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DETERMINATION OF ERYTHRODIOL IN VEGETABLE OILS
Results of a collaborative study and the standardised method

Prepared for publication by

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Determination of erythrodiol in vegetable oils: results of a collaborative study and the standardised method

Abstract - The development, by collaborative study, of a standardised method for the determination of the erythrodiol content of vegetable oils is described. The procedure involves the isolation of the unsaponifiable matter in the oil, its purification by column chromatography and fractionation by thin-layer chomatography. The silanised fraction containing the erythrodiol is then analysed by gas-liquid chromatography and quantitative assessment made by reference to beta-sitosterol or by use of an internal standard (betulin).

INTRODUCTION

Erythrodiol, a triterpenoid glycol (also known as homo-olestranol) was first identified in olive-residue oil by Gracian and Martel (1); it constitutes one of the characteristic components of the unsaponifiable fraction of that oil. Erythrodiol is also found in olive oil, although in significantly smaller quantities, thereby providing a criterion for distinguishing between these two oils. It has also been identified in grapeseed oil and can therefore be used a an identifying characteristic of this oil.

No method of analysis for the determination of the erythrodiol in these oils has so far received the approval of any international organisation. Consequently, when the Committee on Fats and Oils of the Codex Alimentarius Commission considered the question of the desirability of introducing erythrodiol content as a specific characteristic or identity criterion for these oils, it put forward a proposal to the IUPAC Commission on Oils, Fats and Derivatives for the development of a procedure for the determination of erythrodiol which could be adopted as an international standard method.

COLLABORATIVE STUDY AND RESULTS

The basis for the development of the method was the procedure adopted by Ghimeti et al (2), which involves gas-liquid chromatography of the erythrodiol, previously extracted from the oil. This procedure was in fact used as the basis for the relevant official Italian standard (3).

The method of analysis adopted (as a result of the earlier studies) consists of six stages: 1) extraction of the unsaponifiable matter; 2) purification of this extract by treatment with ion-exchange resin; 3) fractionation of the unsaponifiable matter by thin-layer chromatography; 4) recovery of the sterol fraction and triterpenoid glycols; 5) silanisation of the recovered fractions and analysis of their silanised solution by gas-liquid chromatography; 6) quantitative evaluation of the chromatograms.

For stage 1) diethyl ether was chosen in preference to light petroleum to ensure that the sterols and triterpenoid glycols are completely extracted. In stage 2) it is essential to remove any fatty acids and/or soap residues that may have been entrained during extraction since the presence of these results in less defined bands during the fractionation by thin-layer chromatography. The use of an alumina column (as in the official Italian method) was found to be unsatisfactory for standardisation purposes; a double-layer ion-exchange resin was found to be much more reliable. In stage 3) a hexane-diethyl ether-formic acid mixture was chosen for development of the thin-layer plates as this provided the optimum conditions for the separation of the 4-methylsterols from the beta-sitosterol. This separation is critical since the quantity of 4-methylsterols varies considerably from one olive oil to another. In stage 5) it was found that delta 7-avenasterol may overlap with erythrodiol when a packed column is used for the analyses (these two components can readily be separated by capillary columns) but since delta 7-avenasterol is only present in minute quantities in olive oil and olive-residue oil it does not normally have any significant effect on the quantitative results.

Three samples of oil were selected for the collaborative study: a refined grapseed oil, a mixture of refined olive-residue oil and refined olive oil, and a refined olive-residue oil. These three samples can be considered as being representative of a low, average, and high erythrodiol content, respectively.

Table 1 gives the results received from the eight participating laboratories. Unfortunately only four of the participants complied strictly with the study protocol, one of the remaining four submitted only one result for samples 1 and 3 and it was not possible to include all of the the results of the remaining three participants due to various reasons. As a result any conclusions drawn from the statistical evaluation must be viewed as being subject to a relatively wide margin of error.

