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Review

Derivatization of fatty acids and its application for conjugated linoleic acid studies in ruminant meat lipids

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Abstract: Conjugated linoleic acid (*c*9, *t*11 CLA) is a dietary fatty acid produced mainly by ruminant animals and exhibits promising health-promoting biological effects. For lipid fatty acid composition analyses, including CLA, lipids must be pre-treated so that the free and esterified fatty acids (triacylglycerols, phospholipids, etc) are available for determination. The most common treatments involve fatty acid methyl ester derivatives from relatively simple chemical reactions, but this becomes complicated when esterification of CLA is involved because of potential changes in its positional and geometrical isomers by reaction with certain reagents. In this review we explain concisely the advantages and disadvantages of the most popular methods (acid- and base-catalysed methods) generally employed for total fatty acid derivatization and their determination on a gas chromatograph. Based on our experiences we put forward the (trimethylsilyl)diazomethane method as an alternative and successful approach for ruminant tissue lipid determinations.

Keywords: fatty acids; derivatization methods; conjugated linoleic acid; CLA; ruminant lipids

INTRODUCTION

In ruminant meat, as in other meats, the quality of fat is as important as its quantity. Today, health professionals emphasize the association between nutrition and a number of lifestyle diseases in man (particularly heart disease and cancer), and have focused research activities on the role of metabolically important fatty acids. Dietary advice often recommends a reduction in the fat content of the diet, maintenance of the ratio of polyunsaturated to saturated fatty acids (P:S ratio) at about 0.45 or higher and an increase in the intake of *n-3* polyunsaturated fatty acids (PUFA) relative to *n-6* PUFA such that the *n-6:n-3* ratio is less than 4.[1–3]

Food products from ruminants, especially milk fat, represent the major dietary sources of conjugated linoleic acids (CLA), especially with respect to the *cis*-9, *trans*-11 isomer [*cis*-9(*Z*), *trans*-11(*E*) $C_{18:2}$]. Sources of other conjugated fatty acids are also found in plant seed oils ($C_{18:3}$) and marine organisms ($C_{18:4}$, $C_{20:4}$, $C_{22:6}$) with only trace amounts of CLA.[4–9] The *cis*-9, *trans*-11-octadecadienoic acid isomer (referred to as *'rumenic acid'*), accounts for 75–90% of total CLA in ruminant fats.[5,10] Recently, a number of health-promoting biological activities for CLA using animal and *in vitro* models have been demonstrated,[11–16] including changes in body composition and anticarcinogenic, antiatherogenic, antidiabetogenic and immunomodulatory activities. Currently, the evidence for direct beneficial effects in humans is scarce, sometimes contradictory, but promising.[17,18]

Conjugated linoleic acids

'Conjugated linoleic acid' often describes a generic family of positional and geometric isomers of linoleic acid ($C_{18:2}$ *c*9, *c*12) with diverse biological activity, in which the double bonds are conjugated instead of existing in the more typical methylene-interrupted configuration. Several different isomers of CLA have been reported. Of these isomers, the *c*9, *t*11 isoform is believed to be the most common natural form of CLA with biological activity as it is incorporated into the phospholipids of cell membranes.[5,11,15,19]

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However, some authors could not detect *c*9, *t*11 CLA in polar lipids of animal muscle, but found it present in neutral lipids, related to the storage lipid fraction.[20]

Health-promoting properties have also been proposed for the other isomers, even if they are minor in quantity (eg long-chain derivatives of $C_{18:2}$ *t*10, *c*12 appear to be strong inhibitors of prostaglandin biosynthesis[16]). Since there are potential health benefits arising from CLA consumption, there is considerable research effort directed to determining and quantifying these components in ruminant-derived products.

CLA (predominantly the *c*9, *t*11 isomer) is formed during ruminal biohydrogenation of linoleic acid as the first intermediate. It should be noted that *Butyrivibrio fibrisolvens* is thought to be the key bacterium involved in ruminal biohydrogenation and CLA formation.[21] Other bacteria can also convert linoleic acid into CLA, including *Lactobacillus, Propionibacterium* and *Bifidobacterium* spp.[22–25] For more information in the area of biohydrogenation in the rumen, the reader is directed towards more substantial reviews.[26–28] The next step of the pathway involves the hydrogenation of the conjugated diene resulting in *trans*-11-octadecenoic acid (also referred to as *trans*-vaccenic acid, $C_{18:1}$ *t*11). This is the major *trans* isomer of ruminant tissues and milk fat, and further biohydrogenation results in stearic acid ($C_{18:0}$).[29] The *c*9, *t*11 isomer can also be synthesized by animal tissues from $C_{18:1}$ *t*11 if the Δ^9-desaturase enzyme is present,[30–32] this may also occur in humans through similar desaturase enzymes.[33–35] In this way, the concentrations of $C_{18:1}$ *t*11 isomer and *c*9, *t*11 CLA that are available for absorption from the gastrointestinal tract are related to the dietary intake of unsaturated fatty acids and rumen conditions that may affect the growth and activity of the reticulo rumen micropopulation. It is unlikely that *trans* octadecenoic acid availability limits this pathway in ruminants because the concentration of *trans* octadecenoic acid in milk and fat depots is always much higher than CLA. In contrast, changes in the rumen environment (pH, substrate types and concentration, dilution rate), which can lead to changes in microbial activity, will have an effect on CLA formation.

