INTERNATIONAL STANDARD ISO 3594

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION •МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ •ORGANISATION INTERNATIONALE DE NORMALISATION

Milk fat — Detection of vegetable fat by gas-liquid chromatography of sterols (Reference method)

Matières grasses du lait — Détection des matières grasses végétales par chromatographie en phase gazeuse des stérols (Méthode de référence)

First edition — 1976-12-15

UDC 637.127.1 : 543.544.45

Ref. No. ISO 3594-1976 (E)

Descriptors : dairy products, milk, chemical analysis, determination of content, fats, gas chromatographic method analysis.

Price based on 5 pages

FOREWORD

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO Member Bodies). The work of developing International Standards is carried out through ISO Technical Committees. Every Member Body interested in a subject for which a Technical Committee has been set up has the right to be represented on that Committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work.

Draft International Standards adopted by the Technical Committees are circulated to the Member Bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 3594 was drawn up by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the Member Bodies in August 1974.

It has been approved by the Member Bodies of the following countries :

Australia
Austria
Belgium
Chile
Czechoslovakia
France
Germany
Hungary
India
Iran
Ireland
Israel
Netherlands
New Zealand
Poland
South Africa, Rep. of
Spain
Sweden
Turkey
United Kingdom
U.S.S.R.

No Member Body expressed disapproval of the document.

NOTE – This International Standard has been developed jointly with the IDF (International Dairy Federation) and the AOAC (Association of Official Analytical Chemists, U.S.A.) on the basis of an IDF Standard for the purpose of being included in the FAO/WHO Code of Principles concerning Milk and Milk Products and Associated Standards.

The text as approved by the above organizations has also been published by the IDF (IDF Standard No. 54A) and by the AOAC (Official Methods of Analysis).

Printed in Switzerland

Milk fat — Detection of vegetable fat by gas-liquid chromatography of sterols (Reference method)

1 SCOPE AND FIELD OF APPLICATION

This International Standard specifies a reference method for the detection, by gas-liquid chromatography (GLC), of the presence in milk fat of vegetable fat containing β-sitosterol. The limit of detection depends upon the β-sitosterol content of the added vegetable fat.

2 REFERENCES

ISO/R 707, *Milk and milk products — Sampling.*

ISO 3595, *Milk fat — Detection of vegetable fat by the phytosteryl acetate test.*

3 PRINCIPLE

Preparation of sterol digitonides as described in ISO 3595 and dissolution in a mixture of formamide and dimethylformamide. Extraction of the liberated sterols with pentane. Separation of the sterols by gas-liquid chromatography.

If, on the chromatogram, a peak with the retention time of β-sitosterol is obtained, the presence of vegetable fat in the fat sample under investigation is demonstrated. Peaks of other phytosterols may support this conclusion.

4 REAGENTS AND MATERIALS

All reagents shall be of analytical quality.

4.1 Formamide and dimethylformamide, mixture, in equal volumes.

4.2 *n*-Pentane.

4.3 Column packing : 2 to 4 % loading of a methyl silicone gum rubber, stable up to at least 300 °C, on a flux-calcined diatomaceous earth, acid washed and silanized, mesh size 80/100 (175 to 150 μm) or 100/120 (150 to 125 μm).

4.4 Sensitivity test solution : 1 mg of milk fat sterols in 1 ml of *n*-pentane, freshly prepared from milk fat (see 7.2).

4.5 Peak resolution test solution : 0,9 mg of rape seed oil sterols and 0,1 mg of milk fat sterols in 1 ml of *n*-pentane, each sterol being freshly prepared from rape seed oil and milk fat respectively (see 7.2).

4.6 Reference test solution : 1 mg of soyabean oil sterols in 1 ml of *n*-pentane, freshly prepared from soyabean oil (see 7.2).

4.7 Carrier gas : nitrogen.

4.8 Hydrogen.

4.9 Oxygen or air.

5 APPARATUS

Usual laboratory equipment and

5.1 Gas chromatograph, fitted with hydrogen flame ionization detector, silver or glass injection system, or direct-on-column injection device, and recorder.

