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Note

High-performance liquid chromatographic method for the determination of esterified butyric acid in fats

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In addition to its importance as an indicator of the quality of milk fat, butyric acid is widely employed for the direct estimation of milk fat in food products, since this is the only practical source of esterified butyric acid in food manufacture. The butyric acid content of milk fat is sufficiently constant for this purpose, and a figure of 3.6% (w/w) is generally accepted. There are two main methods in common use for the specific determination of butyric acid in milk fat, both of which involve the analysis of underivatized butyric acid by gas–liquid chromatographic (GLC) analysis after saponification by some means[1,2]. There could be selective losses, since sample discrimination effects have been found when mixtures containing components of widely differing volatility are chromatographed[3].

As part of a broad programme to develop Certified Reference Materials for food analysis, the Community Bureau of Reference undertook an interlaboratory comparison of methods for the determination of butyric acid and this revealed certain disparities. It was therefore decided to examine the possibility of employing a high-performance liquid chromatography (HPLC) method, based on one described earlier[4], since this in principle should be free of many of the errors associated with gas chromatographic determinations.

EXPERIMENTAL

Materials

Tributyrin was obtained from Sigma (Poole, U.K.), acetonitrile and hexane were from Fisons (Loughborough, U.K.) and phenethylalcohol was purchased from Fluka (Fluorochem Ltd., Glossop, U.K.). Bovine tallow was part of a bulk shipment from a local supplier and was purified by column chromatography with Florisil™ as the adsorbent. The triacylglycerol fraction was eluted with five column volumes of hexane–diethyl ether (85:15, v/v).

Preparation of phenethyl esters

1 *M* potassium phenethylate in phenethyl alcohol was prepared by dissolving

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freshly-cut potassium metal in the alcohol in an atmosphere of nitrogen. The transesterification procedure was adapted from that of Christopherson and Glass[5].

The milk fat (20 mg) was dissolved in heptane (1 ml) in a tapered centrifuge tube, 1 *M* potassium phenethylate (50 μl) was added and the mixture was swirled gently to effect solution. In about 1 min at room temperature, the solution went cloudy as potassium glycerate was precipitated from the solution. After a reaction time of exactly 5 min, the reaction was stopped by addition of acetic acid (6 μl). The mixture was centrifuged at about 700 *g* in a small laboratory centrifuge to precipitate any inorganic by-products. In order to avoid any possibility of inadvertently injecting particulate matter onto the HPLC column, the supernatant was decanted into a fresh tube.

HPLC analysis

A Spectra-Physics (St. Albans, U.K.) Model 8700 ternary solvent delivery system was used with a Pye-Unicam (Cambridge, U.K.) Model 4025 UV spectrophotometric detector. Peaks were quantified by electronic integration with a Spectra-Physics Model 4270 integrator. The system was equipped with a Rheodyne valve-injection system and a 10-μl loop. A column (250 × 4.5 mm) packed with Spherisorb™ ODS-1 (5 μm particles; Bibby Scientific Products, Stone, U.K.) was utilized and was eluted with acetonitrile–water (3:2, v/v) at a flow-rate of 2 ml/min. The detector was operated at 257 nm, in the range of 0.08 absorption units and with a time constant of 0.1 s. Samples were injected in aliquots of 50 μl to ensure that the loop was filled completely. Under the conditions described, the phenethylate ester of butyric acid eluted in 4.5 min as illustrated in Fig. 1. When samples containing nat-

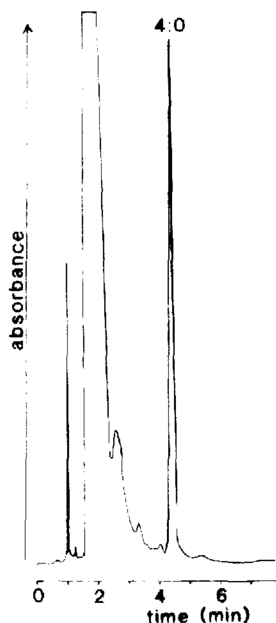

Fig. 1. HPLC separation of the phenethyl ester of butyric acid (4:0) prepared from a milk fat. Separation conditions are given in the text.

ural fats were analysed, the column was flushed with acetonitrile alone after about ten injections to clear longer-chain fatty acid derivatives from the stationary phase.