In general, lipids obtained from animal sources have complex structures and often most of the fatty acids found in meat lipids are bound to either an alcohol (glycerol) via ester bonds (triacylglycerols, phospholipids and sterol esters) or to a long-chain base (sphingosines) via amide bonds (sphingolipids). For fatty acid composition analyses, the complex lipids must be pre-treated so that the free and esterified fatty acids are available for determination. Fatty acids are converted to more non-polar and volatile derivatives by various derivatization methods. Formation of alkyl esters (methyl, ethyl, propyl or butyl esters) is the most standard type of reaction used in analysis of fatty acids,[36,37] in particular is the preparation of fatty acid methyl esters. Esters formed with higher molecular weight alcohols (ethanol, propanol, isopropanol, butanol) are useful in determining very volatile short chain fatty acids as are often found in dairy products and milk. For example, derivatization with isopropanol separates the low molecular analytes from the solvent front and improves the ruggedness for determination of the C_4–C_{10} group of fatty acids by reducing the loss of such acids during the preparation procedure itself. Short chain fatty acids are more volatile than the longer chain C_{12} and greater fatty acids characteristic of ruminant muscle and adipose tissue. The use of isopropanol gives rise to derivatives with higher boiling points (due to longer alkyl chains), which are much easier to handle and separate on a gas chromatograph.[37] The major disadvantage with the use of these higher molecular weight alcohols is the lack of commercially available standards and reference mixtures. Each individual fatty acid would have to be derivatized to allow for identification via similar retention time (flame ionisation detection) or ion chromatographs (mass spectroscopy). In contrast, the availability of methyl esters is very broad indeed (Supelco, Matreya, Larodan, Aldrich, etc). The preparation of the methyl ester derivatives of fatty acids is carried out in a simple reaction, but it becomes more complicated when derivatization of conjugated fatty acids or other potentially labile fatty acids are involved (eg labile functional groups include epoxyl, cyclopropane and cyclopropene rings). The presence of a conjugated bond within fatty acids makes them unsuitable for the most common techniques (acid-catalysed reactions) employed for fatty acid analysis. For example, CLA can easily be isomerized by boron trifluoride (BF_3), thus changing the profile of the original isomer content.[38]

Gas chromatography (GC) with the use of a flame ionization detector (FID), a large split flow (>30:1) and preferably hydrogen as carrier gas at a flow rate of 1.2 ml min^{-1} is the suggested method for the analysis and quantification of fatty acids and their isomers, particularly if high resolution, polar capillary columns are employed (eg 100 m Chrompak CP-Sil88, 100 m Supelco SP-2560, 120 m SGE BPX-70). GC exhibits rapidity, high resolution and sensitivity.[39] However, even under optimal chromatographic conditions, a complete separation of all CLA isomers is not yet achievable and it is recognized that a combination of methods (multicolumn silver ion HPLC coupled with GC, two-dimensional GC, GC-MS) will be required to provide the full isomeric distribution of a sample.[5,40–43]

Derivatization methods for total fatty acid analysis by gas chromatography

Fatty acids of animal origin exist as a heterogeneous group of chemical structures. It is therefore necessary to optimize the derivatization procedure in order to obtain accurate, quantitative and qualitative results

while addressing potential procedural difficulties associated with ester preparation.[44] Common problems include:

- incomplete conversion of the fatty acids to their methyl (or indeed ethyl- or propyl-) ester homologues (often as a result of the presence of water);
- alteration of the original fatty acid profile during esterification, because of chemical mediated changes in the proportion of different positional and/or geometric isomers;
- formation of artefacts which can be wrongly identified as fatty acids (giving rise to spurious peaks);
- contamination and subsequent damage of the GC column resulting from unclean samples (carry over of methoxide, trimethylsilyl, etc).

Various methods are currently employed for the conversion of fatty acids in a complex lipid matrix to fatty acid methyl esters. In this review we will try to explain the advantages and disadvantages of some of the different methods generally used for total fatty acid determinations, particularly in regard to ruminant tissues.

There are basically two mechanisms, which include both acidic and alkaline catalysis:

(1) Methylation following hydrolysis of the fatty acids from the complex lipids involves saponification (alkaline hydrolysis) in which the ester bond between the fatty acid and the glycerol moiety is cleaved (eg triacylglycerols and phospholipids) under conditions of elevated temperature and in the presence of a strong alkali. This is followed by methylation typically performed in the presence of an acidic catalyst in methanol.
(2) Direct *trans*esterification involving alkaline or acidic catalysts in a process where the native alcohol (ie glycerol) is displaced from the lipid by another alcohol in large molar excess under anhydrous conditions.

It is essential to note that the method for preparing methyl esters for GC analysis is critical and has to avoid modifications to the original isomer distribution. Generally, there is no single method that works optimally in all situations and for all kinds of samples. Researchers must know the nature of their sample and select the appropriate method.[45] Some of the most frequently employed methods are described below.

Acid-catalysed methods (Fig 1)

Boron trifluoride (BF_3) in methanol

An aliquot of lipid extract is mixed with BF_3–methanol and heated to a maximum of 100 °C for 2–90 min depending on the type of lipid.[46] After cooling to ambient temperature, water and a non-polar solvent are added, vortexed, and the two phases separated by centrifugation. The upper organic phase containing the methyl esters is carefully removed to a new vial where it is removed under a stream of nitrogen gas (N_2). The remaining residue containing fatty acid methyl esters is dissolved in n-hexane prior to GC analysis.

