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DETERMINATION OF BUTYRIC ACID IN FATS CONTAINING BUTTERFAT
Results of a collaborative study and the standardised method

Prepared for publication by

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Determination of butyric acid in fats containing butterfat: results of a collaborative study and the standardised method

Abstract - The development, by collaborative study, of a standardised method for the determination of the butyric acid content of butterfat and fats containing butterfat is described. The procedure involves saponification of the fat, liberation of the butyric acid by phosphoric acid and determination of the free butyric acid by gas-liquid chromatography in the presence of an internal standard.

INTRODUCTION

The butyric acid content of milkfat and butterfat, although subject to a significant natural variation, can be assumed for practical purposes to be constant within certain limits. This fact allows the butyric acid content of a fat to be used as a criterion for the measurement of the amount of butterfat in the fat. The factor to be adopted for calculating the milk fat/butterfat content of a fat from its butyric acid content is the subject of continuing discussion elsewhere, although it is perhaps appropriate to mention here that a factor of 3.6 has been adopted by a number of enforcement authorities in the European Economic Community. This report confines itself to the development of a method for the determination of butyric acid in milk fat, butterfat either alone or blended with other fats and does not consider the validity of the 3.6 factor or other factors for equating butyric acid to butterfat.

Butyric acid, along with the other fatty acids present in butterfat may be determined by the gas-liquid chromatography of its methyl ester (ref. 1). However, where butyric acid alone is specifically required to be determined, a procedure based on the chromatography of the free acid offers considerable advantages, both in time and precision. For this reason it was decided to confine the study to methods for determining butyric acid in isolation from other fatty acids. The development of the present method for butyric acid by Commission VI.3 was designed to prevent duplication of effort by working groups of different international organisations having a common interest in this field.

1st COLLABORATIVE STUDY AND RESULTS

For the first study (1983/84) the working group was invited to follow a procedure based on that proposed by Phillips and Sanders in 1968 (ref. 2). This procedure had been widely used for several years in the UK by enforcement analysts and others, and had been found to give reliable results; furthermore in 1982 a collaborative study of the procedure in which thirty UK laboratories and two from the Netherlands took part established that the precision of the method was satisfactory - in that study samples of butterfat admixed with lard, with cocoa butter and with coconut oil were analysed for their butyric acid content (ref. 3).

For the first (preliminary) Commission VI.3 study two samples were provided viz. a 100 per cent butterfat together with an "optional" sample containing about 10 per cent of butterfat. Participants were invited to report results based on peak heights and optionally, in addition, results based on peak areas. A statistical report of the results submitted is given in Table 1. Included in the participating laboratories were three members of the IDF/FIL E49 working group (ref. 4) and one member of the OICC/AIFC (now OICCC) (ref. 5) working group which were concerned with the determination of milk fat in milk and other dairy products, and in chocolate products, respectively.

It will be seen from Table 1 that not all participants reported results obtained by both measurement of the peak heights and peak areas but comparison of the results shows that there are no significant differences related to the method of peak measurement in respect of the mean values. In view of the possibility of inaccuracies arising from electronic integration of peak areas, especially where base-line resolution of the butyric acid peak from the solvent peak is not achieved, it appears that peak height measurement is to be preferred - especially in view of its relative simplicity. It will be noted, however, that there is some improvement in the reproducibility when peak area measurement is adopted.

TABLE 1. RESULTS FOR BUTYRIC ACID (expressed as per cent m/m of the sample).

Sample	100 per cent butterfat		10 per cent butterfat	
Method of peak measurement	height	area	height	area
Number of laboratories	13	7	7	5
Number of results retained	24	12	12	10
mean value	3.319	3.252	0.354	0.365
standard deviation of repeatability	0.069	0.063	0.015	0.009
standard deviation of reproducibility	0.232	0.161	0.056	0.018
repeatability value r	0.196	0.178	0.044	0.025
reproducibility value R	0.655	0.456	0.159	0.052

Statistical evaluation of the results remaining after elimination of outliers (Table 1) gave values for standard deviations which were interpreted as confirming that the procedure was adequately "rugged" to warrant a more comprehensive study.

