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Note

Preparative separation of milk fatty acid derivatives by high-performance liquid chromatography

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The method of choice for the isolation of individual fatty acid components for structural analysis or for liquid-scintillation counting until relatively recently has been preparative gas–liquid chromatography[1,2]. With samples such as milk fat where the major fatty acids vary in carbon chain-length from 4 to 18, there is no accepted method for condensing out each component from the carrier gas in a reproducible manner. High-performance liquid chromatography (HPLC) in the reversed-phase mode is being used increasingly for separating fatty acids, either as aliphatic esters when refractive index detection is used, or as aromatic esters when UV detection is possible[1,3]. We required a method for routinely isolating milk fatty acids of relatively low specific activity (^{3}H or ^{14}C) on a small scale (1–2 mg) for liquid-scintillation counting from experiments with goats *in vivo*, and have investigated the possibility of using isocratic HPLC systems for the purpose.

EXPERIMENTAL

All reagents and solvents were Analar or HPLC grade and were supplied by Fisons (Loughborough, U.K.). Lipids were extracted from cows' milk with chloroform–methanol (2:1, v/v)[4].

Methyl ester derivatives were prepared by the following modification of established procedures[5,6]. Milk fat (20 mg) was dissolved in acetonitrile (1 ml) with slight warming. Methyl acetate (25 μl) then 1 *M* potassium methoxide in methanol (50 μl) were added and the mixture left for 5 min before being stopped by the addition of acetic acid (6 μl). The esters were cleaned up by brief centrifugation (2 min at about 1500 *g*) through a column of neutral alumina (1 cm) in a disposable Pasteur pipette. Aliquots of the resultant solution were injected directly onto the HPLC column.

Butyl and benzyl esters were prepared in an analogous way from the appropriate alcoholic base solutions.

A Model 8770 isocratic HPLC pump (Spectra-Physics, St. Albans, U.K.) equipped with a Knauer differential refractometer (Dr. H. Knauer, Oberursel/Taunus, F.R.G.) was used. The column consisted of a 5 cm × 5 mm I.D. guard column and a 25 cm × 5 mm I.D. main column packed with LiChrosorb 10 RP 18 (HPLC Assoc., Macclesfield, U.K.). Isocratic elution conditions are described in the text. Helium degassing of solvents was used.

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RESULTS AND DISCUSSION

The primary problem in the chromatographic separation of the fatty acid components of milk fat is the quantitative conversion to suitable derivatives. For gas chromatographic purposes, methyl ester derivatives are prepared by most workers by base-catalysed transesterification using a method originally described by Christopherson and Glass[5], but which has recently been modified to minimise adventitious

