción Animal, Universidad de Córdoba, Ctra. Madrid-Cádiz
km 396, 14071 Córdoba, Spain

*Corresponding author: Miguel Angel de la Fuente

Instituto de Investigación en Ciencias de la Alimentación, Nicolás Cabrera, 9., 28049
Madrid, Spain

Phone: +34 910017933; Fax +34 910017905. E-mail: mafl@if.csic.es

ABSTRACT

Meat lipids are mostly comprised by triacylglycerols, but small amounts of plasmalogens are also present in intramuscular fat. The purpose of this study was to evaluate the effect of lipid derivatization on the presence of dimethyl acetal (DMA) molecules from plasmalogenic lipids in intramuscular fat samples. Three different methods of methylation were assayed. Acid-catalyzed methanolysis using HCl, the traditional procedure to derivatize meat lipids, was compared to two base-catalyzed methanolysis based on the ISO International standard procedure using either KOH and/or NaOCH₃ which, apparently, are only able to methylate fatty acids from triacylglycerols. DMA compounds were isolated by thin layer chromatography and then identified by gas chromatography-mass spectrometry. The most prominent DMA molecules detected were 16:0 and 18:0, but also minor amounts of monounsaturated and branched-chain DMA were quantified. Acid methylation yielded the highest amounts of DMA. However, the present article demonstrates that ISO standard based methylation procedures could also generate DMA derivatives in considerable quantities, which is not usually considered and may interfere with the determination of fatty acid methyl esters (FAME) from triacylglycerides. The current research warns scientist about possible FAME misidentifying and overestimations in intramuscular fat analysis using basic methylation and the need to consider the presence of DMA in samples that contain plasmalogens.

Key words: plasmalogen, acid-catalyzed methanolysis, fatty acid methyl ester, intramuscular fat.

1. INTRODUCTION

Plasmalogens are a group of glycerophospholipids in which the *sn*-1 position of the glycerol backbone is linked to a fatty aldehyde via an alk-1-enyl ether bond. The presence of the nonhydrolyzable ether bond makes plasmalogens more susceptible to oxidative attacks in comparison to their acyl analogues. This fact has pushed up the idea that plasmalogens could act as scavengers, protecting other lipids from oxidative reactions [1]. Furthermore, changes in plasmalogens levels were shown significant in tissues of various tumor types which makes these molecules good candidates as potential cancer biomarkers [2].

Plasmalogens are constituents of microorganisms [3-4] and animal tissues [5]. Edible meat also contains plasmalogens in different proportions, contributing to the total lipid content of this foodstuff [6-7]. The radical at the *sn*-1 position of meat plasmalogens usually derives from saturated and monounsaturated fatty aldehydes with 16 or 18 carbon atoms [8-10]. These analytes tend to elute before the corresponding fatty acid methyl ester (FAME) derivatives in gas chromatography (GC), which often complicates lipid analysis, and their occurrence seem to be influenced by the derivatization process.

When plasmalogens are treated with acidic transesterification reagents during methylation, the traditional procedure to derivatize all meat lipids, the vinyl ether bond is broken at the *sn*-1 position. The aldehyde that is generated reacts immediately with methanol and yields dimethyl acetals (DMA) molecules. However, it is not so clear the production of DMA under alkali methylation conditions. Although it has been suggested that DMA formation does not occur if methanolysis is carried out with basic catalysts [11-12], some studies have reported the presence of these plasmalogens derivatives in chromatographic analysis after basic transesterification [5,8]. Unfortunately, DMA and

FAME present very similar GC retention times when analyzed using polar stationary phases. As a result, certain compounds could often be inaccurately labeled and overestimated due to the coelutions occurring in routine lipid analysis.

The aim of this study was to compare the effects of two basic-methylation procedures with a conventional acid-transesterification methodology on the occurrence of DMA molecules in intramuscular fat.