No major difficulties with the procedure were experienced by the participants in this first study although some expressed concern about (1) the possible loss of butyric acid during the procedure (i.e. following the acidification by phosphoric acid) and (2) the addition of the internal standard after filtration of the soaps solution.

In view of the successful outcome of the first study a second study was organised for 1984/85. It was agreed that in the second study participants should be invited to analyse the provided samples using both the method studied in 1983/84 and also the Kuzdzal-Savoie procedure (K-S) which had also been published in 1968 (ref. 4) and was in general use by several laboratories in France, since the K-S method appeared to offer certain advantages over the P-S procedure. In the Kuzdzal-Savoie procedure the butyric acid is released from the barium soaps in the presence of the internal standard and this was considered to minimise inaccuracies arising from any loss of the volatile butyric acid. A summary of the main differences between the two procedures is given in Table 2.

TABLE 2. COMPARISON OF THE PHILLIPS & SANDERS AND KUZDZAL-SAVOIE PROCEDURES

	P-S Method	K-S Method
1) Saponification	ethanolic KOH	methanolic $Ba(OH)_2$
2) Sample size	100 mg	200 mg
3) Internal standard addition	after liberation of fatty acids with phosphoric acid	before saponification of sample
4) Calibration curve	0.08 to 2.0 mg butyric acid	0.8 to 20 mg butyric acid

5) Gas-liquid chromatography

The column and operating parameters are the same for both procedures. It should be noted however that the Kuzdzal-Savoie procedure involves the chromatography of more concentrated solutions (about 8-fold) than the Phillips and Sanders method.

Note

The reference standard solutions of butyric acid and the internal standard solutions of valeric acid used are the same for both procedures, but in the case of the Phillips & Sanders procedure the solutions are diluted ten-fold before use.

TABLE 3. RESULTS FOR BUTYRIC ACID (expressed as g butyric acid/100 g sample)

(a) - Using modified Phillips & Sanders procedure

Lab Code	Sample 1	m	Sample 2	m	Sample 3	m	Sample 4	m
01	3.27 3.25	3.26	1.70 1.72	1.71	0.18 0.17	0.18	1.71 1.71	1.71
03	3.41 3.36	3.39	1.84 1.74	1.79	0.18 0.17	0.18	1.73 1.69	1.71
04	3.49 3.12	3.30	1.68 1.74	1.71	0.18 0.18	0.18	1.56 1.58	1.57
06	3.37 3.44	3.41	1.84 1.77	1.81	0.19 0.18	0.19	1.79 1.85	1.82
07	3.22 3.21	3.22	1.75 1.74	1.75	0.14 0.15	0.15	1.67 1.71	1.69
08	3.33 3.32	3.33	1.67 1.68	1.68	0.17 0.16	0.17	1.71 1.72	1.72
09	3.79 3.97	3.88	1.88 2.01	1.95	0.19 0.18	0.19	2.01 2.08	2.05
10	3.76 3.78	3.77	1.72 1.87	1.80	0.23 0.21	0.22	2.13 1.98	2.05
11	3.58 3.78	3.68	1.82 1.91	1.87	0.18 0.19	0.19	2.30 1.80	2.29
13	3.58 3.54	3.56	1.84 1.77	1.81	0.18 0.18	0.18	1.86 1.84	1.85
14	3.18 3.28	3.23	1.71 1.73	1.72	0.22 0.24	0.23	1.68 1.70	1.69

