COMMISSION REGULATION (EC) No 86/94

of 19 January 1994

establishing a reference method for the determination of sitosterol and stigmasterol in butter

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Regulation (EEC) No 804/68 of 27 June 1968 on the common organization of the market in milk and milk products [1], as last amended by Regulation (EEC) No 2071/92 [2], and in particular Article 6 (7) thereof,

Whereas butter may be traced and the traced products have to be controlled according to Articles 3 and 6 of Commission Regulation (EEC) No 570/88 [3], as last amended by Regulation (EC) No 3049/93 [4];

Whereas strict compliance with the conditions relating to the tracing of butter is essential in order to prevent the risk of unauthorized use of subsidized butter;

Whereas taking account of the importance of tracing in the proper functioning of this scheme it is necessary to establish common methods for detection of all tracers required by this scheme applicable in the same way throughout the Community; this would in particular ensure equal treatment of all operators having access to this scheme and eliminate unequal conditions of competition which may result at present, as a consequence of different national methods of analysis;

Whereas a reference method for the determination of stigmasterol and sitosterol in butteroil has been established by Commission Regulation (EEC) No 3942/92 [5];

whereas this method can be applied in a slightly modified form for the determination of sterol tracers in butter;

Whereas the accuracy of the method and the tolerance limits had to be determined;

Whereas the measures provided for in this Regulation are in accordance with the opinion of the Management Committee for Milk and Milk Products,

HAS ADOPTED THIS REGULATION:

Article 1

The reference method of analysis to determine the stigmasterol or β-sitosterol content of butter according to Article 6 of Regulation (EEC) No 570/88 is specified in the Annex.

Butter has been traced correctly if the results obtained are in accordance with the requirements specified in paragraph 8 of this Annex.

Article 2

This Regulation shall enter into force on the third day following its publication in the *Official Journal of the European Communities.*

It shall apply from 1 April 1994.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 19 January 1994.

For the Commission

René STEICHEN

Member of the Commission

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[1] OJ No L 148, 28. 6. 1968, p. 13.
[2] OJ No L 215, 30. 7. 1992, p. 64.
[3] OJ No L 55, 1. 3. 1988, p. 31.
[4] OJ No L 273, 5. 11. 1993, p. 7.
[5] OJ No L 399, 31. 12. 1992, p. 29.

ANNEX

DETERMINATION OF SITOSTEROL OR STIGMASTEROL IN BUTTER BY CAPILLARY-COLUMN GAS CHROMATOGRAPHY

1. SCOPE AND FIELD OF APPLICATION

The method describes a procedure for the quantitative determination of sitosterol or stigmasterol in butter. Sitosterol is taken to be the sum of B-sitosterol and 22 dihydro B sitosterol, other sitosterols are assumed to be insignificant. It is applicable to samples received under Regulation (EEC) No 570/88.

2. PRINCIPLE

The butter is saponified with potassium hydroxide in ethanolic solution and the unsaponifiables are extracted with diethyl ether.

The sterols are transformed into trimethyl-silyl ethers and are analysed by capillary-column gas chromatography with reference to an internal standard betulin.

3. APPARATUS

3.1. 150 ml saponification flask fitted with a reflux condenser having ground-glass joints.

3.2. 500 ml separating funnels.

3.3. 250 ml flasks.

3.4. Pressure equalizing funnels, 250 ml or similar, to collect waste diethyl ether.

3.5. Glass column, 350 mm × 20 mm fitted with sintered glass plug.

3.6. Waterbath or isomantle.

3.7. Reaction vials, 2 ml.

3.8. Gas chromatograph suitable for use with a capillary column, provided with a splitting system consisting of:

3.8.1. a thermostatic chamber for columns capable of maintaining the desired temperature with an accuracy of ± 1 °C;

3.8.2. a temperature-adjustable vaporization unit;

3.8.3. a flame ionization detector and converter amplifier;

3.8.4. an integrator-recorder suitable for use with the converter-amplifier (3.8.3).