2. MATERIALS AND METHODS

2.1. Samples and lipid extraction

This work was carried out using intramuscular fat samples from lamb meat. Samples for lipid analysis were taken from the T13 vertebra section of the *Longissimus thoracis* muscle after 6 days of aging. Intramuscular lipids were extracted using the Bligh and Dyer method [13]. Briefly, muscle was homogenized with a mixture of chloroform and methanol adding BHT as antioxidant. Dilution with chloroform and water separated the homogenate into two layers. The chloroform layer containing all the lipids was collected, and, after removal of the solvent, the total lipids were preserved in amber vials frozen at -17°C until its derivatization.

2.2. Lipid derivatization and fractionation.

Three different methylation procedures were assayed. Acid-catalyzed methylation was carried out by reacting 25 mg of total lipids extracted from muscle with 1 mL of anhydrous HCl/CH₃OH 5% w/v for 1 h at 80°C according to Santercole *et al.* [10].

Methyl esters were also prepared by base-catalyzed methanolysis using two transesterification reagents according to ISO [14]. The first alkali reagent was prepared dissolving 11.2 g of potassium hydroxide (KOH) in 100 mL of methanol. Alternatively, 10.8 g of sodium methoxide (NaOCH₃) were dissolved in the same volume of methanol to obtain the second reagent. Each basic transesterification mixture (0.2 mL) was reacted with 25 mg of total lipids at room temperature. Five minutes after mixing, 130 mg of solid NaHSO₄H₂O were added and the mixture was centrifuged for 3 min at room temperature. Then, an aliquot of the supernatant was removed for GC analysis.

After acid methylation of intramuscular lipids, FAME and DMA were fractionated by thin layer chromatography (TLC) onto a silica-gel plate using dichloromethane as solvent. The two bands containing DMA and FAME were scraped off and analyzed by GC as previously Alves et al. [4] described.

2.3. Gas chromatography analysis.

An Agilent model 6890 N Network System (Palo Alto, CA, USA) equipped with auto injector, fitted with a FID and a CP-Sil 88 fused silica capillary column (100 m x 0.25 mm i.d., Varian, Middelburg, The Netherlands) was used. Injector and detector temperature was 250 °C. Helium was the carrier gas and inlet pressure was set at 194 kPa. The sample volume injected was 1 µL at a split ratio of 1:100. Total chromatographic time was 90 min, including two ramps. Initial oven temperature was 45 °C. After 4 min, it was raised at 13 °C min⁻¹ to 165 °C and held for 35 min, then increased to 215 °C at 4 °C min⁻¹ and maintained for 30 min. Quantification was carried out using 11:0 as internal standard.

The identification of DMA and FAME was accomplished on an Agilent chromatograph (model 7890A) fitted with a mass spectrometry (MS) electronic impact detector (5975C inert MSD). The filament trap current was 400 μ A at 70 eV. Injections were carried out under data system control with an injection volume of 1 μ L and split ratio of 1:20. The remaining chromatographic conditions were similar to those described above for GC-FID analysis. The AOCS Lipid Library (<http://lipidlibrary.aocs.org/>), Wiley 275 and NIST 05 libraries were used to identify the mass spectra.

2.4. Statistical analysis.

Statistical analysis was conducted with SPSS Version 24.0 (SPSS Inc., Chicago, Illinois, USA). A one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test was used to compare DMA levels in lamb intramuscular fat after different methylation procedures. $P < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. Analysis of DMA and FAME by GC

The DMA profile, which reflects the vinyl ether chain of plasmalogens, was analyzed by GC. Catalysis in methanolic media of the lipid samples generated DMA and FAME simultaneously. As it can be observed in Figures 1 and 2 (down chromatograms of total sample), both groups of compounds eluted in the same chromatographic areas, that led to assorted coelutions and quantification challenges. To overcome this issue, a TLC using dichloromethane as developing solvent was carried out to discriminate FAME and DMA analytes. Both fractions were collected and subsequently analyzed with the same GC

program. Fifteen peaks were identified in the DMA fraction and, under our chromatographic conditions, two co-elutions between FAME and DMA were observed in lamb intramuscular fat. *Anteiso* 15:0 methyl ester overlapped with *iso* 16:0 DMA (Figure 1) while *cis*-9 17:1 methyl ester was not well resolved from 18:1 DMA (Figure 2).

