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COMMISSION REGULATION (EEC) No 3942/92
of 22 December 1992
establishing a reference method for the determination of sitosterol and stigmasterol in butteroil

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►**M1**	Commission Regulation (EEC) No 2539/93 of 15 September 1993	L 233	1	16.9.1993
►**M2**	Commission Regulation (EC) No 175/1999 of 26 January 1999	L 20	22	27.1.1999

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THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic 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 thereof,

Whereas butteroil has to be traced and the traced butteroil has to be controlled according to Commission Regulation (EEC) No 3143/85 ([3]) as last amended by Regulation (EEC) No 1264/92 ([4]), and in particular Articles 5 and 6 thereof;

Whereas butteroil may be traced and the traced products have to be controlled according to Commission Regulation (EEC) No 570/88 ([5]) as last amended by Regulation (EEC) No 124/92 ([6]), and in particular Articles 3 and 6 thereof;

Whereas butteroil has to be traced and the traced butteroil has to be controlled according to Commission Regulation (EEC) No 429/90 ([7]), as last amended by Regulation (EEC) No 1264/92, and in particular Articles 10 and 11 thereof;

Whereas strict compliance with the conditions relating to the tracing of butteroil 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 these schemes it is necessary to establish common methods for detection of all tracers required by these schemes applicable in the same way throughout the Community; this would in particular ensure equal treatment of all operators having access to these schemes and eliminate unequal conidtions of competition which may result at present, as a consequence of different national methods of analysis.

Whereas it is difficult to establish such reference methods for all tracers simultaneously; whereas establishing a reference method for the determination of stigmasterol and sitosterol in butteroil constitutes a first step in this direction;

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:

([1]) OJ No L 148, 28. 6. 1968, p. 13.
([2]) OJ No L 215, 30. 7. 1992, p. 64.
([3]) OJ No L 298, 12. 11. 1985, p. 9.
([4]) OJ No L 135, 19. 5. 1992, p. 5.
([5]) OJ No L 55, 1. 3. 1988, p. 31.
([6]) OJ No L 14, 21. 1. 1992, p. 28.
([7]) OJ No L 45, 21. 2. 1990, p. 8.

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Article 1

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The reference method of analysis to determine the stigmasterol content of butteroil according to Article 6 of Regulation (EEC) No 3143/85, Article 6 of ►M2 Regulation (EC) No 2571/97 ◄ or Article 11 of Regulation (EEC) No 429/90 and the β-sitosterol content of butteroil according to Article 6 of ►M2 Regulation (EC) No 2571/97 ◄, is specified in the Annex.

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Butteroil 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 February 1993.

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

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ANNEX

DETERMINATION OF SITOSTEROL OR STIGMASTEROL IN BUTTEROIL 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 butteroil. 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 Regulations (EEC) No 3143/85, (EEC) No 570/88 and (EEC) No 429/90.

2. PRINCIPLE

The butteroil 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 euqalising 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 vaporisation 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 2 °C for two hours.

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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 normalisation. 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 Stigmasterl 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 stigmaasterol 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 Weigh, to the nearest 1 mg, approximately 1 g of butteroil (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 alow 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 an 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

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nitrogen. Wash the flask with two further 2 ml aliquots of diethyl ether, transferring to the vial and removing the ether 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 five 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 optimised 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 may 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 1 µl of silylated standard solution (stigmasterol or sitosterol) and adjust the integrator calibration parameters.

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

Inject 1 µl of silylated sample solution and meaure peak areas. Each chromatographic run must e 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 2b.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.

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6. CALCULATION OF RESULTS

6.1 Determine the area of the sterol peaks and betulin peaks in both standards bracketing 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 10,2 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 3,6 % relative of the mean of the determinations.

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 25,3 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 8,9 % relative of the mean of the determinations.

7.3 Source of precision data.

The precision data were determined from an experiment conducted in 1991 involving nine laboratories and six samples (three blind duplicates) for stigmasterol; six samples (three blind duplicates) for sitosterol.

8. TOLERANCE LIMITS

▼M2 8.1. Three samples must be taken from the traced product in order to check on the correct tracing of the product.

▼B 8.2 Stigmasterol

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

▼M2 8.2.2. The results of the three samples obtained from the analysis of the product is used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (Critical Difference for a 95 % probability level (CrD95) taken into consideration):

— 120,0 mg/kg (95 % of the minimum incorporation rate for 95 % pure stigmasterol),

— 122,0 mg/kg (95 % of the minimum incorporation rate for 85 % pure stigmasterol),

— 84,0 mg/kg (70 % of the minimum incorporation rate for 95 % pure stigmasterol),

— 86,0 mg/kg (70 % of the minimum incorporation rate for 85 % pure stigmasterol).