Since its introduction as a rapid method of preparation of fatty acid methyl esters from lipids,[46–48] BF_3 has found wide application as a good direct *trans*esterification reagent (methylating the fatty acids directly from the acylglycerol parent lipid), and esterification reagent (converting free fatty acids to their methyl ester derivatives). Rapid methyl ester formation is favoured by using an excess of anhydrous methanol and it can be purchased from commercial chemical companies already prepared at the desired concentration. It also has some disadvantages: the BF_3–methanol reagent is toxic and relatively unstable on storage. There have been reports that artefacts can form[49–51] and polyunsaturated fatty acid decomposition can occur[52–54] if the reagent is old or allowed to become too concentrated through evaporation. Some by-product of the degradation of BF_3–methanol reagent (formation of fluoroboron compounds by reaction with atmospheric oxygen[55]) is capable of catalysing the addition of methanol across a double bond and new artefactual peaks have been observed eluting late in GC chromatograms. It can also cleave the rings in cyclopropane fatty acids and

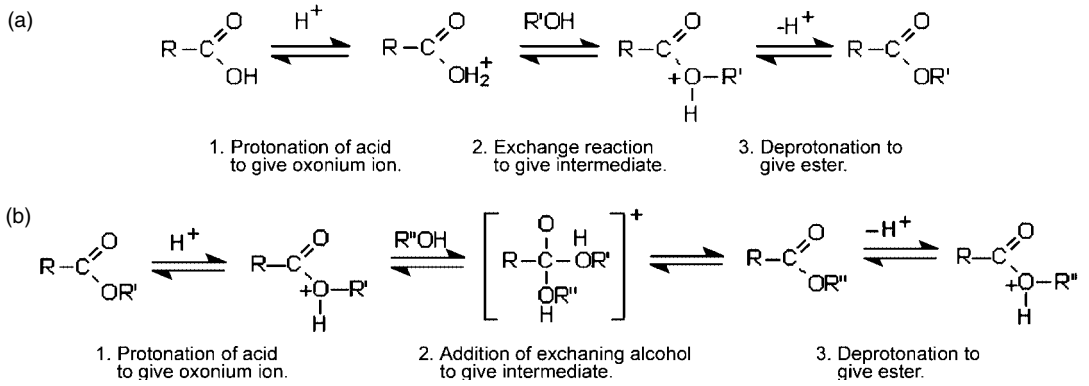

Figure 1. General mechanism of acid-catalysed esterification of fatty acids (a) and *trans*esterification of lipids (b). In the presence of water esterification is incomplete and *trans*esterification results in some hydrolysis of the lipid to give free fatty acids. (Adapted from Christie WW, *Advances in Lipid Methodology — Two*, ed by Christie WW, pp 69–111. Oily Press, Dundee, 1993).

react with the antioxidant butylated hydroxytoluene (BHT) to produce additional unknown peaks in chromatograms.[55,56] BF_3 is to be avoided if it is necessary to analyse fatty acids containing unusual functional groups such as conjugated double bonds as it can alter their configuration.[54,57] It has been reported that CLA exhibits conversion from *c*, *t* or *t*, *c* isomers to *t*, *t* isomers or unknown substances as a consequence of BF_3 methylation.[38] The use of the milder BCl_3–methanol reagent in place of the BF_3–methanol reagent has similar results.[54]

Hydrochloric acid (HCl) in methanol

To the lipid aliquot is added 5% (w/v) anhydrous HCl in methanol and it is heated to 80 °C for 20 min. Then some distilled water is added and the methylated fatty acids extracted with *n*-hexane. The n-hexane is evaporated under a stream of N_2 and re-dissolved in an adequate volume for GC analysis.

HCl–methanol converts both acylglycerols and free fatty acids into methyl esters. It has the advantages of being clean, easily prepared and applicable to all common lipid classes, including N-acyl lipids such as sphingolipids and glycosphingolipids.[58] However, several authors[54,57,59–61] have found that acidic conditions can alter the isomer distribution of conjugated systems, usually seen as an increase in *t*, *t* isomer content. As with other acid-catalysed methylations, allylic methoxy derivatives are formed.[57,61,62] Lowering the methylation temperature can decrease CLA isomerization, but these milder conditions may not be sufficient to methylate all lipids.[38,60,63]

Sulphuric acid (H_2SO_4) in methanol

To the lipid aliquot is added 0.1 M H_2SO_4 in methanol and the tubes heated to 100 °C for 30–60 min. Sodium bicarbonate solution is added to neutralize the reagent and n-hexane to extract the FAMEs. The organic layer is carefully removed, then dried over anhydrous sodium sulphate. Successive *n*-hexane extracts are added and evaporated under a stream of N_2. The residue is re-dissolved in n-hexane for GC analysis.

