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Standard Method for the

DETERMINATION OF TOTAL STEROLS IN FATS AND OILS

(including results of a collaborative study)

Prepared for publication by

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Standard method for the determination of total sterols in fats and oils (including results of a collaborative study)

Abstract - An enzymatic method for the determination of total sterols in fats and oils is described. The method has been standardized following a collaborative study the results of which are given and discussed.

From an analytical point of view, sterols are one of the most important groups among the various components of the unsaponifiable fraction of animal and vegetable oils. Their relative composition (sterolic profile) is easily determined after extraction of the unsaponifiable matter by a combination of TLC and GLC, according to the IUPAC standard method 2.403.

As shown by experience, the sterols composition of a given kind of fat is practically independent of the variety, and consequently much more constant that the fatty acids composition. Consequently the sterolic profile is very often used for the identification of the components of a fatty blend. If a quantitative estimation of the components is needed, a quantitative determination of the total sterols (or of each individual sterol) is necessary. This is possible via the precipitation of insoluble digitonides (ref.1) but the method is rather obsolete and a more modern method has been searched for.

INTRODUCTION

The quantification of the qualitative GLC used for sterolic profile, was first considered. This quantification requires recourse to an internal standard. On account of the difficulty of determining the concise relative response factor of each individual sterol with respect to the internal standard, whatever this might be, the method soon appeared to be insufficiently repeatable and above all to be lacking in reproducibility, to be used. Nevertheless such a method seems to be valuable for the direct determination of one particular sterol only - cholesterol for example.

Another possibility considered is the enzymatic oxidation of sterols by cholesterol oxidase (Note a), a method widely used for the determination of cholesterol level in blood.

Cholesterol oxidase is not specific for cholesterol, but promotes the oxidation of the hydroxyl in 3 when β oriented. Consequently phytosterols can be oxidized easily (ref.2). Contrarily cholesterol oxidase does not react significatively with 4,4'-methylsterols. It rapidly appeared that the repeatability of the method was satisfactory and its reproducibility acceptable.

A collaborative study has been consequently organized and its results statistically interpreted.

PRINCIPLE OF THE METHOD

Cholesterol oxidase oxidizes sterols quantitatively to sterones with quantitative formation of hydrogen peroxide.

A simultaneous oxidation, with catalase (Note b), of methanol by the formed hydrogen peroxide, leads to the quantitative formation of formaldehyde.

Finally, formaldehyde reacts with acetyl acetone, giving quantitatively 3,5 diacetyl-1,4-dihydrolutidine, which is spectrometrically determined at 410 nm.

Note a : Cholesterol oxygen oxidoreductase (EC 1.1.3.6)
Note b : Hydrogen peroxide oxidoreductase (EC 1.11.1.6)

After saponification of the fat or oil to be analysed and decomposition of the soaps thus obtained, the analysis is directly performed without any further fractionation.

From the absorbance of the final solution a simple calculation leads to the number of micromoles of sterols per 100 g of fat or oil. For practical purposes it is easy to express instead the final results in µmol; in milligrams of cholesterol for animal fats and oils; or β-sitosterol for vegetable fats and oils.

If the percentage composition of the sterol fraction is known, the calculation of the content of each sterol present in that fraction is possible.

COLLABORATIVE STUDY

The enzymatic procedure was collaboratively studied.

For the collaborative study, four oils containing various amounts (unknown) of sterols were analysed by 16 laboratories. Participants were required to apply the method without modification. A training sample of known content was also supplied to each participant but the result obtained for this sample was not required.

Sample 1 was an olive oil (low sterol content)
Sample 2 was a corn oil, refined, bleached and deodorized (high sterol content)
Samples 3 and 4 were identical and were a fully refined soyabean oil (medium sterol content).

Each laboratory reported the results of two independent determinations (TABLE 1).

