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Application of radiometric and isotope dilution-mass spectrometric techniques to the certification of edible oil and fat reference materials

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Summary. A method for the isotope dilution-mass spectrometric (ID-MS) determination of butyric acid C_4 in butter fat (RM164) was developed in order to support the data gathered from nine experienced European laboratories within the final certification exercise. The ID-MS results ($3{,}46 \pm 0{,}06$ g C_4/100 g fat) were in very good agreement with those obtained by "classical" GLC and HPLC techniques. (RM164 was finally certified at $3{,}49 \pm 0{,}06$ g C_4/100 g fat). This paper reports briefly on a previous preliminary study undertaken to validate a procedure (agreed by the BCR-sterol group) for the isolation of the sterol from fats and oils. By use of labelled sterols and radiometric measurements it was shown that sterol recoveries were superior to 96%.

The procedure was applied during the 3rd Intercomparison exercise for sterol determination in RM162 (Blend of Soya-Maize oil), for the GLC measurements of cholesterol in RM380 (Whole milk powder) and RM384 (Lyophilized pork muscle) and to the ID-MS determination of cholesterol in RM163 (blend of animal fats) and RM164 (anhydrous milk fat).

Introduction

The use of lipids as Reference Materials (RMs) is of great help for food analysts. Indeed, when certified values are available, the degree of trueness and precision of the analytical measurements can be accurately assessed. Three fatty RMs have been selected in the field of a BCR project: a blend of soya-maize oil (RM162), a tallow-lard mixture (RM163) and anhydrous milk fat (RM164). Their development was undertaken following a three step procedure:

– Study of the stability/homogeneity of ampouled high quality material

– Preliminary intercomparison (determination of the properties for which certification could possibly be achieved)

– Certification of selected properties.

Fatty acid profile of RM162 and 163 was certified in 1988 [1].

Provisionally certified values for 10 fatty acids and for butyric acid of butterfat (RM164) have been recently proposed. Results for other parameters were considered to be satisfactory for citation as indicative values (see Table 1).

The aim of the present study was to apply Isotope Dilution-Mass Spectrometry (ID-MS) to the certification of butyric acid content and to the determinaion of sterols. Several aspects of the analytical procedure for sterol determination were also investigated by use of radiometric techniques.

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Certification of butyric acid in butter fat

The availability of butter fat with certified value for butyric acid content is important for quality control purposes:

– this parameter serves as quality indicator

– custom laboratories (EEC) are required to determine the butter content of processed food on the basis of C_4 level with the goal to make tariff classification [2].

During the final exercise for C_4 certification, we developed a ID-MS technique based on the method of Philips and Sanders [3] which can be summarized as follows:

1. saponification of the milk fat with potassium hydroxide solution followed by acidification with phosphoric acid to liberate the fatty acids, 2. separation of the water soluble fatty acids (including C_4) by filtration, 3. direct determination of butyric acid by GC-MS in the presence of heptadeuterated labelled butyric acid as internal standard.

GC-MS conditions: Column: FFAP-CB (Macherey-Nagel FRG) L = 25 m, ∅ = 0.32 mm, df = 0.2 µm, T° programmation from 55 to 120° C at 20° C then from 120 to 215° C at 30° C. Carrier: Helium 70KPa.-Spectrometer: R10-10C (Nermag France), Delsi (France) DI 700 Chromatograph.-Fragmentometric analysis: Electron impact at 70 eV. The characteristic mass fragments m/e = 60 (C_4) and m/e = 63 (heptadeuterated molecule) were monitored. They result from McLafferty rearrangement.

The work was undertaken in order to support the analytical data obtained by "classical" GLC and HPLC methods in nine European laboratories. The results of the study are summarized in Table 2.

In the optimal chromatographic conditions, ID-MS determinations were fast, reliable and had a good repeatability. The calibration curves were linear. A slight difference of retention time was always observable between

Table 1. Reference materials

	RM162 Soya-maize oil	RM163 Pig-beef fat	RM164 Milk-fat
FA profile	Certified	Certified	Certified
Butyric acid	–	–	Certified
Sterol profile[a]	Indicative values	Indicative values	Indicative values
Cholesterol[a]	Indicative values	Indicative values	Indicative values
USM	Indicative values	–	–
2-position FA	–	Indicative values	–