3.2. DMA identification by MS

The use of MS detector hyphenated to a capillary GC column was very useful to determine the structure of individual DMA as well as other FAME eluting in same chromatographic region. Under electron impact ionization, DMA are fragmented extensively and they show a base peak at $m/z = 75$. It results from the loss of the DMA head group, which is the principal identifier of any DMA moiety. Another characteristic fragmentation is the $[M-31]^+$ ion which is linked to the loss of a methoxyl radical and allows the determination of DMA molecular weight [3]. For instance, mass spectra of 14:0, 15:0, 16:0, and 18:0 DMA exhibited the ion $[M-31]^+$ at 227, 241, 255 and 283, respectively, being supported by the mass spectra from the databases above mentioned (see Material and Methods section) as well as Alves et al. (4) reports.

Five peaks eluting between *iso* 18:0 and 18:0 FAME (Figure 2, Total) presented a similar mass spectra with a $[M-31]^+$ ion at 281 (Figure 3). All of them corresponded to 18:1 DMA moieties but their double bond position and geometry did not generate differences in the mass spectrum. Tentative identification of the 18:1 DMA isomers may be accomplished by analogy with the FAME profile that follows the same elution pattern (Alves et al., 2013). In this way, the highest 18:1 peak in the DMA fraction had to be assigned to *cis*-9 18:1 DMA being preceded by different *trans* 18:1 DMA isomers (Figure 2). It is

important to notice that *cis*-9 17:1 FAME would partially interfere in the determination of plasmalogens as it elutes in the 18:1 DMA region. The rest of DMA after *cis*-9 18:1 DMA would include 18:1 isomers with *cis* unsaturation but where the double bond could be located at higher positions of the carbon chain (Figure 2, DMA).

The elution pattern was also utilized to assign *iso* and *anteiso* 17:0 DMA (Figure 1). It is well known that *iso* and *anteiso* FAME appear at a carbon number of $(n-1 + 0.5)$ and $(n-1 + 0.7)$, respectively, in different GC columns [15]. The present research would confirm that this principle also applies to DMA molecules. *Iso* and *anteiso* 17:0 DMA compounds eluted at 0.5 and 0.7 units, respectively, when considering the distance between 16:0 DMA and 17:0 DMA as a unit. Furthermore, both peaks showed an identical mass spectrum to 17:0 DMA with the characteristic ions at $m/z = 75$ and 269 (Figure 3). Similarly, *iso* 16:0 DMA eluted right in the middle between 15:0 DMA and 16:0 DMA (Figure 1). In this chromatographic region, other two DMA moieties were also assigned to 16:1 structure because their mass spectra presented the characteristic ions $[M-31]^+$ at 253 and the base peak at $m/z = 75$ (Figure 3).

In addition, the utilization of GC-MS was useful to determine the identity of other minor compounds in the chromatogram. The group of tiny peaks eluting after 15:0 DMA and immediately before *iso* 15:0 and *anteiso* 15:0 FAME (Figure 1) corresponded to branched-chain 15:0 FAME with the methyl group located at different positions within the backbone. Likewise branched-chain 17:0 FAME other than *iso* and *anteiso* were assigned to four peaks eluting after 17:0 DMA. Finally, MS detector also confirmed the presence of BHT in the samples (Figure 1) which was added during the extraction process to avoid lipid oxidation.

3.3. Effect of the methylation catalyst

Table 1 compares the content of the 15 individual DMA after three different methylation procedures of the same intramuscular fat sample. The highest contents of DMA were observed under acidic conditions but all catalysts produced these molecules. Methylation using anhydrous HCl/CH₃OH (5%) is known to be complete for all lipids, including alk-1-enyl ethers, after 1 h at 80°C. High temperatures would promote the formation of DMA and, therefore, values measured with this procedure should be considered a reliable reference. In addition, this methodology has been profusely employed in the analysis of lipids from different sources [12,16-17].