This is an acid-catalysed methanolysis or *trans*esterification method, and through its application it is possible to convert both acylglycerols and free fatty acids into methyl esters. Hitchcock and Hammond[64] suggested that H_2SO_4–methanol was a very satisfactory reagent for methylation, but it also has the same disadvantages as other acid-catalysts in changing the isomeric distribution of conjugated fatty acids. Interestingly, H_2SO_4–methanol has been found to be the mildest of the acid-based methods, and, if the reaction conditions are precisely controlled (55 °C for 5 min), little or no isomerization or methoxy artefacts are produced for the unesterified forms of CLA. For esterified lipids, artefacts are still produced (because more severe reaction conditions are required), but at a much lower quantity than those arising from the use of BF_3.[45,59] Some authors showed good results using a traditional base-catalysed methylation, followed by a mild treatment using H_2SO_4–methanol.[45]

In general, acid-catalysed methods can be used in a direct *trans*esterification or in a two-step methylation incorporating a previous hydrolysis or saponification of the lipids to free fatty acids. These methods usually require high temperatures (60–100 °C) for completion of methylation. Attention should be paid to the methylation of CLA isomers because these methods have been reported to change the *c*, *t* and *t*, *c* configurations in favour of the *t*, *t* configuration and generate allylic methoxy artefacts.[57,59,60] In addition, higher temperatures and/or longer incubation times generate considerable amounts of artefact. When acid-catalysed methylations are carried out at a low temperature (60 °C) with a longer reaction time, the extent of isomerization and allylic methoxy derivative formation is greatly reduced.[38,57,65] However, under such conditions, methylation of esterified fatty acids is not always complete, resulting in incomplete quantification. In experiments carried out by some researchers, *t*, *t* isomers were increased by acid-catalysed methods (HCl–MeOH and BF_3–MeOH) but not by base-catalysed ones (NaOMe).[60] In another study, changes in CLA isomer proportions using the H_2SO_4 method were relatively small compared with those observed for the other acid-catalysed methods (BF_3, HCl).[59] This is in agreement with results obtained by Park *et al*.[45] In the BF_3 method there is a time-dependent change in the CLA composition where *t*, *t* isomers increase markedly with time. Unknown peaks also continuously increase during methylation, but the total level observed in the H_2SO_4 method was lower than in the other two methods. Some authors also analysed the influence of antioxidants and dimethylsulphoxide (DMSO) and dimethylformamide (DMF) on CLA isomerization when used in conjunction with the acidic-catalysts. Antioxidants (butylated hydroxytoluene, ascorbic acid, β-carotene, α-tocopherol) at a 1 mM concentration had no preventive effect on the CLA isomer distribution. However, both DMSO and DMF added at the level of one-third of the reaction mixture both exhibited a protective effect against CLA isomerization by BF_3 and H_2SO_4. It is thought that DMSO and DMF interact with the protonated alcohol (by way of their projecting oxygen atom) to prevent that protonated alcohol reacting with the conjugated double bonds in CLA and forming methoxy derivatives that upon further reaction can form *t*, *t* isomers.[59]

A combination of methods appears to be the most suitable for analysis of lipid samples containing conjugated fatty acids. Normally NaOH or KOH in methanol is used at a temperature of 60–100 °C for the initial hydrolysis of acylglycerols. Saponification using sodium hydroxide in methanol followed by esterification with BF_3–methanol was widely used and adopted as the official method for both the American Oil Chemists' Society (AOCS)[66] and the

Official Methods of Analysis of AOAC International.[67] However, it is not suitable for CLA quantification because *cis*, *trans*-conjugated fatty acids are lost (significant increase in the *t*, *t* CLA isomer occurs) and methoxy artefacts are formed.[54]

Base-catalysed methods (Fig 2)

Sodium methoxide ($NaOCH_3$) in methanol

A lipid aliquot (20–40 mg) is dissolved in dry toluene, 2 ml 0.5 M sodium methoxide (metallic sodium in anhydrous methanol) added and the samples heated at 60 °C for 20 min. This esterification step can also be carried out at room temperature (20 °C). A few drops of glacial acetic acid are added followed by 2 ml saturated NaCl solution to eliminate excess methoxide. With 50 ppm butylated hydroxy toluene (BHT) 2 ml n-hexane are added, and vortexed vigorously. The upper organic phase containing the extracted methyl esters is taken, dried over anhydrous sodium sulphate or 100 µl of a water scavenger such as 2, 2 dimethoxypropane added, and dried under a gentle stream of N_2 at 40 °C. The fatty acid methyl esters are resuspended in 2 ml of n-hexane with 50 ppm BHT and injected for GC analysis. Dilution of the sample in n-hexane may be necessary, depending on the GC response.

This *trans*esterification method is the generally accepted method for the conversion of acylglycerides to methyl esters. Nevertheless, this technique is not suitable when large amounts of free fatty acids are present in the sample, as it is unable to methylate free fatty acids and N-linked (amide-bond) fatty acids such as those found in sphingolipids and glycosphingolipids.[68] This is a characteristic of all base-catalysed methylations. Only acid-based reagents and diazomethane can methylate free fatty acids. $NaOCH_3$ does not cause isomerization of conjugated double bonds, nor does it produce methoxy artefacts.[54,57] Reaction conditions need to be strictly anhydrous as the presence of water causes saponification.[54] $NaOCH_3$ is a popular methylating agent in the oil industry, especially for refined oils, where the free fatty acids have already been removed during the refining stage. The removal of excess methoxide before GC analysis is also strongly recommended, as the reagent tends to interfere with analysis. An aqueous wash necessary for the removal of excess reagent can affect some fatty acids. Without due care, losses in short-chain fatty acid methyl esters can render the technique inaccurate for the determination of methyl butyrate (C_4) and caproate (C_6), which are present in considerable amounts in dairy fat.[69]

Potassium methoxide ($KOCH_3$) or potassium hydroxide (KOH) in methanol

The $KOCH_3$ reagent is used in a similar manner to $NaOCH_3$ described in the previous section, but is more reactive and difficult to prepare. KOH in anhydrous methanol may also be used: are the lipids dissolved in n-hexane, KOH methanolic reagent added and the samples heated at 50 °C for 10–15 min. A few drops of glacial acetic acid are added followed by water and n-hexane. The solution is vortexed vigorously and the upper organic phase taken and dried over anhydrous sodium sulphate prior to injection for GC analysis.