TABLE 1. RESULTS FOR ERYTHRODIOL CONTENT

Lab Code*	Sample 1 refined olive residue oil				Sample 2 refined olive residue oil + refined olive oil**				Sample 3 refined grapeseed oil			
	% relative to β-sitosterol		mg/100 g oil		% relative to β-sitosterol		mg/100g oil		% relative to β-sitosterol		mg/100g oil	
1a	48.6		48		27.6		28		7.5		14	
b	46.7	47.8	48	47.6	27.3	27.7	27	27.7	7.5	7.4	15	14.3
c	48.0		47		28.3		28		7.3		14	
1a	47.2		51		27.9		28		7.3		14	
b	48.0	47.3	49	49.3	25.9	26.4	27	27.0	7.1	7.2	13	13.7
c	46.8		48		25.3		26		7.3		14	
2a	39.8		52.2		21.0		35.4		5.7		12	
b	38.1	39.0	52.2	54.1	19.4	20.2	32.5	32.3	5.9	5.8	13.6	13.1
c	39.0		57.9		20.1		28.9		5.8		13.6	
2a	43.9		52.6		20.4		31.1		5.1		14.8	
b	41.4	42.7	57.4	55.0	19.2	19.8	34.4	32.7	5.1	5.2	13.9	14.0
c	42.7		54.9		19.8		32.6		5.2		13.4	
3a	41.1		48.6		22.7		25.3		3.9		7.5	
b	42.8	42.3	47.2	47.3	23.7	22.7	23.8	24.6	4.8	4.3	8.4	7.9
c	43.0		46.1		21.9		24.9		4.8		7.7	
3a	44.0		46.9		23.8		23.6		5.3		8.7	
b	42.7	42.8	47.5	46.8	21.4	22.4	25.1	24.5	4.1	4.7	7.2	7.9
c	41.8		46.1		21.9		24.9		4.8		7.7	
4a			41.6				24.1				8.2	
b			41.6	41.6			24.5	24.4			8.2	8.3
c			41.6				24.6				8.6	
4a			42.2				24.8				8.6	
b			42.8	42.6			24.6	24.7			8.4	8.5
c			42.8				24.8				8.6	
5a	46.0		47.4		23.9		25.1		5.3		12.8	
b	46.5	46.8	48.1	48.2	25.3	25.4	26.3	26.2	4.8	4.9	12.1	12.3
c	47.5		49.0		27.1		27.2		4.5		12.0	
5a	45.8		48.3		29.3		26.0		4.6		7.9	
b	48.8	47.0	50.0	48.6	28.2	29.1	27.4	26.2	4.5	5.0	6.9	7.3
c	46.3		47.4		29.7		25.1		5.9		7.2	
6a	34.8				16.4				2.7			
b	39.9	37.3			16.0	16.2			2.2	2.5		
6a	37.4				23.3				3.2			
b	38.3	37.9			21.8	22.2			4.0	3.6		
c					21.5							
7a	41.9		42.2		23.0		24.7		3.9		7.9	
b	41.3	42.1	42.2	42.7	22.7	22.7	24.4	24.4	3.5	3.6	7.0	7.3
c	43.0		43.7		22.5		24.1		3.5		7.0	
8	30.1		33.0						6.6		0.8	

*for each laboratory two separate determinations, each injected three times

**mixture (1:1, v/v) sample 1 and refined olive oil

Table 2 sets out the results of the statistical analysis and it will be seen that high values for the repeatability and reproducibility of the method are reported i.e. it reveals predictable standard deviations, both on a within- and inter-laboratory basis, that are higher than those normally found for other methods of analysis applied to fats and oils. Nevertheless, it is considered that these values are acceptable, bearing in mind the complex nature of the analytical procedure. (There are many examples of complex methods of analysis whose precision is similar to, or even less than, that of the method described here for the determination of erythrodiol and which, despite their poor precision, have been employed effectively in routine analyses of fats and other commercial products.)

