An Improved Method for the Analysis of Ruminant Fats

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A method is described for the separation of saturated methyl esters from mercuric acetate adducts of unsaturated esters using an ion exchange resin. The esters thus separated were analyzed by gas chromatography on a short wide-bore capillary column containing a bonded polyethyleneglycol liquid phase. Detailed analyses of sheep and beef depot fats can be produced more rapidly and with greater accuracy using this methodology than with that currently available.

Ruminant depot lipids are complex mixtures of glycerides containing saturated and unsaturated fatty acid moieties (1). Saturated moieties consist of normal and methyl-branched components; the latter may be of the *iso* and *anteiso* species as well as those with the branches on even-numbered carbon positions relative to the carboxyl groups (2, 3). Both geometric and positional isomers of unsaturated moieties also are usually present (1). The relative amounts of *n*-saturated, branched-chain and unsaturated components in a fat sample depend on the animal species, its diet and on the location in the animal from which the sample is taken (1, 4).

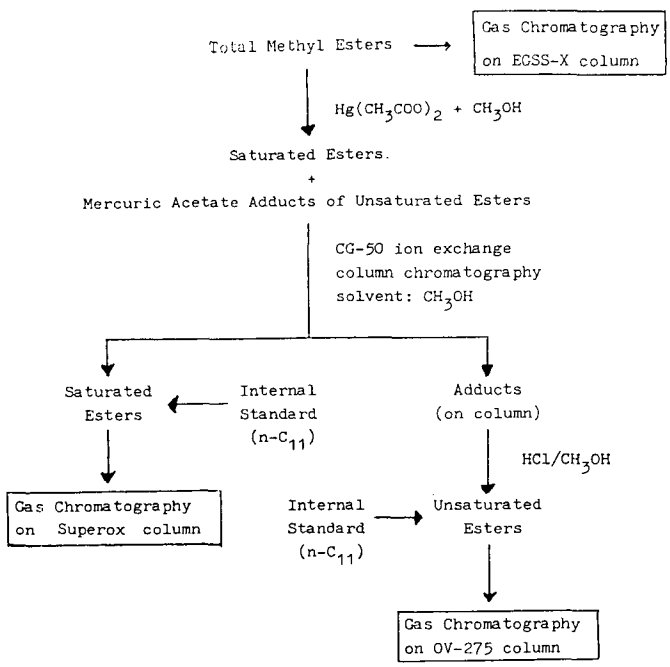
Detailed compositional analyses of fatty methyl esters derived from ruminant lipids are very time consuming. The esters, both prior to and after silver ion or mercuric acetate adduct chromatography, in combination with urea fractionation and hydrogenation, are analyzed by gas chromatography using packed polar and nonpolar columns (1). Peak overlap also occurs during the separation of methyl esters on a narrow-bore capillary column (5).

The investigation described in this paper was directed toward developing an improved method for the analysis of methyl esters derived from ruminant fats. Unsaturated components in the ester mixtures were first converted to their mercuric acetate adducts. Separation of these adducts from the non-adducted materials by ion exchange chromatography yielded the two individual groups of saturated and unsaturated components of the original ester mixtures. An internal standard was added to each group, and they were then analyzed by gas chromatography. This procedure is depicted in Scheme 1.

EXPERIMENTAL

Fat (ca. 100 mg extracted from sheep or beef subcutaneous tissue) was transesterified by reaction with methanolic KOH followed by BF_3/methanol (6). Unsaturated components in the resulting methyl ester mixtures were converted to their mercuric adducts by heating them for one hr at 40°C in one ml of a methanolic solution containing 100 mg of mercuric acetate and two drops of acetic acid. The cooled reaction solution was then applied to a column (10 cm × 1 cm o.d.) containing two g (dry weight) of Amberlite CG-50 type 1 weak cation exchange resin that had been swollen and packed in methanol. Saturated esters were eluted into a 50-ml round-bottomed flask with 20 ml of methanol. Most of the solvent was removed under reduced pressure and the esters dissolved in two ml of petroleum ether (boiling range 30–40°C) containing a known weight (ca. 2 mg) of methyl undecanoate as an internal standard. Water was then added to the flask, with gentle stirring, until the hydrocarbon solution of esters appeared in the neck of the flask. The solution of esters was transferred to a screw cap vial. Adducts on the column were decomposed by passing five ml methanolic hydrogen chloride [prepared by slowly adding, with vigorous stirring and cooling, acetyl chloride to methanol (5% v/v)] through the resin, followed by 20 ml methanol. The resulting acidic methanolic solution of unsaturated methyl esters was treated the same as the corresponding solution of saturated esters.