KOH in anhydrous methanol and $KOCH_3$ are other *trans*esterification reagents commonly employed in the production of methyl esters from oils. In a study carried out involving $NaOCH_3$ and $KOCH_3$ reagents, it was found that $KOCH_3$ was more active than $NaOCH_3$ and produced greater conversion of cholesteryl esters into their corresponding methyl esters under comparable conditions.[70] $KOCH_3$ was used for quantitative determination of conjugated linoleic acid isomers in beef fat.[71] An interesting point about the use of KOH in methanol is its ability to efficiently *trans*esterify lipids directly from dry (lyophilized) tissue samples. Other researchers showed that concentrations of CLA were greatest after direct *trans*esterification of dried muscle tissue with KOH compared with $NaOCH_3$.[61] They further reported similar efficiencies for both reagents when *trans*esterifying extracted total lipid. This is to be expected, as strong alkali is efficient at solubilizing biological tissues.

Tetramethylguanidine (TMG) in methanol

To a dried sample of lipid is added 20% TMG in methanol. The tubes are heated at 100 °C for 10 min. Saturated NaCl solution and n-hexane are added to each tube, then vortexed and centrifuged to separate the phases. After centrifugation the organic layer is removed, and the n-hexane extraction repeated. n-Hexane extracts are combined and dried under a gentle stream of N_2. The residue containing FAMEs is re-dissolved in an adequate volume of solvent for GC analysis.

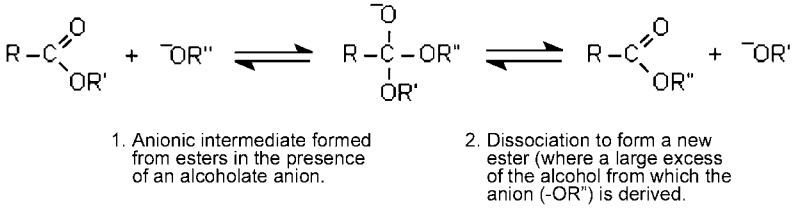

Figure 2. General mechanism of base-catalysed *trans*esterification of lipids. An unesterified fatty acid is converted to a carboxylate ion, $R\text{-}COO^-$, in a basic solution and cannot be esterified. In the presence of water, the intermediate (R″=H) will dissociate irreversibly to the free acid. (Adapted from Christie WW, *Advances in Lipid Methodology — Two*, ed by Christie WW, pp 69–111. Oily Press, Dundee, 1993).

More recently, guanidine and its alkylated derivatives in methanol have been used to catalyse the esterification of oils and fats. Although the original authors of the TMG method[72] claimed that it worked well with acylglycerols and free fatty acids, recent results by other researchers suggest otherwise.[45,54,57] This method is not effective in methylating free fatty acids and N-acyl lipids, and has difficulty with some esterified lipids such as phospholipids.[45,57] In addition, TMG can give undesirable products that extract with n-hexane and interfere with the analyses of short-chain FAME. Therefore, this method is not suitable for samples with high acid values. For clear chromatographic resolution, excess reagent needs to be removed prior to sample injection onto the GC column. The derivatization reaction is quite rapid; the reagent is inexpensive and mild, causing no isomerization of conjugated double bonds. Conditions for TMG–methanol need to be absolutely anhydrous, otherwise, soap formation will result in the loss of fatty acids and it will not be possible to obtain accurate results.[54] Park *et al*[45] obtained good results for methylation of esterified-CLA rich lipids in egg yolk, but only if they also included an additional reaction with H_2SO_4–methanol at 55 °C for 5 min.

Other reagents are available for *trans*esterification with shortened derivatization times. One of these is tetramethylammonium hydroxide in methanol, which *trans*esterifies triglyceride fatty acids to methyl esters.[73,74] This reagent minimizes the losses of $C_{14:0}$ and $C_{15:0}$ fatty acids and unsaturated C_{18} isomers in the analysis of plant oils, margarine, lard and animal tissues.[73]

In general, base-catalysed methods are mild reactions suitable for methylating the fatty acids bound up in acylglycerides, but are not suitable for samples with substantial amounts of free fatty acids. Care must be taken to exclude water from the reaction medium to prevent saponification. This hydrolysis is an unwanted and irreversible side reaction.[52,53]

Diazoalkale (diazomethane) method (Fig 3)