RESULTS AND DISCUSSION

As certification of the butyric acid content of a candidate milk fat reference material could not be achieved until differences in the results obtained by alternative methods were resolved, an HPLC method was adapted to the purpose. Difficulties were experienced initially with the detection of benzyl esters, the derivatives used previously[4], by UV absorbance as artefacts of the reaction with apparently higher molar extinction coefficients than the fatty acid derivatives at the optimum wavelength tended to interfere with the determination of butyric and other fatty acids. No such problems were encountered in the analysis of butyric acid when phenethyl esters were prepared by reaction of milk fat with potassium phenethylate solution in an inert solvent. Heptane was found to be the best solvent for the reaction, and quantitative yields of the required esters were obtained in a very short time. If the reaction time was inadvertently prolonged, slow hydrolysis occurred. HPLC analysis of the butyrate derivative was then straightforward under the conditions described as butyryl phenethylate eluted in a region of the chromatogram well away from interfering substances, including the derivatives of fatty acids of higher molecular weight. The hexanoic acid derivative eluted after about 8 min, and it was usually advisable to wait until this had cleared the column before the next sample was injected. Longer-chain fatty acid derivatives eluted only slowly and did not interfere, provided that the column was flushed out at regular intervals with acetonitrile.

Initially, the repeatability of injections of the same extract was assessed by preparing the phenethylate esters of a sample of milk fat and injecting five aliquots onto the column in sequence. The standard deviation was only 0.6% of the mean. The short-term repeatability of the whole procedure was assessed by making five complete determinations (including derivatization) on milk fat and six complete determinations on tributyrin. The standard deviations were then 0.8% of the mean. Similar results were obtained for the day to day repeatability.

The nature of the detector response was studied by carrying out the complete procedure with a range of concentrations of a tributyrin standard, and the results were expressed in terms of integrator counts *versus* the concentration of tributyrin in each injection. The detector response was found to be rectilinear over the range 1 to 25 μg, and it passed through the origin. The correlation coefficient was 0.9995, and 10 μg of tributyrin gave 204 000 integrator counts.

To assess the effect of co-extractives on the procedure, triacylglycerols were purified from commercial bovine tallow by column chromatography. No detectable butyric acid was present when this material was subjected to the derivatization procedure. Three mixtures of tallow triacylglycerols with added tributyrin were prepared gravimetrically, in relative proportions close to that found in milk fat. Following derivatization, the predicted and actual results, obtained by means of the calibration curve, were compared (Table I). Again there was good agreement, although the calculated results were 1.9% higher in relative terms than expected.

The method was applied to a standard milk fat (Community Bureau of Reference RM 164) and six complete determinations were made. The butyric acid content

TABLE I

COMPARISON WITH ACTUAL (GRAVIMETRIC) AND DETERMINED TRIBUTYRIN LEVELS IN TALLOW-TRIBUTYRIN MIXTURES

The results are the means and standard deviations of 5 determinations.

Tributyrin (%, w/w)	
Actual	*Determined*
3.48	3.56 ± 0.051
3.98	4.06 ± 0.039
4.48	4.55 ± 0.085

of this material was found to be 3.53% ± 0.09 (n = 6). When this was repeated six months later by a different analyst, the result was 3.54% ± 0.12. In an interlaboratory study with the same standard milk fat[6], the median value obtained by one of the GLC procedures[1] was 3.46% and by the other procedures[2], it was 3.53%.

In addition, it is possible that the precision of the method could be improved further by making use of an internal standard. The obvious standard to use appeared to be trivalerin, but this could not be obtained from any of the usual purveyors of biochemicals. However, an overall accuracy of 2% of the true value in the determination of butyric acid in milk fat appeared acceptable for most purposes. The procedure is extremely sensitive. If the butyric acid content of butter fat could be assumed to be relatively constant under most conditions, the method could be used to detect as little as 1% of butter fat in admixture with other fats.

The method described above for the determination of butyric acid in milk fat has been adapted to HPLC from an established GLC procedure, designed to minimize losses of the short-chain fatty acids. There was no extraction step in which losses of butyric acid, which is relatively soluble in water, could occur. Nor was there a solvent evaporation step, in which losses of the volatile butyric acid derivatives could occur. Also in comparison to the GLC methods, there was no vaporization in a heated injection port, where again selectivity effects could be observed, and there was no need for factors to correct for a differential response.

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