Methyl esters thus fractionated were analyzed by gas chromatography using a Hewlett-Packard Model 5840 chromatograph, fitted for use with packed columns, with the injector and the flame ionization detector temperatures set at 230°C and nitrogen as the carrier gas. The esters were separated on a 10-m × 0.53-mm i.d. fused silica capillary column containing either bonded Superox polyethylene glycol or RSL-150 (NON-PAKD, Alltech/Applied Science Laboratories) that was temperature programmed from 110 to 200°C at 2°C/min with the carrier gas flow rate set at 2 ml/min. A narrow-bore glass liner was used in the injection port. Nitrogen (at a flow rate of 40 ml/min) from a second port was used as the make-up gas, being introduced through a tee-piece between the end of the column and the detector. The end of the column passed



SCHEME 1. Methodology for the analysis of methyl esters derived from ruminant fats.

through the tee-piece to minimize dead-space in the carrier flow. Two 2-m × 3.2-mm o.d. stainless steel columns containing either EGSS-X (10% w/w on DMCS treated Chromosorb W) at 180°C or Apiezon L grease (3% w/w) at 200°C with a carrier gas flow rate of 30 ml/min were also used in this study. Identification of methyl ester isomers was based on their co-chromatographing with commercially available authentic methyl esters and on published relative retention data (7).

RESULTS AND DISCUSSION

Ester fractionation. An example of the separation of saturated from unsaturated fatty methyl esters derived from a sheep (ewe) subcutaneous fat sample is illustrated in Figure 1. There was no cross-contamination of the two fractions. This was confirmed by treatment of the separated saturated and unsaturated fractions with hydrogen over Adam's catalyst. Both methanolic aqueous hydrochloric acid (8) and methanolic hydrogen chloride were used for decomposing the adducts. The latter reagent was preferred, to minimize the amount of water in the solvent. Thus the possibility of precipitation and hydrolysis of the esters in the column was decreased.

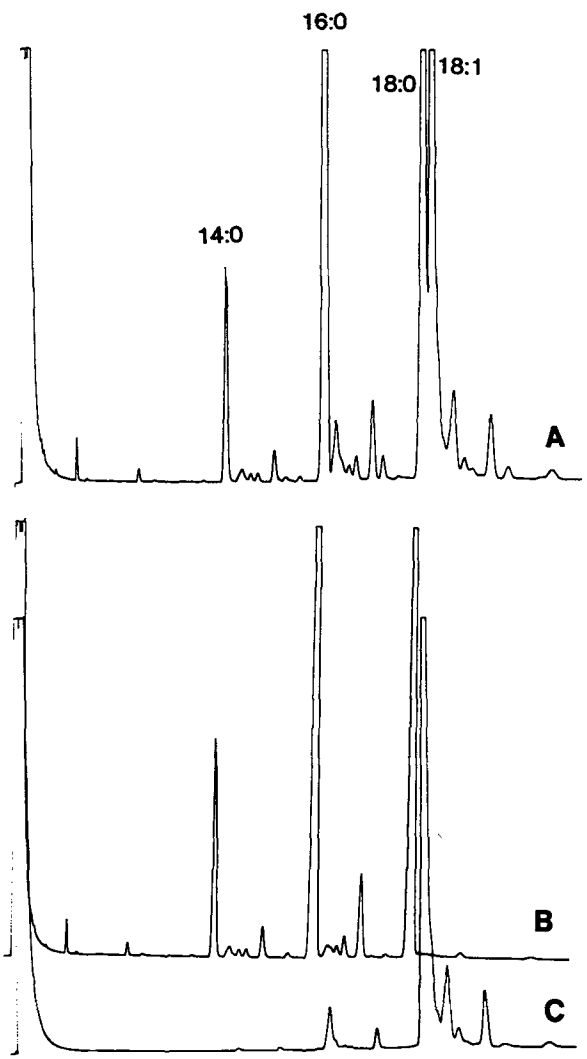


FIG. 1. Gas chromatograms, using a wide-bore capillary column containing Superox, of methyl esters derived from sheep (ewe) subcutaneous fat. A, Total esters; B, saturated esters; C, unsaturated esters.