(b) - Using modified Kuzdzal-Savoie procedure

Lab Code	Sample 1	m	Sample 2	m	Sample 3	m	Sample 4	m
01	3.58 3.63	3.61	1.92 1.92	1.92	0.22 0.22	0.22	1.92 1.96	1.94
03	3.51 3.44	3.48	1.84 1.83	1.84	0.06* 0.12*	0.09	1.87 1.90	1.89
04	3.39 3.45	3.42	1.91 1.74	1.83	0.19 0.18	0.19	1.83 1.96	1.90
05	3.22 3.69	3.46	1.62 1.69	1.66	0.11 0.11	0.11	1.90 1.93	1.92
06	3.32 3.54	3.43	1.75 1.71	1.73	0.25 0.25	0.25	1.88 1.89	1.89
07	3.53 3.49	3.51	1.68 1.68	1.68	0.16 0.16	0.16	1.79 1.73	1.76
08	3.43 3.45	3.44	1.73 1.74	1.74	0.22 0.19	0.21	1.78 1.78	1.78
09	3.61 3.51	3.55	1.50 1.71	1.61	0.17 0.18	0.18	1.81 1.80	1.81
11	3.88 3.56	3.62	1.82 1.90	1.86	0.22 0.22	0.22	2.09 1.90	2.00
12	3.41 3.39	3.40	1.80 1.74	1.77	0.05 0.06	0.06>	1.53 1.34	1.44>
13	3.62 3.71	3.65	1.89 1.95	1.92	0.21 0.22	0.22	-	-
14	3.67 3.46	3.57	2.25 2.28	2.27°	0.13 0.11	0.12	0.95+ 1.38+	1.17>

*Cochran outlier; +Cochran straggler; °Dixon straggler; >Dixon outlier; Lab 2 excluded; Lab 5 (P-S) single results reported; Lab 10 no results for K-S; Lab 12 no results for P-S;

2nd COLLABORATIVE STUDY AND RESULTS

For the second study four samples were provided: (1) a sample of 100 per cent butterfat (this was identical to that provided for the first study, although its identity was not revealed to the participants); (2) a blend of refined tallow with tributyrin containing 1.82 per cent of butyric acid; (3) a sample of refined tallow containing 5 per cent m/m of Sample 1; and (4) an optional sample of a commercial blend of cream and vegetable oil which was claimed by the manufacturer to contain about 50 per cent milkfat. Results from this second study are given in Table 3.

The samples provided for this study were designed to test the precision of the procedures over the range of butyric acid equivalent to that found in samples containing from 5% through 50% to 100% butterfat. From the statistical analysis of the results (Table 4) it will be seen that the theoretical amount of butyric acid in sample 2 (1.82%) was accurately determined by both procedures studied, a mean of 1.79% being obtained when using the P-S method and a mean of 1.82% using the K-S method.

On the basis of the mean results obtained for sample 1 (3.46% and 3.52% by the P-S and K-S methods respectively) the calculated levels of butyric acid in sample 3 would be 0.173% and 0.176% (again respectively). These figures compare satisfactorily with the overall mean values found for sample 3 i.e. 0.185% and 0.182% for the two methods, respectively.

The mean result of 3.46 by the P-S method for Sample 1 which, as indicated above, was identical to the sample provided for the first study, also compared satisfactorily with the mean overall value of 3.40% obtained for this sample by nine laboratories, using the same procedure, in the first study.

TABLE 4. STATISTICAL ANALYSIS OF RESULTS FOR BUTYRIC ACID - 2ND STUDY

Method	P-S	K-S	P-S	K-S
Sample No.	1	1	2	2
Number of laboratories	13	13	13	13
Number of accepted results	22	24	22	24
Mean value (per cent m/m)	3.46	3.52	1.79	1.82
Repeatability standard deviation	0.102	0.137	0.076	0.063
Repeatability coefficient of variation	2.95%	3.9%	4.2%	3.4%
Reproducibility standard deviation	0.242	0.141	0.095	0.179
Reproducibility coefficient of variation	7.0%	4.0%	5.3%	9.8%
Repeatability value r (95) ISO 5725	0.29	0.39	0.21	0.18
Reproduciblity value R (95) ISO 5725	0.69	0.40	0.27	0.51

Method	P-S	K-S	P-S	K-S
Sample No.	3	3	4	4
Number of laboratories	13	13	11	11
Number of accepted results	22	20	22	18
Mean value (per cent m/m)	0.185	0.182	1.79	1.87
Repeatability standard deviation	0.008	0.008	0.044	0.058
Repeatability coefficient of variation	4.5%	4.9%	2.4%	3.1%
Reproducibility standard deviation	0.024	0.04	0.155	0.087
Reproducibility coefficient of variation	12.9%	22.6%	8.7%	4.6%
Repeatability value r (95) ISO 5725	0.02	0.025	0.12	0.16
Reproduciblity value R (95) ISO 5725	0.07	0.12	0.44	0.25