TABLE 2. STATISTICAL ANALYSIS OF RESULTS

	results expressed as % relative to β-sitosterol			results expressed as mg/100g oil		
Sample No.	1	2	3	1	2	3
Number of labs.	7	6	7	7	6	6
No. accepted values	11	11	12	12	11	10
Mean value (x)	42.0	23.2	5.1	46.4	26.8	10.5
Repeatability						
S_r	0.33	2.27	0.42	0.71	0.27	0.40
CV_r %	0.8	9.8	8.3	1.5	1.0	3.8
r (95%)	0.9	6.4	1.2	2.0	0.8	1.1
Reproducibility						
S_R	5.60	3.15	1.49	6.11	3.18	3.0
CV_R %	13.3	13.6	29.4	13.2	11.9	28.6
r (95%)	15.9	11.0	4.4	17.4	9.0	8.7

In order to ascertain if a relationship exists between the repeatability (r) and the reproducibility (R) of the method and the respective mean values at the three levels studied, the coefficients of correlation (C_r) and (C_R) were also calculated. The values obtained for these coefficients are given in Table 3.

TABLE 3. COEFFICIENTS OF CORRELATION BETWEEN THE VALUES FOR REPEATABILITY AND REPRODUCIBILITY AND THE CORRESPONDING MEANS

Quantitative criterion	C_r	C_R
mg erythrodiol/100 g oil	0.725	0.91
% (m/m) apparent β-sitosterol content	- 0.05	0.99

Although these values are no doubt insufficient to draw concrete and precise conclusions, the values indicated appear to point to a direct correlation between erythrodiol content and the accuracy of the analysis, both in within- and inter-laboratory terms; the correlation is actually more pronounced in the latter. A highly anomalous result was found for the repeatability of the method where the erythrodiol/beta-sitosterol ratio was employed as the quantitative criterion in that the correlation is virtually non-existent. In contrast, although the value calculated for C_r in the same analyses using betulin as the internal standard is not very good, it is far from being the non-existent value found in the former case. Some distorting factors have quite obviously been at play in one or more of the analyses, originating from the quantification of the beta-sitosterol, and which are not reflected, or at least to a much lesser extent, when the second criterion is employed. It is

somewhat surprising, moreover, to discover that the correlation for the reproducibility values is much better, obtaining good coefficients of 0.91 and 0.99. This could be interpreted that systematic mistakes have been made in each laboratory which were then evened out when the inter-laboratory means were established. But the study protocol adopted in order to simplify the laboratories' work does not allow any analysis of variance in this respect.

ELEMENTARY ANALYSIS OF THE VARIANCE

On the basis of the information available from the laboratory analyses, the variance of the results obtained by the collaborating laboratories can be divided into two distinct parts: the variance for the manipulations involved in the analytical procedure, designated below as s_a^2, and the variance corresponding to the gas-liquid chromatographic determination, designated s_c^2. It is considered that it is worthwhile determining the latter value in view of evidence, inferred from practical experience, that it is an important factor in the discrepancies between the analytical results.

The formula used for this calculation is:

$$s_r^2 = \frac{s_c^2}{kn} + \frac{s_a^2}{n}$$

where:

s_r^2 is the variance between replicates for a complete analysis, the values for which are given in Table 2

s_a^2 is the derived variance for the manipulations involved in the overall analytical process

s_c^2 is the derived variance for the gas-liquid chromatographic determination

k is the number of replicates in the chromatographic analysis (in this case 3)

n is the number of replicates in the complete analysis (in this case 2)

The total values calculated are given in Table 4 and point to the significant part played by the error introduced by the gas-liquid chromatographic determination in the total error for the analysis. Therefore, in order to improve the results obtained, two or three consecutive injections of the silanised solution would appear advisable, taking the mean values of such determinations as the final result.

TABLE 4. PRECISION OF THE METHOD

Sample (**)	relative to β-sitosterol						in mg/100 g of sample					
	S^2_r	S_r	S^2_c	S_c	S^2_a	S_a	S^2_r	S_r	S^2_c	S_c	S^2_a	S_a
1	0.11	0.33	1.69	1.30	-0.34	0	0.50	0.71	2.23	1.49	0.26	0.51
2	5.15	2.27	0.94	0.97	9.98	3.16	0.07	0.27	0.58	0.76	-0.04	0
3	0.18	0.42	0.16	0.40	0.30	0.55	0.16	0.40	0.33	0.57	0.21	0.46

**(see text for explanation of these symbols)

CONCLUSIONS

1) It is submitted that the method of analysis subjected to collaborative study has produced acceptable results which would be difficult to improve to any significant extent.