Trimethylsilyldiazomethane [$(CH_3)_3SiCHN_2$] method

The lipids are saponified with strong base such as 5 M KOH at a temperature of 60 °C for 60 min (a high concentration of base gives more efficient saponification; the authors routinely use 6 ml of 5 M KOH in 50% methanol with samples containing approximately 20–40mg of total lipid, eg 1 g of muscle tissue). NaOH may also be used and gives identical fatty acid profiles, but reducing the concentration of base below 5 M reduces the overall efficiency of saponification, requiring longer incubation times at 60 °C. Following saponification, the reaction mixture is diluted with 12 ml of 0.5% NaCl and a non-polar solvent (5 ml petroleum spirit) added to remove non-saponifiable lipid components (cholesterol, N-acyl lipids). The samples are vortexed and the upper solvent layer discarded. The samples are acidified using glacial acetic acid (typically 3 ml) and solvent added to partition the (protonated) free fatty acids into the solvent phase. The tubes are vortexed and the non-polar phase collected, then 100 µl of 2, 2-dimethoxypropane (a water scavenger) are added and the mixture is vortexed. The solvent is removed by drying under a stream of nitrogen (N_2) at 40 °C. For esterification, the residue is re-dissolved in 1 ml toluene–methanol (1:2 v/v) and trimethylsilyldiazomethane (TMS-DM) added in molar excess (about 120–160 µl of 2 M TMS-DM in hexane depending on fatty acid content). The diazomethane solution is yellow and this is lost as the methylation reaction proceeds at 40 °C for a maximum of 10 min. In addition, in the presence of free fatty acids there is a rapid liberation of N_2 gas as the reaction proceeds, so it is important not to cap the reaction tubes. There is no need to quench the reaction with acetic acid. Excess solvent and diazomethane are removed under a gentle stream of nitrogen at 40 °C. Finally, the residue is dissolved in 2 ml of *n*-hexane containing 50 ppm butylated hydroxy toluene (BHT), centrifuged at 20 000 × g for 5 min and 1 µl of supernatant transferred to GC for chromatographic analysis. Within-sample precision is very good with typical relative standard deviations of 5–10% for each fatty acid methyl ester (bovine muscle tissue), the higher values associated with those fatty acids in low or variable abundance such as the branched chain fatty acids. The use of an internal standard such as $C_{23:0}$ methyl ester can give variable results (60–85% recovery), but the authors consider this due to solubility issues with the saponification solution. This serves to highlight one of the inherent difficulties with fortification of samples with analytical standards—that is, the standard may not always behave in the same way as native components under the extraction procedure. This is not of much concern

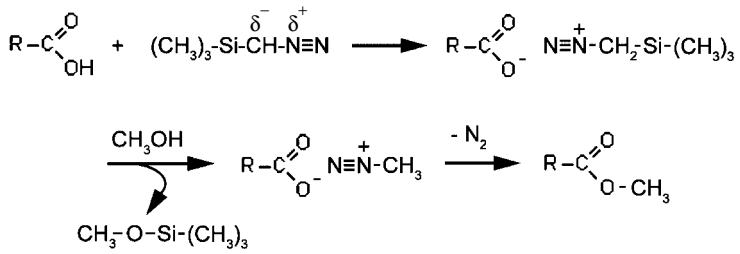

Figure 3. General mechanism of trimethylsilyldiazomethane mediated methylation of free fatty acids. The reaction must have methanol present to get good yields of the desired methyl ester. Adapted from Hashimoto *et al*.[85].

since measurement of the recovered fatty acid residue weight per unit weight tissue agrees well with total lipid content of the tissue as measured by other procedures such as solvent extraction and microwave drying of samples (procedure adapted from Elmore *et al*,[75] by Murray, BE personal communication).

Diazomethane can easily convert free fatty acids to methyl esters in high yield in the presence of methanol, but cannot act on acylglycerides. It is therefore essential to hydrolyse or saponify lipid samples first.[76] Diazomethane, for all its advantages (short incubation times, mild reaction conditions, high methyl ester yield), has several preparative problems (toxic, explosive, potential carcinogen) and reportedly may give rise to artefacts associated with its use as indicated by some authors,[77] even though evidence appears to be lacking in the literature about this in relation to fatty acid methyl ester formation. This, perhaps, contributes to reluctance amongst laboratories to use it as a general procedure in the preparation of fatty acid methyl esters. There was an interesting report concerning unreliable estimates of serum non-esterified fatty acids with the use of diazomethane;[78] however, a subsequent report[79] was able to clarify that solvolysis of serum lipids took place to increase the free fatty acid content. In fact, numerous publications have used diazomethane quite satisfactorily for the methylation of their fatty acids.[57,65,75,80–84] Trimethylsilyldiazomethane (TMS-DM) is an alternative, convenient and commercially available source of diazomethane. It is safer to handle and more stable with minimal side reactions. It can be obtained as a 2.0 M solution in diethyl ether or hexane. The esterification proceeds nearly instantaneously and quantitatively when an excess of TMS-DM is used in the presence of methanol. The reaction conditions are typically very mild (40 °C, 10 min) and the reaction can be easily monitored by the disappearance of its yellow colour and liberation of nitrogen gas (which drives the reaction in favour of methyl ester formation). Excess reagent can be easily eliminated by evaporation under a stream of nitrogen gas and spillages rendered harmless by treatment with acetic acid.