It will be noted that for samples 1, 2 and 4 the K-S method returned slightly higher values for the butyric acid content and this could be interpreted as indicating that some loss of butyric acid occurs before the addition of the internal standard when the P-S procedure is followed. However the difference in the overall mean results obtained by the two methods is less than the standard deviation of both methods and therefore on the basis of the results received it was concluded that there was insufficient justification for claiming that either method had a greater precision than the other over the whole range of butyric acid levels likely to be encountered in fats containing butterfat.

CONCLUSIONS

1) The repeatability and reproducibility values determined from a statistical analysis of the results (Table 4) indicate that the determination of butyric acid (as the free acid) by packed-column GLC, can be carried out to an acceptable degree of precision. The mean results for the butyric acid content of a sample containing a known amount of butyric acid indicates that the accuracy of the procedure when analysing fats containing about 5 per cent butterfat is satisfactory for a standard method.

2) It is possible to obtain comparable results using columns with stationary phases other than FFAP or SP-1220 (the two phases cited in the methods studied) although it is recommended that whenever possible one of the two named phases should be used. It was found that the presence of 1% phosphoric acid could improve the resolution of the butyric acid peak from the solvent peak. Details of the columns and the gas-liquid chromatographic conditions under which they were used are given in Table 5.

3) It was found that particular attention must be paid to the conditioning of the column before any quantitative analyses are attempted and that the resolution efficiency of the column could often be improved by injecting aliquots of phosphoric acid solution or by silanisation.

4) In view of the advantages of the P-S method in terms of simplicity, both in the procedure and the apparatus required, together with the shorter time required for the analyses, the majority of participants appeared to favour the P-S method.

5) On the basis of the comparable results obtained by both procedures and taking into account what is stated above regarding the advantages of the P-S method, the Commission decided to adopt the procedure based on the Phillips and Sanders method and the text of the standardised analytical procedure is given on the following pages.

TABLE 5. GAS-LIQUID CHROMATOGRAPHY OPERATING PARAMETERS 1984/85

Lab Code	Column length (m)	Column i.d. (mm)	Phase	%	Temp. °C
01	2	2	FFAP + 1% phosporic acid	10	135
02	4	3	FFAP	10	180
03	2	2	SP1220 + 1% phosphoric acid	15	140
04	2	3	Carbowax 20M TPA acid	5	140
05	2	2.2	DEGS	20	135
06	2	4	FFAP	15	135
07	2	2	FFAP + 1% phosphoric acid	10	135
08	2	4	FFAP	10	135
09	2	4	FFAP	10	135
10	2	?	DEGS + 3% phosphoric acid	10	115
11	2	2	FFAP	5	135
12	2	2	Supelco SP-1000*	10	135
13	1.5	3	SP1000	10	135
15	2	3	Carbowax 20M	5	135

*equivalent to FFAP

2.310 *DETERMINATION OF BUTYRIC ACID*

1. SCOPE AND FIELD OF APPLICATION

This Standard describes a method for the determination of the butyric acid content of milkfat or butterfat or mixtures of fats containing milkfat or butterfat.

2. PRINCIPLE

Saponification of the fat with potassium hydroxide solution followed by acidification with phosphoric acid to liberate the fatty acids. Separation of the water and water soluble fatty acids by filtration. Direct determination of butyric acid by gas-liquid chromatography in the presence of an internal standard.

3. DEFINITION

The butyric acid content of milkfat or butterfat or mixtures of fats containing milkfat and butterfat is the quantity, expressed as a percentage by mass, of butyric acid, determined in this sample by the present method.