3.9. A fused-silica capillary column entirely coated with BP1 or equivalent in a uniform thickness 0,25 µm; the column must be capable of resolving trimethyl silyl derivatives of lanosterol and sitosterol, a BP1, length 12 m, internal diameter 0,2 mm is suitable.

3.10. A 1 µl gas chromatography microsyringe with hardened needle.

4. REAGENTS

All reagents must be of recognized analytical grade. The water used must be distilled water or water of at least equivalent purity.

4.1. Ethanol, at least 95 % pure.

4.2. Potassium hydroxide, 60 % solution, dissolve 600 g potassium hydroxide (minimum 85 %) in water and make up to one litre with water.

4.3. Betulin of at least 99 % purity.

4.3.1. Internal standard solutions. Betulin in diethyl ether (4.4).

4.3.1.1. The concentration of betulin solution used for sitosterol determination should be 1,0 mg/ml.

4.3.1.2. The concentration of betulin solution used for stigmasterol determination should be 0,4 mg/ml.

4.4. Diethyl ether, analytical purity (free from peroxides or residue).

4.5. Sodium sulphate, anhydrous, granular previously dried at 102 °C for two hours.

4.6. Silylating reagent, for example TRI-SIL (available from Pierce Chemical Co, Cat No 49001) or equivalent. (WARNING : TRI-SIL is flammable toxic, corrosive and a possible carcinogen. Laboratory personnel must be familiar with TRI-SIL safety data and take the appropriate precautions.)

4.7. Lanosterol.

4.8. Sitosterol, of known purity not less than 90 % pure (P).

Note 1 : The purity of standard materials used for calibration must be determined using the method of normalization. Assume that all sterols present in the sample are represented on the chromatogram the total area of the peaks represents 100 % of the sterol constituents and that the sterols give the same detector response. Linearity of the system must be validated over the concentration ranges of interest.

4.8.1. Sitosterol standard solution — prepare a solution containing, to the nearest 0,001 mg/ml, approximately 0,5 mg/ml (W_1) sitosterol (4.8) in diethyl ether 4.4).

4.9. Stigmasterol, of known purity not less than 90 % pure (P).

4.9.1. Stigmasterol standard solution — prepare a solution containing, to the nearest 0,001 mg/m, approximately 0,2 mg/ml (W_1) stigmasterol (4.9) in diethyl ether (4.4).

4.10. Resolution test mixture. Prepare a solution containing 0,05 mg/ml lanosterol (4.7) and 0,5 mg/ml sitosterol (4.8) in diethyl ether (4.4).

5. PROCEDURE

5.1. Preparation of standard solutions for chromatography. The internal standard solution (4.3.1) must be added to the appropriate sterol standard solution at the same time as it is added to the saponified sample (see 5.2.2).

5.1.1. Sitosterol standard chromatographic solution ; transfer 1 ml of sitosterol standard solution (4.8.1) to each of two reaction vials (3.7) and remove the diethyl ether with a stream of nitrogen. Add 1 ml of internal standard solution (4.3.1.1) and remove the diethyl ether with a stream of nitrogen.

5.1.2. Stigmasterol standard chromatographic solution ; transfer 1 ml of stigmasterol standard solution (4.9.1) to each of two reaction vials (3.7) and remove the diethyl ether with a stream of nitrogen. Add 1 ml of internal standard solution (4.3.1.2) and remove the diethyl ether with a stream of nitrogen.

5.2. Preparation of the unsaponifiables.

5.2.1. Melt the butter sample at a temperature not exceeding 35 °C, mix the sample thoroughly by stirring, weigh, to the nearest 1 mg, approximately 1 g of butter (W_2) into a 150 ml flask (3.1). Add 50 ml ethanol (4.1) and 10 ml potassium hydroxide solution (4.2). Fit the reflux condenser and heat at approximately 75 °C for 30 minutes. Detach the condenser and cool the flask to approximately ambient temperature.