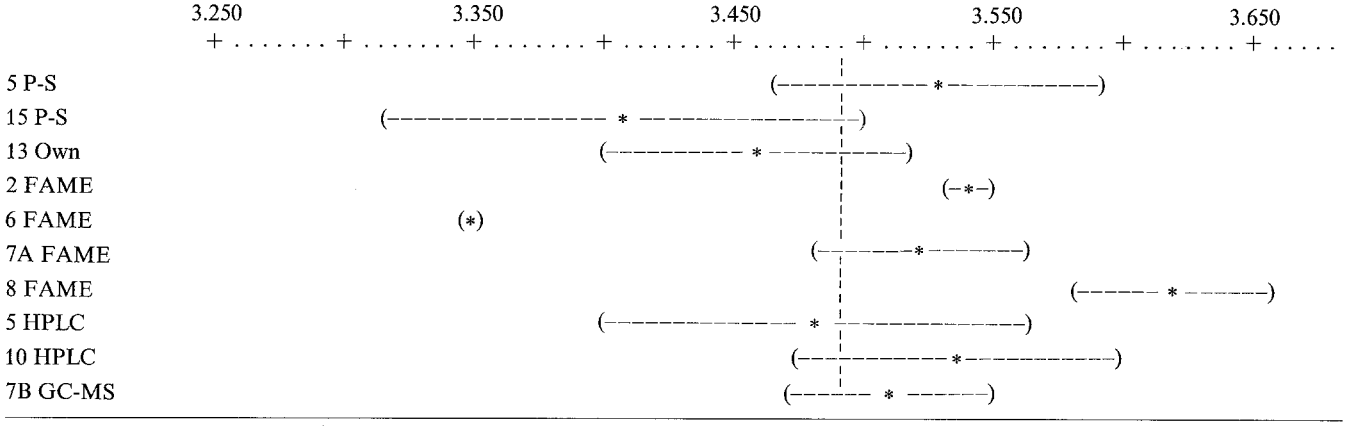
PUFAs, Tocopherols and 12 values are also cited as "indicative" for RM162
[a] Ongoing study

Table 2. Butyric acid content of a test mixture (Tributyrin-Tallow) and of RM164. A. Values from our laboratory. B. Values from certification exercise. C. Supporting measurements (ID-MS). Results expressed in g C_4/100 g fat. FAME = fatty acid methyl ester, der. = derivative

	Method		Control[a]	RM164
A	GLC	Philips-Sanders	3.46 ± 0.03 (n = 3)	3.41 ± 0.06 (n = 7) 3.52 ± 0.03 (n = 8)
		FAME	3.45 ± 0.02 (n = 3)	3.52 ± 0.04 (n = 6) 3.54 ± 0.05 (n = 8)
B	GLC	Philips-Sanders	3.40 ± 0.11 (n = 6)	3.47 ± 0.10 (n = 12)
		Own method	3.41 ± 0.01 (n = 3)	3.46 ± 0.06 (n = 6)
		FAME	3.40 ± 0.06 (n = 12)	3.51 ± 0.10 (n = 24)
	HPLC	Bromophenacyl der.	3.47 ± 0.06 (n = 3)	3.48 ± 0.08 (n = 6)
C	HPLC	Phenethyl der.	ND	3.54 ± 0.01 (n = 12)
	ID-MS	Philips-Sanders	3.39 ± 0.03 (n = 12)	3.46 ± 0.07 (n = 16)

[a] Theoretical value: 3.41

Table 3. Results (set means and 95% confidence intervals) accepted for the certification of the butyric acid content of anhydrous milk fat RM164. FAME fatty acid methyl ester. Butyric acid content mass fraction (g/100 g)



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butyric acid (5.07 min) and the heptadeuterated molecule (5.04 min).

The values achieved for the test mixture (blend of tributyric and refined beef tallow) and for butter fat by ID-MS are in very good agreement with those obtained in our laboratory by using "classical" GLC methods (C_4 measured directly or as methyl ester). They support very well the analytical data gathered from nine experienced European laboratories (Table 3). RM164 (anhydrous milk fat) was finally certified at 3.49 ± 0.06 g/100 g fat.

Table 4. General analytical scheme for sterol determination in fatty materials. The arrows show the different steps investigated by radiochemical analyses

Saponification of the fat after addition of ISTD (Betulin or cholestane) ⇦
↓
Extraction of unsaponifiable (USM) with diethyl ether ⇦
↓
Water washings of the extract ⇦
↓
Fractionation of USM (Preparative TLC)
↓
Isolation of sterols from the gel
↓
Derivatization (TMS)
↓
Capillary GLC analysis

Table 5. Sterol recoveries after saponification, extraction of unsaponifiable and washings

Fatty material	Sterol recoveries (%) after 6 extractions with diethyl ether	Losses by washings with water (%)
Sunflower + 3H-cholesterol	96.2 ± 1.3 (n = 6)	1.0 ± 0.2
Sunflower + 3H cholesteryl oleate	100.7 ± 0.7 ($n = 7$)	0.7 ± 0.1
Butter oil + 3H cholesterol	99.2 ± 1.2 ($n = 3$)	0.7 ± 0.1
Butter oil + 3H cholesteryl oleate	102.7 ± 0.2 ($n = 3$)	0.3 ± 0.2

Each radioactivity counting was repeated 5 times

Sterol analysis

The certification of sterol profile and cholesterol content is of capital importance for nutritional standpoints and for the research of adulteration.