4. APPARATUS

4.1 50 ml beaker

4.2 Test tubes, 10 ml, with ground glass stoppers

4.3 2 - 5 ml graduated pipettes

4.4 1 µl microsyringe

4.5 Gas-liquid chromatograph, with flame-ionisation detector, on-column or all glass injection system, and recorder

4.6 Column, glass, approximately 2 m long and 3 mm internal diameter to fit the chromatograph (4.5), filled with 10% - 15% stationary phase suitable for free fatty acid analysis (Note 1) on an 80/100 acid-washed silanised support (Note 2) conditioned and stabilised for use at an analysis temperature of about 130 - 135°C (Note 3)

4.7 Fast filter paper

4.8 Glass beads

4.9 Watch-glass

4.10 Water bath, maintained at boiling temperature.

5. REAGENTS

5.1 Potassium hydroxide solution, approximately 0.5N in ethanol: dissolve 4.5 g potassium hydroxide in 100 ml ethanol.

5.2 o-Phosphoric acid, 5% (m/V) aqueous solution.

5.3 n-Butyric acid, reference standard, 0.4 mg/ml aqueous solution: dissolve 400 mg n-butyric acid in 100 ml of water and dilute 10 ml of this solution to 100 ml (Note 4).

5.4 n-Valeric acid, internal standard, 0.25 mg/ml aqueous solution: dissolve 250 mg n-valeric acid in 100 ml of water and dilute 10 ml of this solution to 100 ml (Note 4).

6. PROCEDURE

6.1 Construction of a calibration curve

By means of graduated pipettes (4.3) transfer to individual test tubes (4.2) 0.2, 0.5, 1.0, 2.0, 3.5, and 5.0 ml butyric acid solution (5.3). To each test-tube add by pipette (4.3) 2.0 ml valeric acid solution (5.4) and, respectively, 4.8, 4.5, 4.0, 3.0, 1.5, and 0 ml water. Stopper the test tubes and gently mix the solutions.

The solutions in the test tubes contain respectively 0.08, 0.2, 0.4, 0.8, 1.4, and 2.0 mg butyric acid and 0.5 mg valeric acid.

Stabilise the column (4.6) for at least 30 minutes at the analysis temperature (Note 3).

By means of a microsyringe (4.4) inject about 1 µl of the prepared standard solutions in turn. Measure the heights of the butyric acid and valeric acid peaks to the nearest 0.5mm. Plot the ratio of these heights (butyric acid/valeric acid) against the corresponding weight of butyric acid (Note 5).

6.2 Determination of the butyric acid content

Weigh accurately about 100 mg of the sample into a beaker (4.1). Add, by pipette (4.3), 3 ml of the ethanolic potassium hydroxide solution (5.1) and a few glass beads (4.8). Cover the beaker with a watch-glass (4.9) and place on a boiling water bath (4.10). Heat for at least 10 minutes or until fat globules are no longer visible on the surface.

Remove the watch-glass and continue heating until the ethanol has completely evaporated.

Allow the beaker to cool.

Add, by pipette (4.3), 5.0 ml of water. Cover with a watch-glass and swirl gently to completely dissolve the soap (Note 6).

Add, by pipette (4.3), 5.0 ml phosphoric acid solution (5.2). Swirl gently to coagulate the precipitated fatty acids. Filter through a small fluted fast filter paper (4.7). By pipette (4.3), transfer 5.0 ml of the filtrate to a test tube (4.2). Add, by pipette (4.3), 2.0 ml of the valeric acid solution (5.3). Stopper and mix.

Stabilise the column (4.6) for at least 30 minutes at the analysis temperature (Notes 3, 7, 8).

By means of a microsyringe (4.4), inject about 1 µl of the final solution onto the column (4.6). Measure to the nearest 0.5 mm the peak heights of butyric acid from the sample and the valeric acid added as an internal standard (Notes 5, 9).

6.3 Number of determinations

Carry out two determinations in rapid succession.

7. CALCULATION AND EXPRESSION OF RESULTS

7.1 Calculate the peak height ratio of butyric acid/valeric acid obtained from the analysis of the sample and read off from the calibration curve the mass of butyric acid equivalent to the peak height ratio.