5.2.2. Add 1,0 ml of internal standard solution (4.3.1.1) to the flask if sitosterol is to be determined, or (4.3.1.2) if stigmasterol is to be determined. Mix thoroughly. Transfer the contents of the flask quantitatively into a 500 ml separating funnel (3.2) washing the flask in turn with 50 ml water and 250 ml diethyl ether (4.4). Shake the separating funnel vigorously for two minutes and allow the phases to separate. Run of the lower aqueous layer and wash the ether layer by shaking with four successive 100 ml aliquots of water.

Note 2 : To avoid formation of an emulsion, it is essential that the first two water washes are carried out gently (10 inversions). The third wash can be shaken vigorously for 30 seconds. If an emulsion is formed it can be destroyed by the addition of 5-10 ml of ethanol. If ethanol is added it is essential to carry out a further two vigorous water washes.

5.2.3. Pass the clear, soap free ether layer through a glass column (3.5) containing 30 g anhydrous sodium sulphate (4.5). Collect the ether in a 250 ml flask (3.3). Add one anti-bumping granual and evaporate to near dryness in a water or isomantle, taking care to collect the waste solvents.

Note 3: If sample extracts are taken to complete dryness at too high a temperature sterol losses may occur.

5.3. Preparation of trimethyl silyl ethers.

5.3.1. Transfer the ether solution remaining in the flask to a 2 ml reaction vial (3.7) with 2 ml diethyl ether and remove the ether using a stream of nitrogen. Wash the flask with two further 2 ml aliquots of diethyl ether, transferring to the vial and removing the enter with nitrogen each time.

5.3.2. Silylate the sample by addition of 1 ml TRI-SIL (4.6). Close the vial and shake vigorously to dissolve. If dissolution is incomplete, warm to 65-70 °C. Allow to stand for at least five minutes before injecting into the gas-chromatograph. Silylate standards in the same way as samples. Silylate the resolution test mixture (4.10) in the same way as samples.

Note 4: Silylation must be affected in a water free environment. Incomplete silylation of betulin is indicated by a second peak close to that of betulin.

The presence of ethanol at the silylation stage will interfere with silylation. This may result from inadequate washing at the extraction stage. If this problem persists a fifth wash, shaken vigorously for 30 seconds, may be introduced at the extraction stage.

5.4. Gas-Chromatographic analysis.

5.4.1. Choice of operating conditions.

Set up the gas-chromatograph according to the manufacturers instructions.

The guideline operating conditions are as follows:

— column temperature 265 °C

— injector temperature 280 °C

— detector temperature 300 °C

— carrier gas flow rate 0,6 ml/minute

— hydrogen pressure 84 kPa

— air pressure 155 kPa

— sample split 10 : 1 to 50 : 1, the split ratio must be optimized in accordance with the manufacturers instructions and linearity of detector response then validated over the concentration range of interest.

Note 5: It is especially important that the injection liner is regularly cleared.

— amount of substance injected 1 µl of TMSE solution.

Allow the system to equilibrate and obtain a satisfactory stable response before commencing any analysis.

These conditions must be varied in the light of column and gas-chromatograph characteristics so as to obtain chromatograms which meet the following requirements:

— the sitosterol peak must be adequately resolved from lanosterol. Figure 1 shows a typical chromatogram which should be obtained from a silylated resolution test mixture (4.10),

— the relative retention times of the following sterols should be approximately:

Cholesterol	1,0
Stigmasterol	1,3
Sitosterol	1,5
Betulin	2,5,

— the retention time for betulin should be approximately 24 minutes.

5.4.2. Analytical procedure.

Inject µl of silylated solution (stigmasterol or sitosterol) and adjust the integrator calibration parameters.

Inject a further 1 µl of silylated standard soluton to determine the response factors with reference to betulin.