5.2 Gas chromatographic column, glass, U-shaped or coiled, length 100 to 200 cm, inside diameter 2 to 4 mm.

NOTE — Stainless steel should not be used as some types cause false results by deterioration of sterols.

5.3 Micro-syringe, capable of delivering a volume of up to 5 or 10 μl.

6 SAMPLING

See ISO/R 707.

7 PROCEDURE

7.1 Preparation of test sample

See ISO 3595.

7.2 Preparation of sterols

Dissolve about 10 mg of sterol digitonide, prepared as described in ISO 3595, in 0,5 ml of the formamide and dimethylformamide mixture (4.1) in a small test tube, if necessary with gentle heating. Add 2,5 ml of *n*-pentane (4.2) to the cooled solution, stopper the tube and shake. Let the layers separate and use the clear upper pentane layer, containing the liberated sterols, for gas chromatographic analysis. This solution contains about 1 mg of sterol per millilitre.

7.3 Gas-liquid chromatographic conditions

Column temperature : 220 to 250 °C.

Temperature of injection system, if it can be separately heated : 20 to 40 °C above column temperature.

Nitrogen flow rate : 30 to 60 ml/min.

Disconnect the detector and operate new columns under these conditions for 16 to 24 h, so that equilibrium is reached. Connect the detector, ignite the flame and regulate the hydrogen and oxygen or air flow rates so as to obtain appropriate flame height and detector sensitivity. Start the recorder at a suitable chart speed and adjust the zero setting and attenuator. If the base line is steady, the apparatus is ready for use.

7.4 Sensitivity test

Inject 3 to 5 µl of the sensitivity test solution (4.4). Only one distinct peak of cholesterol will appear on the gas chromatogram. Adjust the attenuator so as to obtain approximately full-scale deflection on the recorder (see figure 1).

7.5 Peak resolution test

Inject 3 to 5 µl of the peak resolution test solution (4.5). Peaks of cholesterol, brassicasterol, campesterol and β-sitosterol will appear on the gas chromatogram (see figure 2). Measure the retention distances (distance from sample injection to maximum peak height) of the peaks, d_{CH} for cholesterol, d_B for brassicasterol, d_C for campesterol, and d_s for β-sitosterol, and the peak base widths (retention dimension between intersections of base line with tangents to the points of inflection on the front and rear sides of the peak), w_{CH} for cholesterol and w_B for brassicasterol. The peak resolution, $PR = 2\,(d_B - d_{CH})/(w_B + w_{CH})$, shall be at least 1.

NOTE — To facilitate the measurement of the base widths, extend the longest straight portion of each side of the peak until it intersects the base line; the base width will be the distance between the points of intersection corresponding to each of the two sides.

Calculate the relative retention times (cholesterol = 1,00) for brassicasterol, campesterol, and β-sitosterol.

7.6 Reference test

Inject 3 to 5 µl of the reference test solution (4.6). Peaks of campesterol, stigmasterol, and β-sitosterol will appear on the gas chromatogram (see figure 3). Measure the retention distances of the peaks, d_C for campesterol, d_{ST} for stigmasterol, and d_S for β-sitosterol.

Calculate the relative retention times, which are approximately as follows :

cholesterol	1,00 (about 15 min)
brassicasterol	1,13 to 1,15
campesterol	1,32 to 1,34
stigmasterol	1,44 to 1,46
β-sitosterol	1,66 to 1,68

7.7 Analysis

Switch the attenuator to an attenuation factor four times (usually two steps) lower and inject the same volume of the sterol solution (7.2) as used in 7.4. Record the gas chromatogram.

8 EXPRESSION OF RESULTS

If, on the gas chromatogram, a peak with the relative retention time of β-sitosterol and a height of at least 2 % of the full scale is observed, the presence of β-sitosterol is indicated and the laboratory sample under investigation, from which the sterols have been isolated, is considered to contain vegetable fat.

The presence on the gas chromatogram of peaks of other phytosterols such as campesterol or stigmasterol may support the conclusion.