The first interlaboratory trials undertaken within the BCR project revealed an insufficient level of agreement of the results for absolute sterol content. Nevertheless the sterol profiles (expressed in % of total sterols) were satisfactory [4]:

Additional investigations suggested that a source of error could be associated to the initial steps (lipid saponification, extraction and washings of unsaponifiable) of the method (Table 4).

Finally, a procedure for the isolation of sterols was agreed by the BCR-Sterol group and consequently validated by use of labelled sterols and radiometric measurements. The recovery of both free (3H cholesterol) and esterified (3H cholesteryl oleate) sterols was investigated because the two forms are present in food lipids. Spiked sunflower and butter oil were used in order to evaluate the influence of different kind of soaps on the recovery of sterols.

The design of the work and all experimental conditions were reported in detail in a previous paper [5].

The results of this study are summarized in Table 5.

The radiochemical investigations have shown that:

– cholesterol was quantitatively extracted regardless of the lipid tested (recovery >96%),

– cholesteryl oleate was quantitatively saponified,

– losses by washings did not exceed 1%,

– there were no appreciable amounts of sterol degradation products (TLC and radiodensitometric scanning).

The whole procedure was applied to different reference materials:

– determination of sterol content of soya-maize oil (RM162) (3rd intercomparison), measurement of cholesterol absolute level in two additional BCR's reference materials: RM380 (milk powder) and RM384 (lyophilized pork muscle) and determination of cholesterol in RM163 and RM164 by ID-MS. The ID-MS conditions were the following:

Column: RSL-300 from RSL (Belgium) L = 25 m, ∅ = 0.32 mm, df = 0.3 µm, isothermal analysis at 265°C. Carrier: Helium 70 KPa. Spectrometer: R10-10C (Nermag France), Delsi (France) DI700 Chromatograph.

Fragmentometric analysis: Electron Impact at 70 eV, molecular ions at m/e = 386 (cholesterol) and me/e = 387 (ISTD) were monitored.

The results of the work are presented in Table 6.

Table 6. Sterol content of several BCR reference materials

Sterols in RM162. Mean values (mg/100 g RM) and rsd of results from 9 labs. (BCR studies)

	Total sterols	Cholesterol	Campesterol	Stigmasterol	B-sitosterol
2nd Intercomparison [4]	698 (18)	4.6 (59)	148 (15)	68 (18)	434 (18)
3rd Intercomparison	656 (5)	3.0 (23)	141 (6)	61 (8)	418 (5)

Cholesterol level of 4 RMS (mg cholesterol/100 g RM). Results from our laboratory.

RM	Method for quantitation	Cholesterol content	Values from BCR studies
RM 380 (Milk Powder)	GLC (Betulin as ISTD)	92 ± 2 ($n = 6$)	N.D.
RM 384 (Lyophilized Pork Muscle)	GLC (Betulin as ISTD)	225 ± 1 ($n = 6$)	N.D.
RM 163 (Pig-beef fat blend)	ID-MS (13 C-Cholesterol as ISTD)	138.7 ± 0.2 ($n = 3$)	100 to 140 [1]
RM 164 (Anhydrous milk fat)	ID-MS (13C-Cholesterol as ISTD)	275.2 ± 0.8 ($n = 3$)	*275 ± 30 [6]*

The 3rd intercomparison yielded improved but not entirely satisfactory results. A fourth exercise is planned for spring 1990 in which special attention will be paid to the analytical conditions (use of ISTD concentration at the level of sterols to be measured, column resolution etc.). Cholesterol analyses in RM380 (whole milk powder) and RM384 (pork muscle) were repeatable and in agreement with those commonly found in the literature. Meanwhile a certification study, the results can be considered as indicative values.

In the ID-MS study, cholesterol was analysed without derivatization on a medium polar column. Free cholesterol and ISTD co-eluted at 9.30 min. The repeatability of injections was very good (srd $< 0.6\%$). The results were acceptable and sufficiently repeatable in comparison with those from previous BCR ring-tests (Table 6). Nevertheless, due to natural ^{13}C isotopic contribution of cholesterol to the ISTD peak, the calibration curves were not linear. To overcome this drawback deuterium multilabelled cholesterol will be used in the next study.

The certification of the sterol profile of soya-maize oil (RM162), pig beef fat (RM163) and butter fat (RM164) marked with phytosterols, is in progress in different European laboratories and will lead to GC-MS identification of individual sterols present in the chromatogram.

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References

1. Pocklington WD, Wagstaffe PJ (1988) The certification of the fatty acid profile of two oil and fat reference materials. Community Bureau of References report EUR 11002 EN
2. Official Journal of the European Communities, EEC regulations 4056/87 and 4154/87 (22/12/1987)
3. Pocklington WD, Hautfenne A (1986) Pure Appl Chem 58:1419–1428
4. Pocklington WD (1988) Fresenius Z Anal Chem 332:674–678
5. Lognay G, Dreze Ph, Wagstaffe PJ, Marlier M, Severin M (1989) The Analyst (London) 114:1287–1291

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