Inject 1 µl of silylated sample solution and measure peak areas. Each chromatographic run must be bracketed by an injection of standards. As a guide, six injections of sample should be included in each bracketed run.

Note 6: Integration of the stigmasterol peak should include any tailing as defined by points 1, 2 and 3 in Figure 2 b.

Integration of the sitosterol peak should include the area of the 22 dihydro B sitosterol (stigmastanol) peak which elutes immediately after sitosterol, see Figure 3b, when evaluating total sitosterol.

6. CALCULATION OF RESULTS

6.1. Determine the area of the sterol peaks and betulin peaks in both standards brackerting a batch, and calculate R_1 :

$$R_1 = \frac{\text{Average peak area of sterol in standard}}{\text{Average peak area of betulin in standard}}$$

Determine the area of the sterol peak (stigmasterol of sitosterol) and betulin peak in the sample and calculate R_2 :

$$R_2 = \frac{\text{Peak area of sterol in sample}}{\text{Peak area of betulin in sample}}$$

W_1 = sterol content of the standard (mg) contained in 1 ml of standard solution (4.8.1 or 4.9.1).

W_2 = weight of sample (g) (5.2.1)

P = purity of standard sterol (4.8 or 4.9)

$$\text{Sterol content of the sample (mg/kg)} = \frac{R_2}{R_1} \times \frac{W_1}{W_2} \times P \times 10.$$

7. ACCURACY OF THE METHOD

7.1. Repeatability.

7.1.1. Stigmasterol.

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material shall not exceed 19,3 mg/kg.

7.1.2. Sitosterol

The difference between the results of two determinations carried out within the shortest feasible time interval, by one operator using the same apparatus on identical test material shall not exceed 23,0 mg/kg.

7.2. Reproducibility.

7.2.1. Stigmasterol.

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material shall not exceed 31,9 mg/kg.

7.2.2. Sitosterol.

The difference between the results of two determinations carried out by operators in different laboratories, using different apparatus on identical test material shall not exceed 8,7 % relative of the mean of the determination.

7.3. Source of precision data.

The precision data were determined from an experiment conducted in 1992 involving eight laboratories and six samples (three blind duplicates) for stigmasterol ; six samples (three blind duplicates) for sitosterol.

8. TOLERANCE LIMITS

8.1. Three samples must be taken from the traced product in order to check on homogeneity.

8.2. Stigmasterol

8.2.1. The incorporation for stigmasterol is 150 grams of at least 95 % pure stigmasterol per tonne of butter, i.e. 142,5 mg/kg ; or 170 grams of at least 85 % pure stigmasterol per tonne of butter, i.e. 144,5 mg/kg.

8.2.2. Taking the Critical Difference for a 95 % probability level (CrD_{95}) into consideration, the mean of the three results shall not be less than

in the case of incorporation of 95 % pure stigmasterol, 126,0 mg/kg,

in the case of incorporation of 85 % pure stigmasterol, 128,0 mg/kg.

8.2.3. In addition to the criteria given above, the lowest result obtained from analysis of the product is used to check homogeneity of tracer distribution. This is done by a comparison with the following limits :

— 116,4 mg/kg (95 % of the minimum incorporation rate for 95 % pure stigmasterol, single sample CrD_{95} taken into consideration)

— 118,3 mg/kg (95 % of the minimum incorporation rate for 85 % pure stigmasterol, single sample CrD_{95} taken into consideration)

— 95,1 mg/kg (80 % of the minimum incorporation rate for 95 % pure stigmasterol, single sample CrD_{95} taken into consideration)

— 96,7 mg/kg (80 % of the minimum incorporation rate for 85 % pure stigmasterol, single sample CrD_{95} taken into consideration).

The tracer concentration in the sample giving the lowest result is used in conjunction with interpolation respectively between 116,4 mg/kg and 95,1 mg/kg or 118,3 mg/kg and 96,7 mg/kg.