9 SENSITIVITY

The presence of β-sitosterol contents as low as 0,5 % can be detected by the method described in this International Standard. The limit of detection of vegetable fat in milk fat cannot be given since this depends on the β-sitosterol content of the fat used for admixture, i.e. upon the nature of the fat or mixture of fats added to the milk fat.

10 TEST REPORT

The test report shall mention the method used and the results obtained. It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, as well as any circumstances that may have influenced the results.

The report shall include all details required for the complete identification of the sample and shall be accompanied by the recorded gas chromatogram.

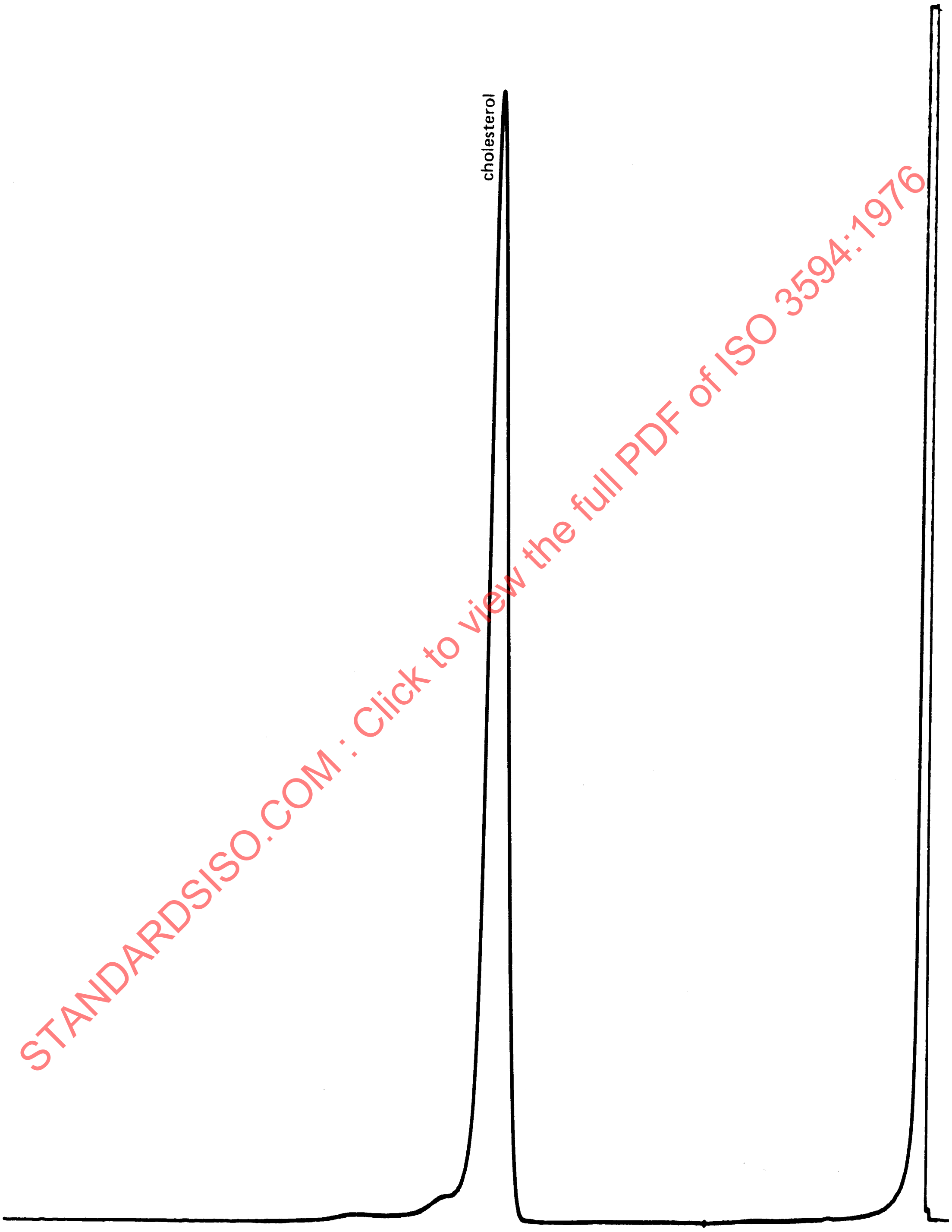

FIGURE 1 — **GLC of milk fat sterols**

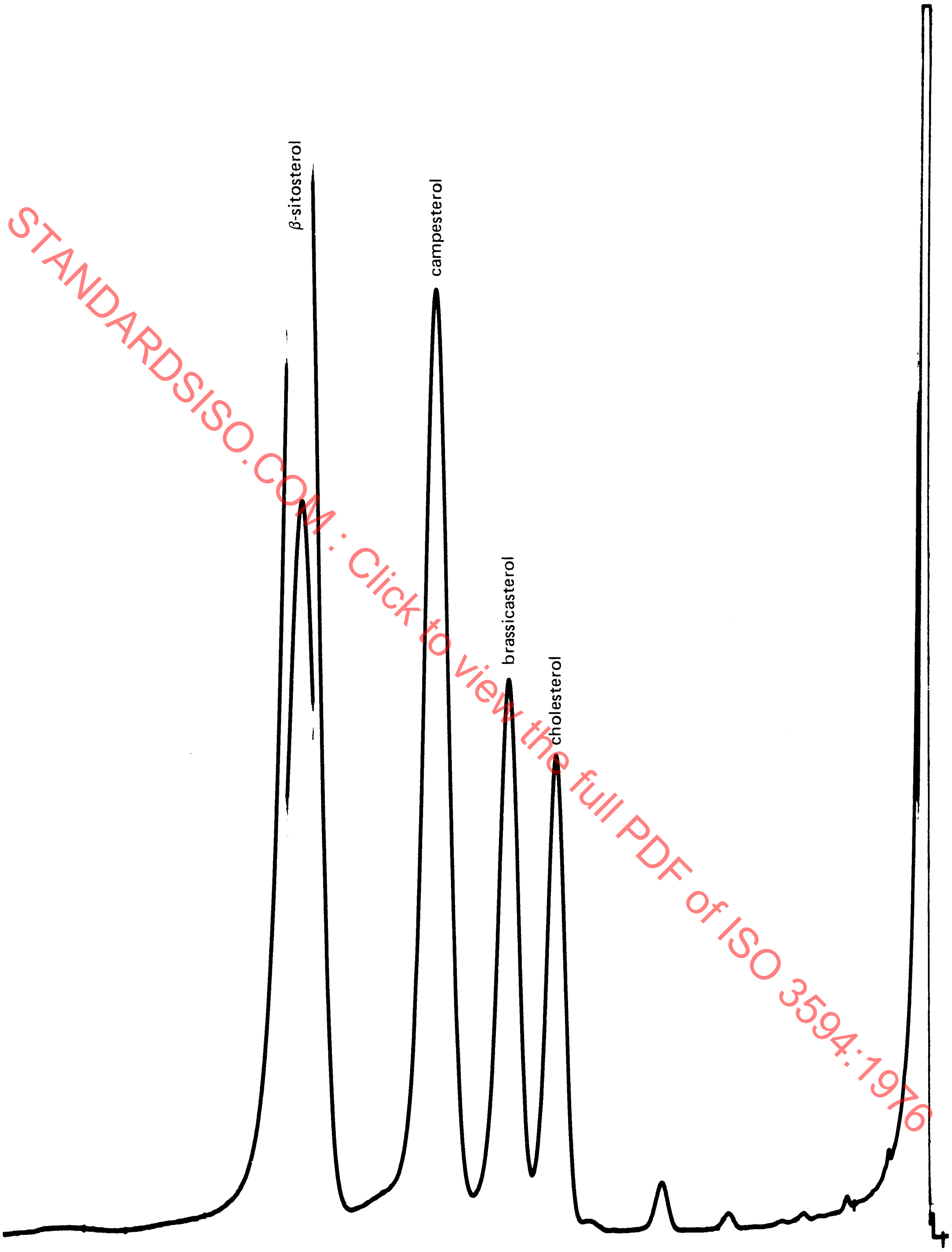

FIGURE 2 – **GLC of rape seed oil sterols and milk fat sterols**