TABLE 1. Enzymatic determination of total sterols

Lab.	Sample 1			Sample 2			Sample 3			Sample 4		
			Mean			Mean			Mean			Mean
1	115	117	116	660	667	663	253	258	255	185	184	184
2	119	117	118	643	641	642	248	246	247	183	184	183
3	146	150	148	554	560	557	220	224	222	218	224	221
4	155	158	157	733	721	727	264	271	267	271	285	278
5	145	144	144	762	776	769	252	254	253	268	265	266
6	123	124	123	543	522	532	189	183	186	251	228	239
7	159	150	155	717	708	712	260	267	263	261	256	258
8	125	127	126	610	600	605	225	230	227	207	218	212
9	128	122	125	620	621	620	209	212	210	215	210	212
10	158	150	154	880	892	886	233	239	236	345	339	342
11	161	137	149	498	570	534	230	201	215	205	226	215
12	83	98	90	562	598	580	221	212	217	198	219	208
13	148	148	148	760	755	757	260	255	257	258	260	259
14	155	155	155	864	856	860	276	285	280	277	277	277
15	139	125	132	726	724	725	257	251	254	274	231	253
16	170	164	167	742	784	763	257	271	264	267	280	273
Average value			138			683			240			242

At first sight, it appeared that the repeatability was good but reproducibility less satisfactory. In order to confirm (or otherwise) this preliminary observation, a statistical evaluation of results was made according to the ISO Standard n° 5725 (ref.3). The use of Cochran's test led to the elimination only of laboratory n° 11.

The statistical evaluation of the 15 remaining laboratories is given in TABLE 2.

As previously stated, the intralaboratory repeatability is good (coefficient of variation lower than 5%). The interlaboratory reproducibility is only acceptable (coefficient of variation around 15%); this last result is probably due to the fact that most of the participants were not familiar enough with the method.

TABLE 2. Statistical evaluation of results

	Sample n°			
	1	2	3	4
. Number of laboratories	15	15	15	15
. Number of results	30	30	30	30
. Mean	137	693	243	245
Repeatability				
. Standard deviation S_r	4,7	12,0	4,9	10,7
. Coefficient of variation v_r	3,5%	1,7%	2,0%	4,4%
. Repeatability r (95%) (2.85 S_r)	13	34	14	30
Reproducibility				
. Standard deviation S_R	21	106	26	43
. Coefficient of variation v_R	15%	15%	11%	17%
. Reproducibility R (95%) (2.85 S_R)	59	300	73	121

On the basis of the above reported results and of the conclusion of the statistical analysis, the Commission decided to adopt the method. The full text of the method is given below.

STANDARDIZED METHOD (n° 2.404)

1. Scope

This Standard describes a method for the determination of the whole of the sterols, either free or esterified in fats and oils.

2. Field of application

This Standard is applicable to animal or vegetable fats and oils, crude or purified, pure or in mixture.

3. Definition

The total sterols content is the quantity of sterols determined by the present method and expressed arbitrarily - in milligrams per 100 g - either as cholesterol for animal fats or as β-sitosterol for vegetable fats (Note 1).

4. Principle

Saponification of the fat or oil to be analysed and decomposition of the obtained soaps. Enzymatic oxidation of the sterols with cholesterol oxidase to sterones, with quantitative formation of hydrogen peroxide (Note 2). Simultaneous enzymatic oxidation, with catalase, of methanol by the formed hydrogen peroxide with quantitative formation of formaldehyde. Reaction of formaldehyde with acetylacetone giving quantitatively lutidine which is spectrometrically determined.

5. Apparatus

5.1. Test tubes, diameter 18 mm.
5.2. Filter funnels.
5.3. Filter paper, fast for filter funnels (5.2).
5.4. 25 - 50 and 100 ml volumetric flasks.
5.5. 0.1 - 0.5 and 1 ml pipettes.
5.6. 5 ml graduated pipettes.
5.7. Saponification flasks and vertical condenser or any apparatus convenient for the saponification of fats and oils.
5.8. Tubes for haemolysis, glass, tightly stoppered.
5.9. Spectrophotometer (allowing, if possible, a measurement in optical density to the nearest 0.001).
5.10 1 cm cells for spectrophotometer (5.9).
5.11 Thermostatic water bath which can be regulated near 40°C.
5.12 Refrigerator regulated near 4°C.
5.13. Glass beads.