Methylation procedures based on ISO standard [14] yielded significantly lower amounts of DMA than HCl derivatization (Table 1). In comparison to acid methanolysis, total DMA content decreased by 44% and 62% with methanolic KOH and NaOCH₃, respectively (Table 1). These results are not in agreement with those generally reported in the literature where is often believed that the alk-1-enyl ethers present in phospholipid molecules are not derivatized under base-catalyzed methanolysis [11-12]. Nevertheless, data from the present research reveal that the vinyl ether bond of plasmalogens could be broken, at least in part, and the aldehydes released to the media would be subsequently converted to DMA with basic catalyzed transesterification. A possible explanation could come from the addition of NaHSO₄H₂O to the transesterification mixture as the ISO procedure [14] recommended. It is added just before the centrifugation to avoid the saponification of preformed esters but, as NaHSO₄H₂O is very acidic, it may also favor the conversion of plasmalogens to DMA molecules. Salih *et al.* [8] previously reported the presence of 16:0 and 18:0 DMA in turkey breast muscle phospholipids after sodium methoxide derivatization but, unfortunately, no details of the procedure were described.

The main problems associated to the generation of DMA molecules in the methylation process are the potential errors of identification and quantification that could occur in the analysis of samples with relevant levels of plasmalogenic lipids. For such reason, GC oven temperature program need to be adequately selected and peak identity finely checked in this type of samples. Under our chromatographic conditions, overlapping between FAME and DMA were minimized but still, two very minor FAME (*anteiso* 15:0 and *cis*-9 17:1) coeluted with DMA molecules. In practical terms, a previous recognition of individual DMA in samples containing plasmogens would be highly encouraged to avoid FAME overestimations and inaccurate assignments on each particular working condition.

There are few studies informing about the presence of DMA in sheep intramuscular fat. In lamb chops, Crackel et al. [11] reported around 12% of total lipids as DMA. In contrast, Santercole et al., [10] supplied an interval of DMA varying from 1.9 to 2.4% in Sarda sheep meat. The fatty aldehyde at the *sn*-1 position of the plasmalogen consisted mainly of saturated and monounsaturated alkenyl moieties without the presence of 18:2 DMA, which is consistent with the current research. 16:0 and 18:0 were the major DMA moieties and *cis*-9 18:1 DMA would be the most prominent monounsaturated fatty aldehyde [5,10]. This could be explained by the fact that plasmalogen alkenyl chains are derived from the alcohol produced from the corresponding fatty acids and 16:0 and *cis*-9 18:1 are the most abundant fatty acid in lamb meat [18].

4. CONCLUSIONS

Fifteen individual molecules of alk-1-enyl ethers were identified and quantified in lamb intramuscular fat under 3 different derivatization procedures. Acid methylation, the

procedure commonly used to derivatize all meat lipids, yielded the highest amounts of DMA. Transesterification based on the ISO standard methodology, normally used to determine only fatty acid from triacylglyceride, also lead to the formation of DMA derivatives but in lower quantities than acid derivatization. Thus, a previous analysis and recognition of DMA molecules in samples containing plasmalogenic lipids would be required to overcome certain complexities related to the coelution of DMA and FAME in the same regions of the chromatogram. This should sensitize scientists performing studies on fatty acids analysis in ruminant meat and possibly integrate the TLC separation of DMA and FAME into the workflow to avoid overestimation in case of some minor fatty acids.

Fundings: This study was funded by the Spanish Ministry of Economy and Competitiveness (Project AGL2016-75159-C2-2-R).

Declarations of interest: None

5. REFERENCES

- [1] N.E. Braverman, A.B. Moser, Functions of plasmalogen lipids in health and disease, *Biochim. Biophys. Acta* 1822, (2012) 1442–1452.
- [2] M.C.F. Messias, G.C. Mecatti, D.G. Priolli, P.D. Carvalho, Plasmalogen lipids: functional mechanism and their involvement in gastrointestinal cancer. *Lipids Health Dis.* 17 (2018) 41.

248 [3] T.S. Oberg, R.E. Ward, J.L. Steele, J.R Broadbent, Identification of plasmalogens in
 249 the cytoplasmic membrane of *Bifidobacterium animalis* subsp. *Lactis*, *Appl. Environ.*
 250 *Microbiol.* 78 (2012) 880-884.