8.3. Sitosterol

8.3.1. The incorporation rate for sitrosterol is 600 grams of at least 90 % pure sitosterol per tonne of butter, i.e. 540 mg/kg.

8.3.2. Taking the CrD_{95} into consideration the mean of the three results shall not be less than 514,4 mg/kg.

8.3.3. In addition to the criterion given above, the lowest result obtained from analysis of the product is used to check homogeneity of tracer distribution. This is done by comparison with the following limits.

— 485,1 mg/kg (95 % of the minimum incorporation rate for 90 % pure sitosterol, single sample CrD_{95} taken into consideration)

— 404,1 mg/kg (80 % of the minimum incorporation rate for 90 % pure sitosterol, single sample CrD_{95} taken into consideration).

The tracer concentration in the sample giving the lowest result is used in conjunction with interpolation between 485,1 mg/kg and 404,1 mg/kg.

Figure 1 Chromatogram of resolution test mixture.

Complete resolution is preferable, i.e. the peak trace for lanosterol should return to baseline before leaving for the sitosterol peak although incomplete resolution is tolerable.

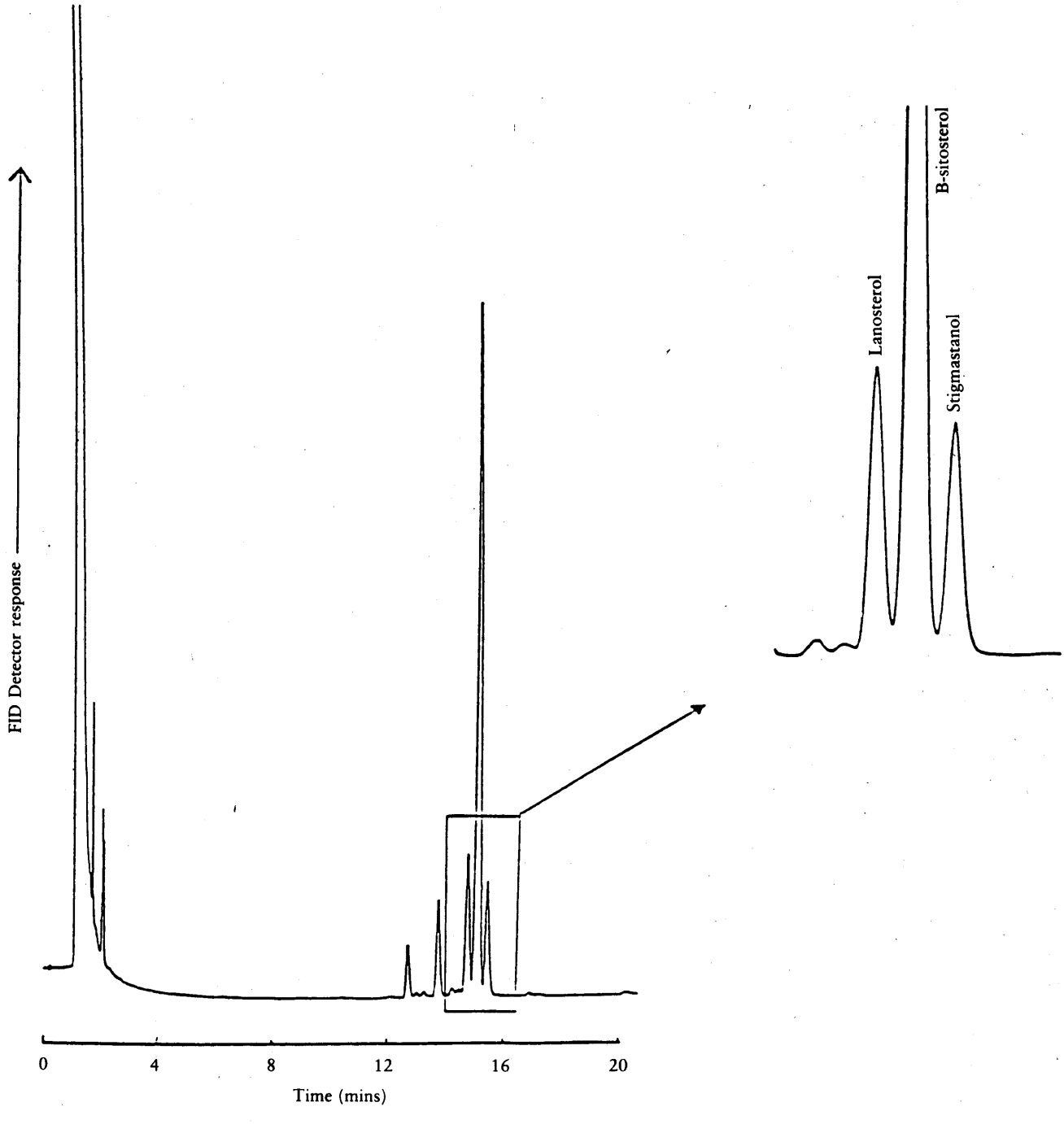


Figure 2a
Stigmasterol standard

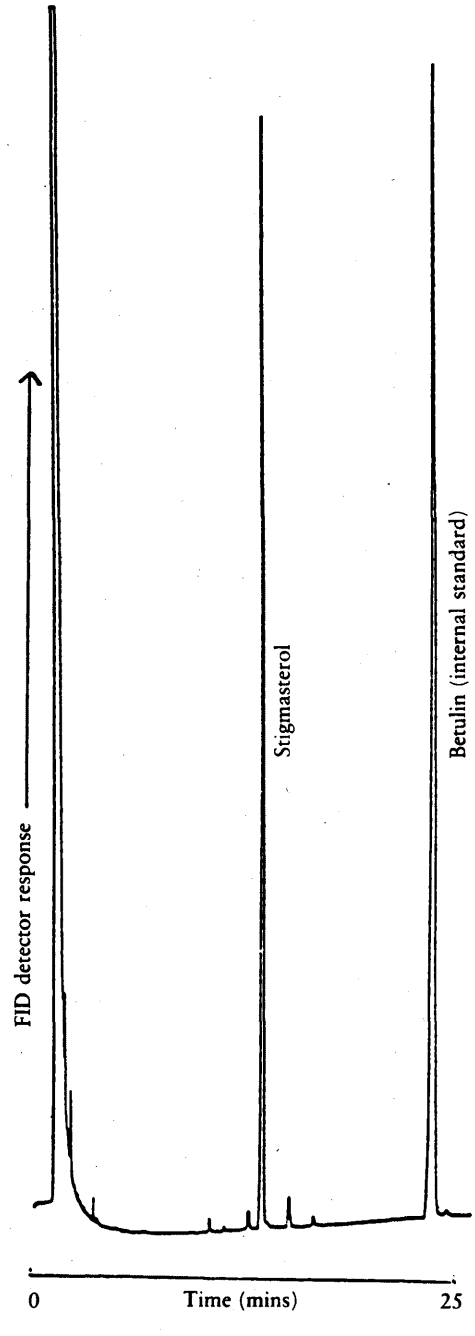
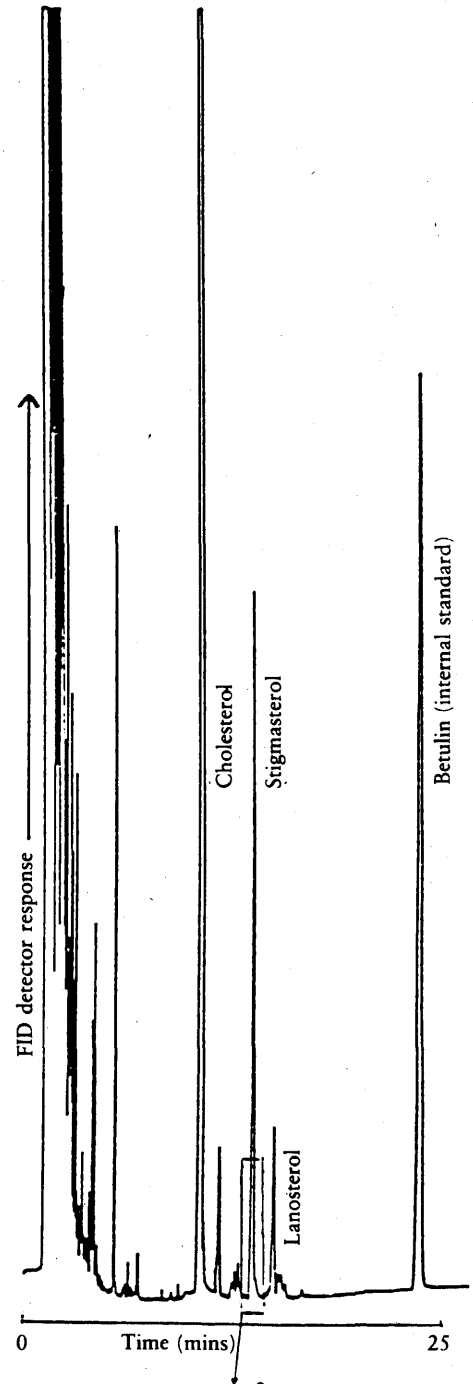
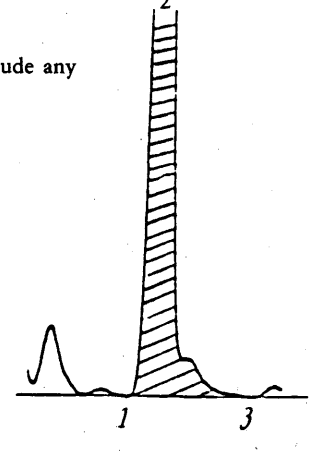