It was demonstrated that the methyl esters prepared from a mixture of fatty acids by reaction with TMS-DM and methanol was quantitative and gave chromatograms which were completely identical to those of methyl esters prepared by the action of pure diazomethane.[85] Work carried out in the laboratory of one of the authors also demonstrates that saponification followed by use of TMS-DM as a methylating reagent gives identical fatty acid profiles to the general base-catalysed method represented by sodium methoxide in methanol when analysing identical lipid samples from a number of sources such as beef fat, muscle and menhaden fish oil (Fig 4). In addition, the usefulness of the technique described previously, allows for the analysis of a wide range of tissue and sample matrices without the need for extensive lipid extraction with the use of solvents (Fig 5). It is the opinion of these authors that TMS-DM promises a non-hazardous routine procedure for the preparation of methyl esters of fatty acids.

TMS-DM does not change the original isomer distribution nor alter the geometric configuration of conjugated double bonds; it also does not produce the methoxy artefacts associated with acid-catalysed methylations as reported by some researchers.[57,59–61] However, in a recent study, Park *et al*[60] found a small amount of artefacts attributable to TMS-DM (stated as new peaks generated from the sample by the procedure) and a group of impurities (stated as peaks that originated from reagents) at low retention times. They suggest that these are trimethylsilyl fatty acid derivatives and trimethylsilyl, respectively, and that they will interfere with short-chain fatty acids in GC. In our view, the comments relating to TMS-DM artefacts[60] need to be carefully considered, especially in view of the lack of detail in how the samples were processed. It must be stated that these authors also

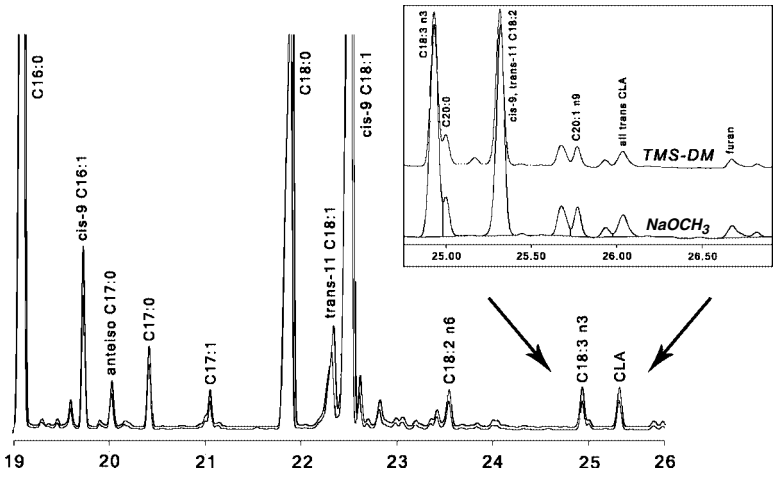

Figure 4. Chromatogram excerpts (overlaid) illustrating the direct *trans*esterification method using $NaOCH_3$ vs the TMS–diazomethane method for the methylation of fatty acids from lipid extracted from beef adipose tissue. Samples were methylated in duplicate and gave identical profiles. Insert is a magnified view of the $C_{18:3}$ to CLA region. Column used was a Phenomenex BPX-70 (SGE), 120 m, 0.25 mm id, H_2 carrier gas, temperature programme for 42 min. Timebase given in minutes.

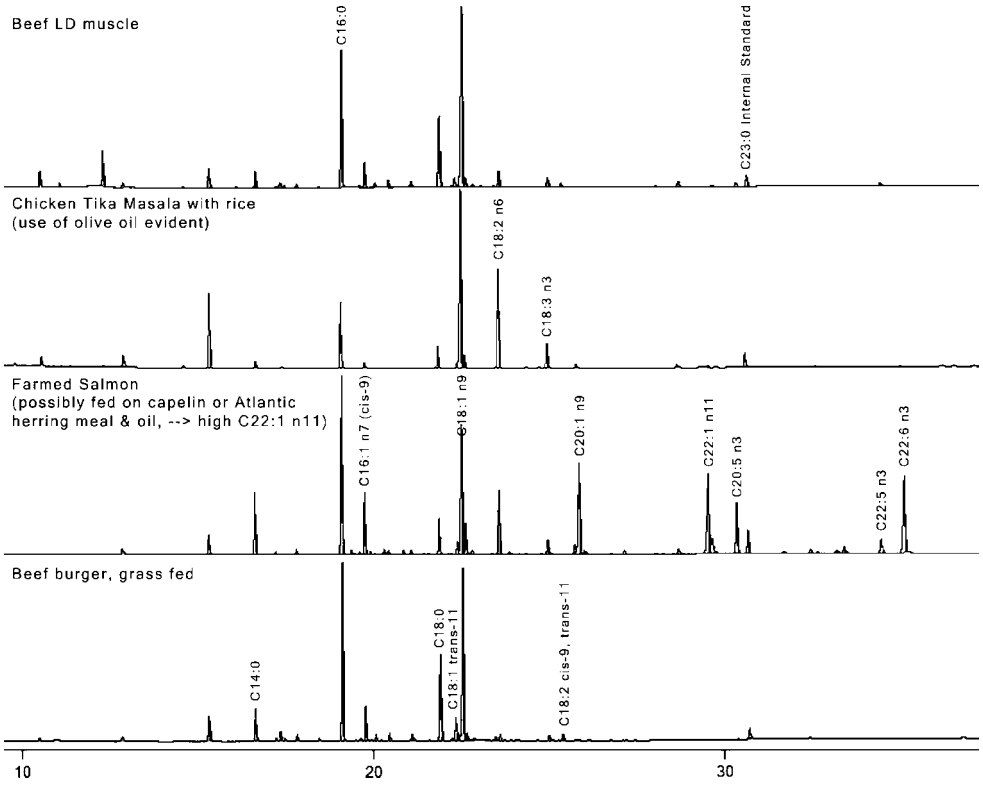

Figure 5. Chromatograms illustrating the range of different foodstuffs (sample matrices) that are easily processed using the saponification/TMS-DM procedure outlined in this review. Column used was a Phenomenex BPX-70 (SGE), 120 m, 0.25 mm id, H_2 carrier gas, temperature programme for 42 min. Timebase given in minutes.