251 [4] S.P. Alves, J. Santos-Silva, A.R.J. Cabrita, A.J.M. Fonseca, R.J.B. Bessa, Detailed
 252 dimethylacetal and fatty acid composition of rumen content from lambs fed lucerne or
 253 concentrate supplemented with soybean oil, *PLoS ONE* 8 (2013) e58386.

254 [5] R.L. Wolff, Characterization of trans-monounsaturated alkenyl chains in total
 255 plasmalogens (1-O-alk-1'-enyl-2-acyl glycerphospholipids) from sheep heart, *Lipids* 37
 256 (2002) 811-816.

257 [6] J. Kraft, J.K.G. Kramer, F. Schoene, J.R. Chambers, G. Jahreis, Extensive analysis of
 258 long-chain polyunsaturated fatty acids, CLA, trans-18:1 isomers, and plasmalogenic
 259 lipids in different retail beef types, *J. Agric. Food Chem.* 46 (2008) 4775-4782.

260 [7] N. Aldai, P. Lavín, J.K.G. Kramer, R. Jaroso, A.R. Mantecón, Breed effect on quality
 261 veal production in mountain areas: emphasis on meat fatty acid composition, *Meat Sci.*
 262 92 (2012) 687-696.

263 [8] A.M. Salih, J.F. Price, D.M. Smith, L.E. Dawson, Identification and quantitation of
 264 dimethyl acetals of hexadecanal and octadecanal in turkey breast muscle phospholipids,
 265 *J. Food Sci.* 53 (1988) 654-655.

266 [9] D. Dannenberger, S. Lorenz, G. Nuernberg, N. Scollan, K. Ender, K. Nuernberg,
 267 Analysis of fatty aldehyde composition, including 12-methyltridecanal, in plasmalogens
 268 from Longissimus muscle of concentrate- and pasture-fed bulls, *J. Agric. Food Chem.* 54
 269 (2006) 182-188.

270 [10] V. Santercole, R. Mazzette, E.P.L. de Santis, S. Banni, L. Goonewardene, J.K.G.
 271 Kramer, Total Lipids of Sarda sheep meat that include the fatty acid and alkenyl
 272 composition and the CLA and trans-18:1 isomers, *Lipids* (2007) 42, 361-382.

273 [11] R.L. Crackel, D.J. Buckley, A. Asghar, J.I. Gray, A.M. Booren, Comparison of four
 274 methods for the dimethylacetal-free formation of fatty acid methyl esters from
 275 phospholipids of animal origin, *J. Food Sci.* 53 (1988) 1220-1222.

276 [12] C. Cruz-Hernández, J.K.G. Kramer, J. Kraft, V. Santercole, M. Or-Rashid, Z. Deng,
 277 M.E.R. Dugan, P. Delmonte, M.P. Yurawecz, Systematic analysis of trans and conjugated
 278 linoleic acids in the milk and meat of ruminants, in: M.P. Yurawecz, J.K.G. Kramer, O.
 279 Gudmundsen, M.W. Pariza, S. Banni (Eds.), *Advances in Conjugated Linoleic Acid*
 280 *Research*, Vol. 3, AOCS Press, Champaign, USA, 2006, pp. 45-93.

281 [13] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification. *Can.*
 282 *J. Biochem. Physiol.* 37 (1959) 911-917.

283 [14] ISO-IDF, Milk fat-Preparation of fatty acid methyl esters. International Standard ISO
 284 15884-IDF 182:2002.

285 [15] P. Gómez-Cortés, V. Rodríguez-Pino, M. Juárez, M.A. de la Fuente, Optimization
 286 of milk odd and branched-chain fatty acids analysis by gas chromatography using an
 287 extremely polar stationary phase. *Food Chem.* 231 (2017) 11-18.