Procedures for the separation of saturated fatty esters from mercuric adducts of unsaturated esters by column chromatography using either alumina (8, 9) or silica acid (10) have been reported, but clean separations were difficult to achieve. Mercuric acetate (11, 12) or mercuric bromide (13–16) adducts can be separated by thin-layer chromatography (TLC), but these methods are not suitable for the analysis of large numbers of samples.

Fractionation of the esters based on their degree of unsaturation was attempted in this study. Saturated esters were removed from the ion exchange column with methanol, followed by most of the monoene adducts by 10% (v/v) acetic acid in methanol. Diene and triene adducts were removed from the column by adding increasing amounts of acetic acid to the solvent, but they could not be separated. Finally, elution of the column with methanolic hydrogen chloride yielded some more (ca. 5% of the original) monoenoic material. These results indicate that this method is of limited use for detailed fractionation of unsaturated esters. Irreversible adsorption of mercuric adducts on alumina has been reported (9).

The ion exchange column could be used for more than one sample if the top three cm of resin material was replaced after each separation. Failure to effect this replacement results in partial decomposition of the adducts, possibly by mercury compounds of undetermined composition deposited on top of the column, leading to contamination of the saturated esters with unsaturateds.

The method of separation described above was found to be most suitable for the rapid separation of saturated from unsaturated fatty esters. A further advantage is that all reactions leading to the mercuric adducts were carried out in sealed test tubes. Whereas the carboxyl-substituted ion exchange resin (Amberlyte CG-50) retained the mercuric adducts, application of the adducts to a sulfonate-substituted macroreticular resin (Amberlyst 15) resulted in their quantitative decomposition to the corresponding unsaturated esters.

Partial analysis of fats. Chromatograms of saturated methyl esters derived from beef and sheep subcutaneous fats on the wide-bore Superox capillary column are shown in Figure 2.

Beef fat showed a relatively simple pattern of *n-*, *iso-* and *anteiso*-esters (Fig. 2A). These esters were also well separated on the EGSS-X column. However, *iso-* and *anteiso*-ester peaks overlapped when these compounds were chromatographed on the nonpolar columns.

The ewe fat had branched-chain components that did not correspond to the *iso* or *anteiso* series of compounds (Fig. 2B). These "other" branched-chain esters appeared as a broad peak, or as a series of overlapping peaks, between the *iso*-ester and the next lower *n*-ester. The ram fat contained an even greater proportion of the "other" branched-chain esters, resulting in the overlapping of these with the *iso-* and *anteiso*-esters (Fig. 2C).