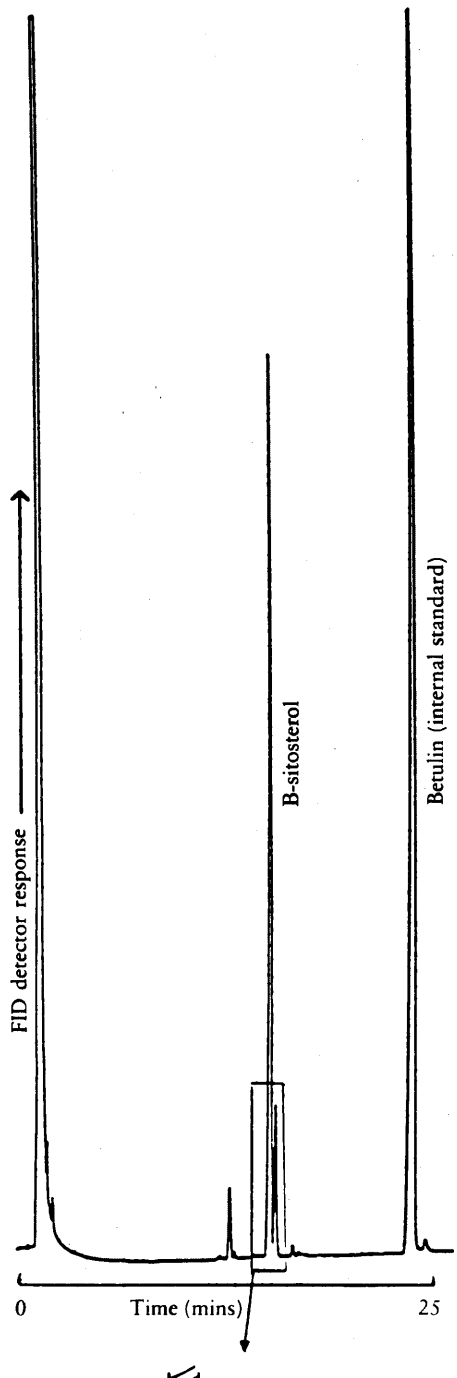
Figure 2b
Butter sample denatured with stigmasterol





Note: Integration of the stigmasterol peak should include any tailing as defined by points 1, 2 and 3.