288 [16] J.K.G. Kramer, V. Fellner, M.E.R. Dugan, F.D. Sauer, M.M. Mossoba, M.P.
 289 Yurawecz, Evaluating acid and base catalysts in the methylation of milk and rumen fatty
 290 acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids* 32
 291 (1997) 129-1228

- 292 [17] W.W. Christie, Lipid Analysis. Isolation, Separation, Identification and Structural
293 Analysis of Lipids, 3rd ed., The Oily Press, Bridwater, UK, 2003
- 294 [18] B. Gallardo, P. Gómez-Cortés, A.R. Mantecón, M. Juárez, T. Manso, M.A. de la
295 Fuente, Effects of olive and fish oil Ca soaps in ewe diets on milk fat and muscle and
296 subcutaneous tissue fatty acid profiles of suckling lambs. *Animal* 8 (2015) 1178-1190

Table 1. Mean content (mg/mL) and standard deviation of dimethyl acetal (DMA) compounds in lamb intramuscular fat using different methylation catalysts (KOH, NaOCH₃, HCl/CH₃OH).

DMA	KOH	NaOCH ₃	HCl/CH ₃ OH
14:0	0.005 ± 0.001	0.005 ± 0.001	0.006 ± 0.001
15:0	0.004 ± 0.001 ^b	0.003 ± 0.001 ^b	0.008 ± 0.001 ^a
<i>iso</i> 16:0 ¹	0.081 ± 0.014	0.087 ± 0.010	0.084 ± 0.003
16:0	0.595 ± 0.105 ^b	0.343 ± 0.126 ^b	1.127 ± 0.072 ^a
16:1	0.026 ± 0.009	0.025 ± 0.002	0.024 ± 0.004
16:1	0.005 ± 0.001 ^b	0.004 ± 0.0003 ^b	0.009 ± 0.001 ^a
<i>iso</i> 17:0	0.013 ± 0.003 ^b	0.009 ± 0.004 ^b	0.027 ± 0.002 ^a
<i>anteiso</i> 17:0	0.020 ± 0.004 ^b	0.010 ± 0.004 ^c	0.043 ± 0.002 ^a
17:0	0.009 ± 0.002 ^b	0.004 ± 0.002 ^b	0.034 ± 0.005 ^a
18:0	0.198 ± 0.057 ^b	0.089 ± 0.045 ^b	0.508 ± 0.036 ^a
<i>trans</i> -18:1	0.065 ± 0.018 ^b	0.032 ± 0.015 ^b	0.160 ± 0.011 ^a
<i>trans</i> -18:1 ²	0.332 ± 0.048	0.287 ± 0.018	0.329 ± 0.025
<i>cis</i> -9 18:1	0.105 ± 0.023 ^b	0.067 ± 0.021 ^b	0.242 ± 0.019 ^a
<i>cis</i> -18:1	0.009 ± 0.002 ^b	0.007 ± 0.002 ^b	0.016 ± 0.001 ^a
<i>cis</i> -18:1	0.017 ± 0.001	0.016 ± 0.001	0.015 ± 0.003
TOTAL	1.485 ± 0.258 ^b	0.988 ± 0.248 ^b	2.632 ± 0.175 ^a

¹ Coelutes with *anteiso* 15:0 methyl ester. ² Coelutes with *cis*-9 17:1 methyl ester.

^{a-c} Values with different superscripts are significantly (P < 0.05) different

FIGURE CAPTIONS

Figure 1. Partial GC chromatograms (from 26 to 39 min) of lamb intramuscular fat prior TLC (Total) and after separation into DMA and FAME lipid fractions. *Aiso*: *anteiso*; BHT: butylhydroxytoluene; DMA: dimethylacetal; FAME: fatty acid methyl ester; *unidentified.

Figure 2. Partial GC chromatograms (from 41 to 50 min) of lamb intramuscular fat prior TLC (Total) and after separation into DMA and FAME lipid fractions. DMA: dimethylacetal; FAME: fatty acid methyl ester.

Figure 3. Mass spectra of 16:1, 17:0, and 18:1 dimethyl acetals in lamb meat intramuscular fat analyzed by GC-EI/MS into a 100-m CP-Sil 88 capillary column.