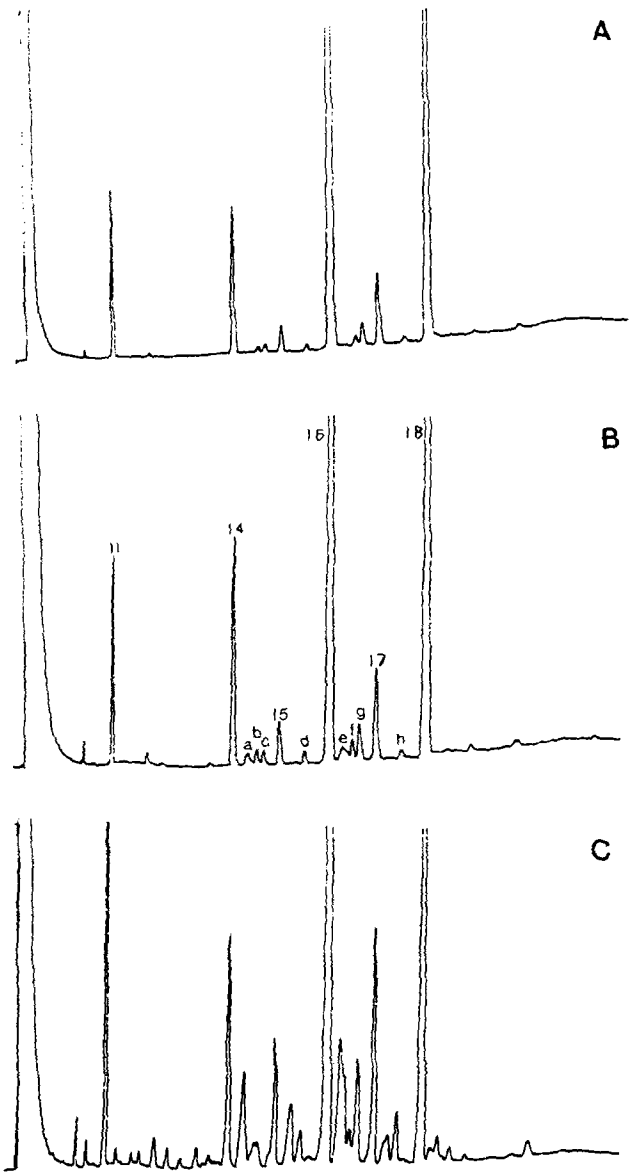


FIG. 2. Gas chromatograms, using a wide-bore capillary column containing Superox, of methyl esters derived from bovine and ovine subcutaneous fats. A, Beef fat; B, ewe fat; C, ram fat. a, "other" branched chain C_{15} esters; b, iso C_{15}; c, anteiso-C_{15}; d, iso-C_{16}; e, "other" branched chain C_{17} esters; f, iso-C_{17}; g, anteiso-C_{17}; h, iso-C_{18}; 11, n-C_{11} internal standard.

Ovine esters could not be satisfactorily analyzed on the EGSS-X column alone as there was extensive overlapping of the *n-*, *iso-* and "other" esters. On the Apiezon L column, the "other" esters appeared as a peak between the *n*-ester and the completely overlapping *iso-* and *anteiso*-esters. Thus the Superox capillary column replaced the EGSS-X and Apiezon L columns for the analysis of ovine fats (1).

A beef fat (4 × 0.1 g) was analyzed by the modified method described above, and the total methyl esters from the fat were also analyzed (4 ×) on an EGSS-X column. Results of analyses of components showing minimal overlapping with other branched-chain or unsaturated components in the "total" chromatogram are shown in Table 1. These analyses yielded similar results.

TABLE 1

Partial Composition of Methyl Esters Derived from a Beef Subcutaneous Fat (expressed as area % from the gas chromatograms)

Fatty ester	Method 1[a]	Method 2[b]
14:0[c]	2.32 ± 0.05	2.34 ± 0.18
15:0	0.45 ± 0.04	0.46 ± 0.04
16:0	24.0 ± 0.4	24.2 ± 0.2
17:0	1.20 ± 0.03	1.31 ± 0.10
18:0	14.0 ± 0.3	14.3 ± 0.3
18:1	42.6 ± 0.6	42.9 ± 0.6

[a]Total methyl esters analyzed by gas chromatography on an EGSSX-column (see text), mean ± SEM for 4 chromatograms (1 sample).

[b]Methyl esters separated as described in text and analyzed by gas chromatography on the Superox column, Mean ± SEM for 4 chromatograms (4 samples).

[c]Chain length: number of double bonds.

Methyl esters derived from bovine fats can be analyzed, without a preliminary separation, on the polar wide bore capillary column if *cis-trans* separations are not required. These fats do not contain the "other" branched chain isomers which overlap on the gas chromatograms with the next lower monoenes. There was no overlap of these monoenes with the *iso* and *anteiso* branched chain isomers on this column.

This methodology has been used for the analyses of ovine and bovine fats from animals fed pasture and grain diets. Results from these analyses will be published separately.

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