6. Reagents

6.1. 2-Propanol (isopropyl alcohol).

6.2. Acetone.

6.3. 2,4-Pentanedione (acetylacetone).

6.4. Cholesterol oxygen oxidoreductase (EC 1.1.3.6) of Nocardia Erythropolis containing 15 U per ml (cholesterol oxidase) (Note 3).

6.5. Hydrogen peroxide oxidoreductase (EC 1.11.1.6) from beef liver (catalase-suspension 1) (Note 3).

6.6. Water obtained by distillation or deionized in an all-glass apparatus.

6.7. Hydrochloric acid 8 N.
Mix 60 ml concentrated pure HCl (d: 1.18) and 30 ml of water (6.6).

6.8. Potassium hydroxide, methanolic solution 0.5 N.
Dissolve 2.8 g of potassium hydroxide in a small quantity of methanol by heating. Cool. Dilute with methanol to 100 ml.

6.9. Solution 1
In a 100 ml volumetric flask (5.4), to 50 ml of the buffer solution (6.12) add 19.1 ml of acetone and a quantity of catalase (6.5) corresponding to 230 000 units. Adjust with the buffer solution.

6.10 Solution 2
In a 50 ml volumetric flask (5.4), to 25 ml of water (6.6) add 0.26 ml of 2,4-pentane -dione (6.3) and 1.10 ml of acetone (6.2). Make up with water (6.6).

6.11 Solution 3
Immediately before use mix 3 volumes of solution 1 (6.9) with 2 volumes of solution 2 (6.10) (Note 4).

6.12 Ammonium phosphate buffer solution adjusted to pH 7.

7. Procedure

7.1. Saponification. Weigh accurately, to within 1 mg, 1 to 2 g of the oil or fat into the saponification flask (5.7) (Note 5). Add 10 ml of the methanolic potassium hydroxide (6.8) and some glass beads (5.13) (Note 6). Heat and reflux for 25 min.

After slight cooling, transfer the still warm contents of the flask to a 25 ml volumetric flask (5.4) with the aid of a few ml of isopropyl alcohol (6.1). Rinse the flask with isopropyl alcohol (6.1). Add 1 ml of hydrochloric acid (6.7). Make up to volume with isopropyl alcohol (6.1) (Note 5). Shake. This solution is known as the test solution. Place in the refrigerator (5.12) at 4°C for approximately 20 min.

Filter as rapidly as possible using filter paper (5.3). The filtrate is immediately used for the enzymatic determination.

7.2. Determination of sterols. Transfer 5 ml of solution 3 (6.11) and 0.4 ml of the filtrate to a test tube (5.1). Mix carefully. Transfer to a haemolysis tube (5.8) 2.5 ml of this mixture and add 0.02 ml of catalase - suspension 1 (6.5). Mix carefully. The remainder of the contents of the test tube is also transferred to a second haemolysis tube (5.8) and constitutes the blank solution. Stopper tightly the blank and the sample tubes and incubate for 60 min in the thermostatic bath (5.11) regulated between 37° and 40°C.

Allow to cool to ambiant temperature.

Transfer to the spectrophotometer cells (5.10). By means of the spectrophotometer (5.9), measure the difference of absorbance at 405 nm between the sample and the blank.