The tracer concentration of the sample giving the lowest result is used in conjunction with interpolation respectively between 120,0 mg/kg and 84,0 mg/kg or 122,0 mg/kg and 86,0 mg/kg.

▼B 8.3 Sitosterol

8.3.1 The incorporation rate for sitosterol is 600 grams of at least 90 % pure sitosterol per tone of butteroil, ie 540 mg/kg.

▼M2 8.3.2. The results of the three samples obtained from the analysis of the product is used to check the rate and the homogeneity of tracer incorporation and the lowest of these results is compared with the following limits (Critical Difference for a 95 % probability level (CrD95) taken into consideration):

— 486,0 mg/kg (95 % of the minimum incorporation rate for 90 % pure sitosterol),

— 358,0 mg/kg (70 % of the minimum incorporation rate for 90 % pure sitosterol).

The tracer concentration in the sample giving the lowest result is used in conjunction with interpolation between 486,0 mg/kg and 358,0 mg/kg.

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Figure 1 Chromatogram of resolution test mixture.

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

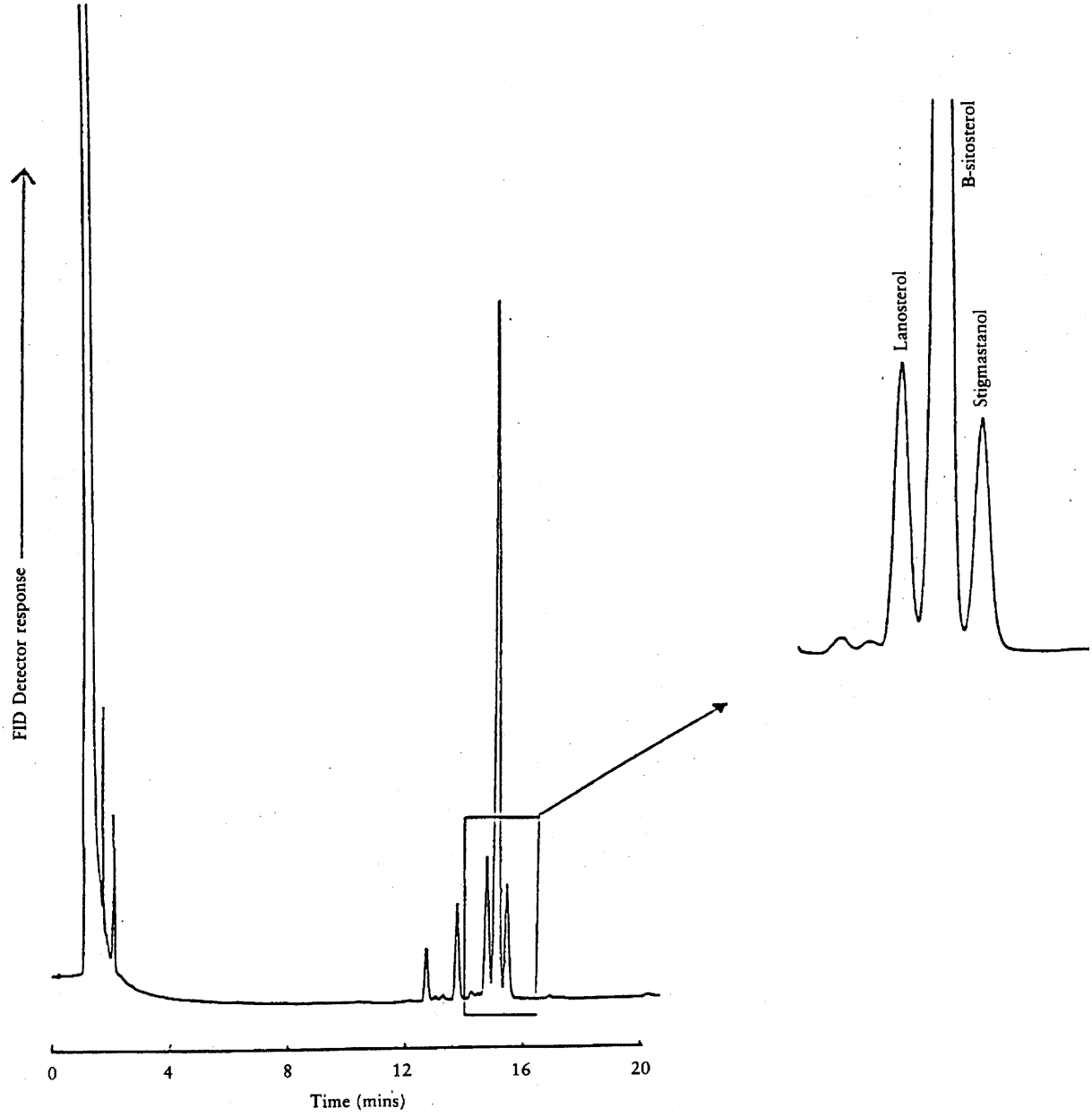

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Figure 2a

Stigmasterol standard

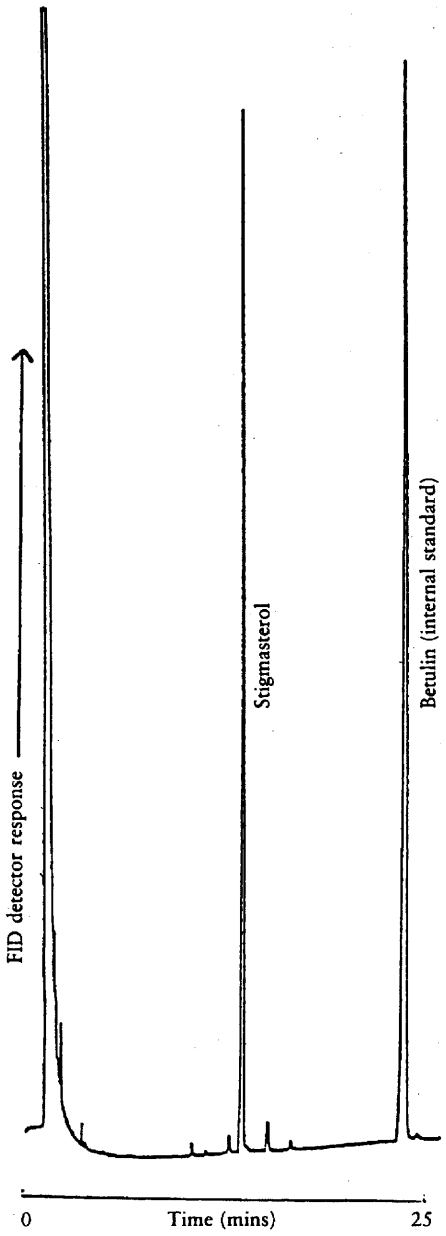

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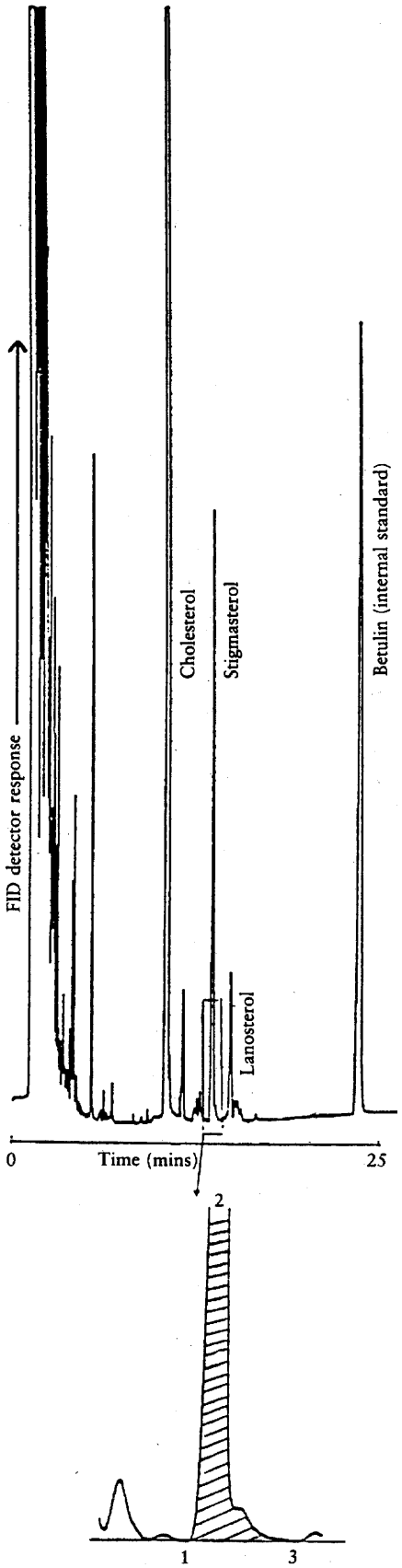
Figure 2b