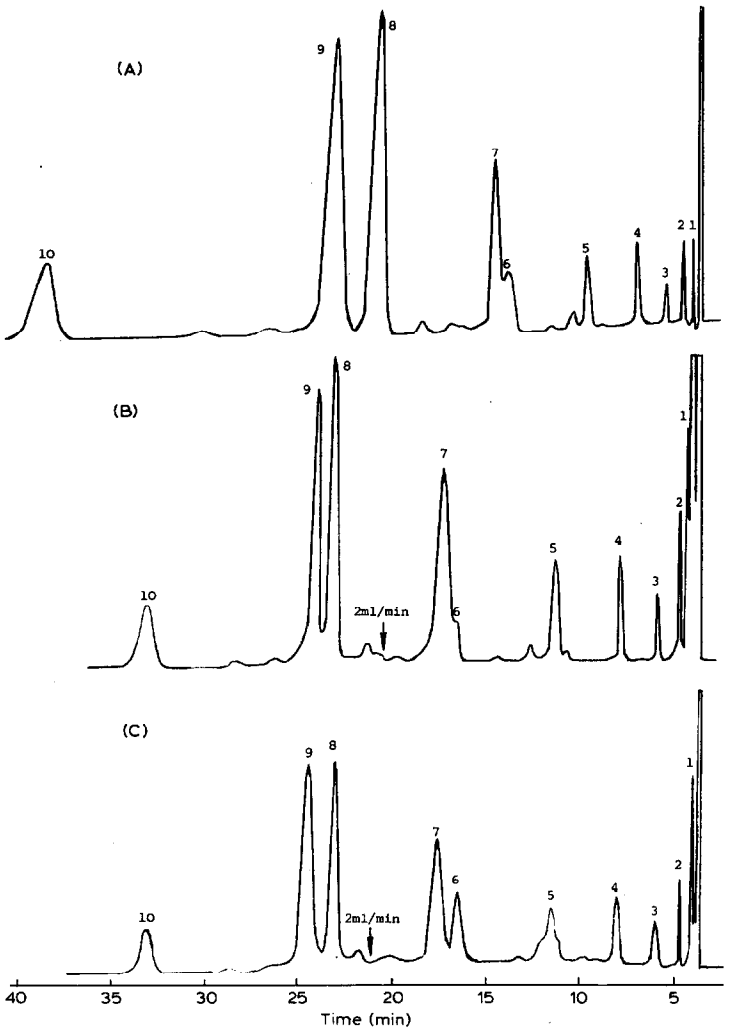

Fig. 1. Reversed-phase HPLC of milk fatty derivatives (sample equivalent to 1 mg of milk fat per analysis) with differential refractive index detection. (A) Methyl esters eluted with acetonitrile–water (95:5, v/v) at 1 ml/min. (B) Benzyl esters eluted with acetonitrile–water (95:5, v/v) at 1 ml/min initially then at 2 ml/min. (C) Butyl esters eluted with acetonitrile–water (98:2, v/v) at 1 ml/min initially then at 2 ml/min. Column and equipment as detailed in the Experimental section. Fatty acid identification: 1 = 4:0; 2 = 6:0; 3 = 8:0; 4 = 10:0; 5 = 12:0; 6 = 16:1; 7 = 14:0; 8 = 18:1; 9 = 16:0; 10 = 18:0.

hydrolysis[6]. In this method, the milk fat dissolved in a solvent is reacted with the minimum reagent, and there are no work-up steps that involve evaporation of solvents or aqueous washes where short-chain components could be lost. As acetonitrile is the most widely-used solvent for reversed-phase HPLC of fatty acid derivatives, methylations were attempted with this as the solvent and were found to proceed satisfactorily if the mixture was warmed slightly to ensure that the milk fat dissolved. Butyl and benzyl esters were prepared successfully by an analogous procedure.

Satisfactory resolution of the methyl ester derivatives of milk fatty acids from C_4 to C_{18} was obtained by reversed-phase HPLC with isocratic elution with acetonitrile–water (95:5, v/v) as shown in Fig. 1A. In particular, methyl butyrate was clearly resolved from an initial peak consisting of methanol and methyl acetate (from the reaction mixture). Only the 14:0 and 16:1 fatty acids were poorly resolved. Separations were carried out on the 1–2 mg scale to ensure sufficient radioactivity counts with samples labelled *in vivo*. Quantitative recovery from the column was obtained with isopically-labelled fatty acid derivatives, if components were eluted directly into scintillation vials and a toluene-based scintillant added without evaporation of the eluting solvent; no significant quenching was caused by the eluting solvent. No "memory" effects were observed. With acetonitrile only as the eluting solvent better resolution was obtained, and the separation time was only 20 min, but methyl butyrate eluted with the reagent peak. For preparative purposes with labelled materials, however, this might be the elution system of choice.

Benzyl esters were also prepared and were eluted under comparable conditions to methyl esters (Fig. 1B), although resolution of the longer-chain components was not as good (the flow-rate was increased after the 14:0 fatty acid to speed up the separation). Unfortunately benzyl butyrate was not clearly resolved from an artefact peak. Butyl esters were particularly well separated (Fig. 1C) by isocratic elution with acetonitrile–water (98:2, v/v), and these would be the preferred derivatives for preparative purposes.

The purpose of this investigation was to devise a small-scale isolation procedure for milk fatty acid derivatives and no attempt was made to assess the potential of the procedure for analytical use. Gas chromatography is undoubtedly the method that most workers would use at present although there are some doubts as to the precision with the more volatile components[7,8]. The HPLC procedures described here could undoubtedly be adapted to the analysis of milk fatty acids, and there should not be difficulties associated with volatility. Benzyl (or other UV-absorbing) esters would then probably be the derivatives of choice since with UV-detection the response would be directly related to the molar proportion of each fatty acid present. With small samples, gradient elution and 3-μm packing materials, excellent resolution should be attainable.

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