8. Expression of results

The total sterols content (in mg per 100 g) expressed arbitrarily either as cholesterol for animal fats or as β-sitosterol for vegetable fats (Note 1) is given by the formula :

$$\frac{C \times V \times M \times 100}{m}$$

where:

C is the concentration - in mmoles of sterols per ml - of the solution submitted to the spectrophotometric measurement, given by the simplified formula :

$$C = 1.824 \times 10^{-3} \times \Delta E$$

where ΔE is the difference of absorbance at 405 nm between the sample and the blank in 1 cm cells (Note 7).

m is the mass in g of the test portion.

M is the molecular mass of the sterol in which the result is arbitrarily expressed

$$M_{\text{cholesterol}} = 386.64 \quad ; \quad M_{\beta\text{-sitosterol}} = 414.69.$$

V is the total volume in ml of the test solution (Note 8).

9. Notes

1. From the percentage composition of the sterol fraction, it is possible to calculate the content of each of the sterol present in that fraction, using the formula given for calculation of the results. In this case it is necessary to take into account the appropriate molecular weight of each sterol.

2. The activity of cholesterol oxidase is not absolutely specific for cholesterol. In fact this enzyme promotes the oxidation of the hydroxyl in 3, when it is β oriented.

3. These solutions can be supplied - ready for use - by Boehringer in Mannheim. The order must refer to the kit for "Determination of cholesterol in food chemistry - test combination for 25 determinations ref. 139050".

4. Solutions supplied ready to use by Boehringer are stable for a year if they are kept at 4°C. Solution 3 which has to be prepared is stable for 3 months if kept at 4°C in amber bottles and if it has been prepared under sterile conditions.

5. To obtain a convenient spectrophotometric measurement, the concentration of sterols in the reaction solution (sample solution) must be between 0.02 and 0.4 g/l. It is necessary to take this in consideration either when weighing the test portion, or during the dilution with isopropyl alcohol.

6. It is generally easier, in case of liquid oils, to work with small quantities of oils, as, after saponification and then acidification, liberated fatty acids are not completely solidified in the cold and can cause difficulties during the determination, by formation of turbidity when the methanolic solution is added.

   In the case of highly coloured fats like crude palm oils, the addition of approximately 5% (by weight of the fat) of activated carbon during saponification enables most of the colour to be eliminated.

   In the case of margarines, the addition of anhydrous sodium sulphate enables a total saponification to be obtained by fixing water.

7. The concentration in sterols of the submitted to the spectrophotometric measurement test solution, expressed in mmoles/ml, is given by the general formula :

$$C = \frac{V_t}{\varepsilon \times d \times V_f} \times \Delta E$$

where :

d is the optical path lenght in cm of the cell,

V_f is the volume in ml of the filtrate of the test solution used for the determination of sterols,

V_t is the volume in ml of liquid in the test tube used for the determination of sterols,

ε is the extinction coefficient at 405 nm of lutidine (7 400 ml/mmole.cm),

ΔE is the difference of absorbance at 405 nm between the sample and the blank.

According to the present procedure d = 1 cm; V_f = 0.4 ml; V_t = 5.4 ml and the formula can be simplified to

$$C = 1.824 \times 10^{-3} \times \Delta E.$$

8. According to the present procedure this volume is generally equal to 25 ml. If the test solution has been diluted F times in order to obtain a convenient spectrophotometer reading, the value of V is equal to 25 ml multiplied by the dilution factor F.

REFERENCES

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2. J. Rietsch and B. Entressangles, Revue française des Corps gras 27, 185 (1980).
3. ISO International Standard n° 5725 (1981).

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Errata

Volume, Issue and Year	Page no. and location	Correction
56, 8 (1984)	1102 Solvent: Molten Salts Column 1, item 6	for $CuCl_2/KCl$ read $CaCl_2/KCl$
57, 6 (1985)	903 Section 6.4 line 1	for cholesterol oxygen oxidoreductase read cholesterol oxidase suspension
	903 Section 6.5 line 1	for (catalase-suspension 1) read (catalase-suspension)
	903 Section 7.2 line 3	for catalase-suspension 1 (6.5) read cholesterol oxidase suspension (6.4)