Butteroil sample denatured with stigmasterol



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

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Figure 3a

Sitosterol standard

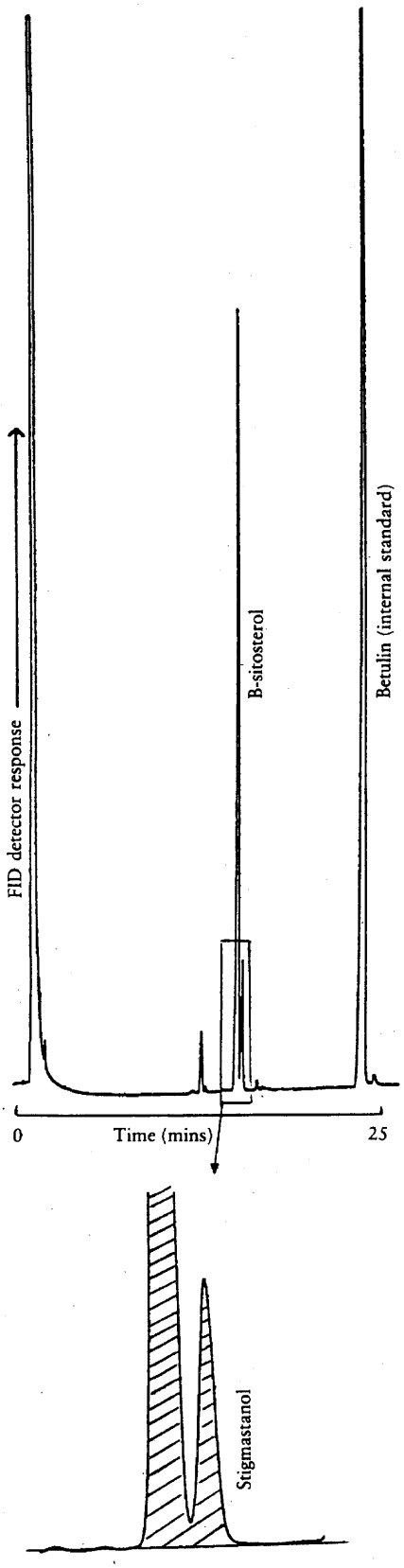

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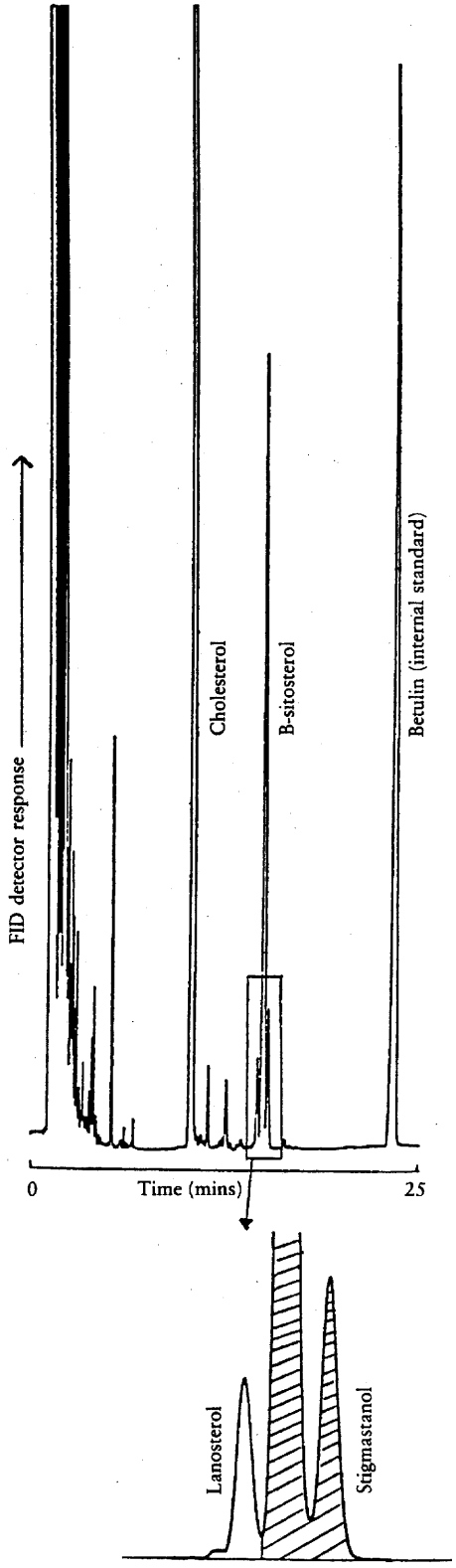
Figure 3b

Butteroil sample denatured with B-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.