7.2 The butyric acid content, expressed as a percentage (m/m) of the sample is given by:

$$\frac{m_b \times 200}{m_s}$$

where

m_b is the mass, in mg, of the butyric acid read from the calibration curve,

m_s is the mass, in mg, of the test portion

Report as the final result the mean of the results of the two determinations, provided the requirements for repeatability (7.3) are met. If the requirements for repeatability are not met, discard the results and carry out a further two determinations on the test sample.

7.3 **Repeatability value.** When the mean of the duplicate determinations lies between any two of the mean values shown in the table in the **Appendix** the difference between the results of the two determinations, carried out in rapid succession by the same operator, using the same apparatus for the analysis of identical test material, should not be greater than the value indicated in the table for the **repeatability value (r)** which corresponds to the higher of the two mean values.

7.4 **Reproducibility value.** When the means of the duplicate determinations, obtained in two different laboratories using this standard method for the analysis of identical test material, lie between any two of the mean values shown in the table in the **Appendix**, the difference between the mean results obtained by those laboratories should not be greater than the **reproducibility value (R)** which corresponds to the higher of the two mean values.

8. NOTES

1. FFAP and SP-1220 with 1% phosphoric acid are suitable.

2. Chromosorb W is suitable.

3. The column should be conditioned at about 180°C for at least 48 hours; If on-column injection is not used the injection port should be set at not less than 175°C. If base-line separation of the butyric acid and valeric acid peaks is not obtained it may be found that the resolution of columns containing a phosphoric acid stationary phase can be improved by the injection of 1 µl quantities of 2.5% (m/V) phosphoric acid solution onto the column while it is heated at the analysis temperature of about 130°C to 135°C. The carrier-gas flow rate should be adjusted so that the butyric acid has a retention time of about 5 minutes.

If tailing of peaks is experienced, even after conditioning of the column, this can sometimes be reduced or eliminated by injecting 2 µl of trimethyl-chlorosilane (TMCS) onto the column.

4. The solutions of valeric acid and butyric acid must be freshly prepared.

5. The amplifier attenuation should be adjusted so that the height of the butyric acid peak for the highest butyric acid standard (6.1) is about 80% full-scale recorder reading.

6. It may be found necessary to warm the mixture gently to achieve complete solution of the soaps.

7. The syringe should be rinsed thoroughly with water between every two analyses and at the completion of analyses should be rinsed with a diluted soap solution in order to minimise any corrosion due to phosphoric acid.

8. After a series of sample injections it is recommended that injections of one or more of the solutions of butyric acid/valeric acid standards (6.1) be made and the calibration curve checked against the corresponding butyric acid/valeric acid peak height ratios obtained from the standard solutions.

9. Peaks for caproic acid and caprylic acid may appear after valeric acid on the chromatogram and interfere with subsequent analyses if care is not taken to ensure that these acids have eluted before another sample solution is injected.

APPENDIX

STATISTICAL ANALYSIS OF RESULTS FOR BUTYRIC ACID

Level*	A	B	C	D
Number of laboratories	13	7	11	13
Number of accepted results	22	14	22	22
Mean value (per cent m/m)	0.185	0.354	1.79	3.46
Repeatability standard deviation	0.008	0.015	0.044	0.102
Repeatability coefficient of variation	4.5%	4.4%	2.4%	2.95%
Reproducibility standard deviation	0.024	0.056	0.155	0.242
Reproducibility coefficient of variation	12.9%	15.9%	8.7%	7.0%
Repeatability value r (95) ISO 5725	0.02	0.04	0.12	0.29
Reproducibility value R (95) ISO 5725	0.07	0.16	0.44	0.69

*Levels A, B, C and D represent the levels of butyric acid to be found in fats containing about 5, 10, 50 and 100 per cent m/m butterfat respectively.

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The Community Bureau of Reference is currently preparing a standard material (RM 164) of anhydrous milk fat (butterfat) in which the level of the butyric acid will be certified, and the IUPAC Oils, Fats and Derivatives Commission gratefully acknowledges the provision of this reference material for analysis in both collaborative studies.

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