reported artefacts due to the other methods employed in their study. In our experience, TMS-DM is a very satisfactory method for the methylation of free fatty acids with very little evidence of artefact formation (there are some contaminants found eluting early, well before $C_{12:0}$; however we find no evidence of artefacts eluting later than this fatty acid). It is crucial that methanol is present in large concentrations for the reaction to proceed completely and vigorously under mild conditions in a solvent system that adequately dissolves complex mixtures of fatty acids from tissue or lipid extracts (eg toluene—methanol at 1:2 v/v). We typically dissolve 30–40 mg total fatty acid in 1 ml of the above solvent with the addition of small amounts of TMS-DM (100–200 µl of a 2.0 M solution in hexane). With lower concentrations of methanol or its complete absence, artefacts are generated in the $C_{18:0}$–$C_{23:0}$ region of our chromatograms. Presumably these artefacts represent partial or intermediate reaction products as well as trimethylsilyl derivatives.

SUGGESTED DERIVATIZATION

Gas chromatography is the primary method of choice for the analysis of the fatty acid content of lipid samples from many different sources because of its rapidity, high resolution and sensitivity.[39] Fatty acids must first be converted to non-polar, volatile derivatives such as methyl esters. However, care must be taken when deciding upon a suitable derivatization procedure, particularly if the analyst is also interested in determining conjugated fatty acids such as CLA. No single procedure exists which simultaneously methylates all lipid components (triacylglycerides, free fatty acids, N-acyl lipids such as sphingolipids and glycosphingolipids, and very short chain fatty acids). The best method or combination of methods will depend almost entirely on the nature of the sample to be analysed. The papers by Shantha *et al*[54] and Kramer *et al*[57] have excellent summaries of the many varied methods available, particularly when referring to CLA analysis, and the reader is encouraged to refer to these publications.

Traditionally, when analysing lipid samples with a significant CLA content (milk and dairy products, ruminant meats) the recommendations clearly advise base-catalysed *trans*esterification, often involving the use of sodium methoxide in methanol. However, a few reports also suggest that acid-catalysed methylations may be used if mild conditions are employed, often shortened times (5–20 min) at reduced temperatures (50 °C).[45,59,60]

In this review, the authors have no problems in suggesting a slightly different approach which works well on both extracted lipid and oils, but especially so on different tissue matrices (eg adipose tissue, muscle tissue, fish, etc). However, the analysis of extracted lipid and pure oils by TMS-DM is not quite as efficient as methods based on sodium methoxide. Qualitatively, results are identical, all fatty acids are derivatized, but peak heights tend to be lower for samples processed by the saponification/TMS-DM

procedure by about 30%–40%. This difference may be a result of the difference in solubility of oils and lipids (or their respective solvents) in 5 M KOH 50% methanol vs 0.5 M sodium methoxide ($NaOCH_3$). In support of this is the observation that different solvents effect saponification—non-polar solvents such as diethylether and petroleum spirit reduce the efficiency of saponification whereas toluene–methanol solutions give the highest efficiency. In every case the smallest volume of added solvent ensures the highest efficiency of saponification. The saponification/TMS-DM procedure, however, has a large advantage over sodium methoxide in that it is not as sensitive to water in the samples. Hence, it is not necessary to lyophilize tissue and complex matrices prior to derivatization. Up to 1g of tissue or 50 mg of oil or lipid extract can be successfully saponified using strong alkali in aqueous methanol. Following a simple set of clean up steps, the extracted fatty acids are dissolved in 1 ml of toluene–methanol (1:2 v/v) and converted to their methyl esters using 200–400 µmol TMS-diazomethane. There is no need to quench the reaction with acetic acid and after dissolving in n-hexane with 50 ppm of butylated hydroxytoluene (BHT) they are ready for injection onto the GC. This procedure will enable the analysis of all the esterified fatty acids as well as the free fatty acids ($C_{11:0}$ to $C_{22:6}n$-3, branched chain fatty acids, cyclopropane fatty acids, furans, conjugated fatty acids, long chain PUFA, etc) in a sample, but it is important to realize that losses would be incurred for the volatile very small chain fatty acids and N-acyl lipids.

For very small chain fatty acids (as present in milk and butter fats) it would be wise to also produce the fatty acid isopropyl ester (FAIPE) analogues by the methods using isopropanol and H_2SO_4.[86,87] For analysis of N-acyl lipids the acid-catalysed methods (HCl, BF_3, acetyl chloride and H_2SO_4), eg HCl in anhydrous methanol at 80 °C for 1 h,[88] are appropriate, but at the expense of correct CLA isomer determinations. For the determination of fatty acids in pure oils then the sodium methoxide method is more than appropriate.[54,57,68]

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