Figure 3a
Sitosterol standard



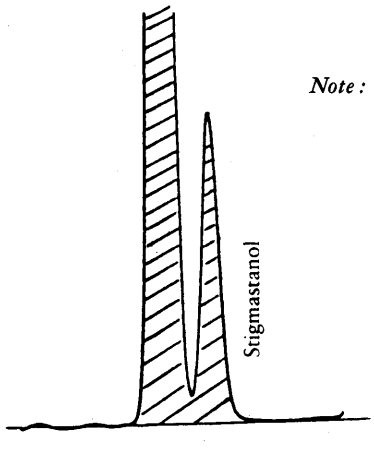
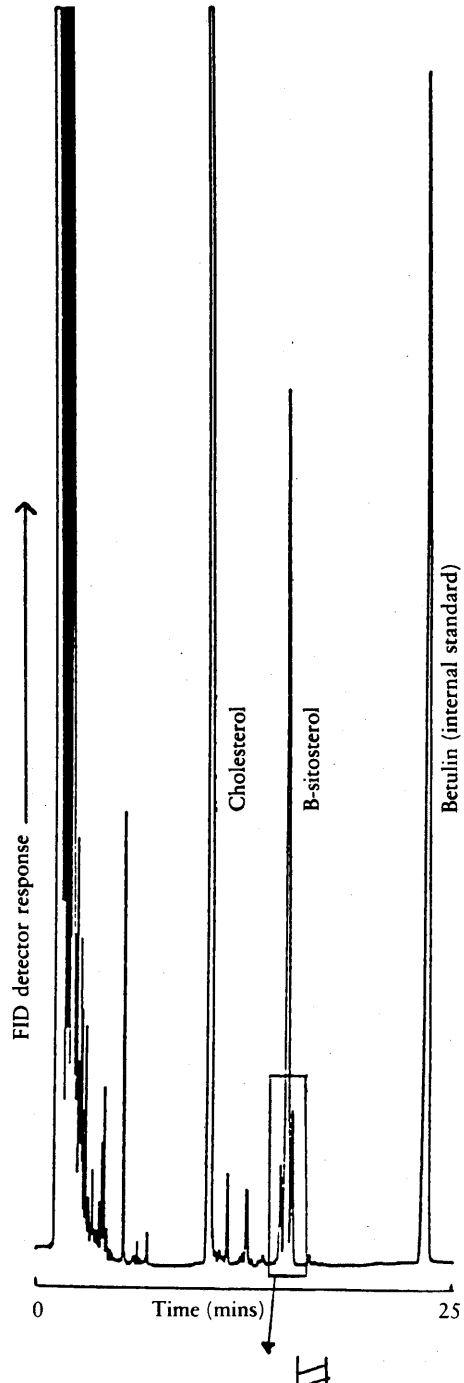
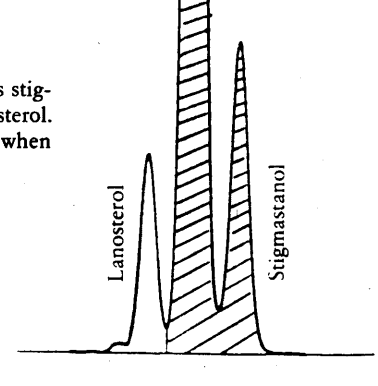


Figure 3b
Butter sample
denatured with β-Sitosterol





Note: B-sitosterol often contains an impurity (identified as stigmastanol) which elutes immediately after B-sitosterol. The areas of these two peaks should be summed when evaluating the total B-sitosterol present.