2) In view of the complexity of the analysis, it is essential that the operator must be experienced in its application. It is advisable to perform the determinations in duplicate, analysing each duplicate by not less than three gas-liquid chromatographic injections.

3) On the basis of the results of the collaborative study, and in full recognition of the shortcomings of the procedure, the Commission decided to adopt the method. The text of the standardised procedure is given on the following pages.

2.431 DETERMINATION OF THE ERYTHRODIOL CONTENT

1 SCOPE

This Standard describes a method for the determination of the erythrodiol content of an oil (Note 1).

2 DEFINITION

The erythrodiol content is the quantity of erythrodiol determined by the present method and expressed either relative to the β-sitosterol content of the oil or as a percentage by mass of the oil (absolute concentration).

3 FIELD OF APPLICATION

This Standard is applicable to vegetable oils.

4 PRINCIPLE

Saponification of the oil. Extraction of the unsaponifiable matter, if desired in the presence of an internal standard (betulin), followed by purification. Isolation of the sterols and triterpenoid glycols fraction of the unsaponifiable matter by thin-layer chromatography.
Analysis of the combined sterol and triterpenoid glycol fractions, after silanisation, by gas-liquid chromatography.
Calculation of the concentration of erythrodiol relative to the β-sitosterol content of the oil, otherwise calculation of the absolute concentration of erythrodiol if an internal standard is used.

5 APPARATUS

5.1 Extraction and purification of the unsaponifiable matter

5.1.1 The equipment described in 5.2 of method 2.401 except items 5.2.4 and 5.2.6. (Note 2)

5.1.2 25 ml flasks, pear-shaped, for use with rotary evaprator (5.1.5).

5.1.3 Column for chromatography, about 30 cm long and 15 mm internal diameter, preferably with a sintered glass filter, N° 2 porosity.

5.1.4 Magnetic agitator.

5.1.5 Rotary vacuum evaporator with water bath.

5.2 Isolation of the sterols and triterpenoid glycols by thin-layer chromatography (TLC)

5.2.1 250 ml conical flasks.

5.2.2 5 and 10 ml flasks, pear-shaped, for use with rotary evaporator (5.1.5.).

5.2.3 Spreader for the preparation of plates.

5.2.4 Glass plates 20 x 20 cm.

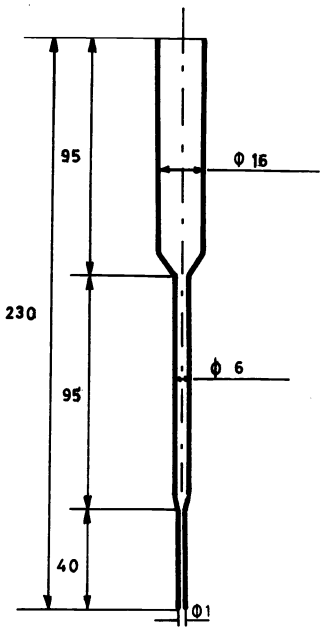
5.2.5 Glass developing tank with ground glass lid, suitable for the glass plates (5.2.4).

5.2.6 Metal rack for holding and transporting plates (5.2.4).

5.2.7 Cabinet or desiccator for storing and keeping plates (5.2.4) dry.

5.2.8 Micro-pipette or micro-syringe 50 µl for dispensing 0.3 - 0.4 µl drops (Note 3).

5.2.9 Atomiser for applying visualising reagent to plates.



Elution column (dimensions in mm)

5.2.10 Column, glass, about 30 cm long and 6 mm internal diameter, plugged at the end with glass yarn (5.2.11) as illustrated in the figure, for the elution of the fractions separated by TLC (Note 4).

5.2.11 Glass yarn, chromatographic grade.

5.2.12 Oven, regulated at 103 ± 2°C.

5.2.13 Ultra-violet light source.

5.3 Analysis of the sterols and triterpenoid glycols by gas-liquid chromatography

5.3.1 Gas-liquid chromatography, with column (5.3.2) and flame-ionisation detector, preferably equipped with facilities for on-column injection and with an integrator.

5.3.2 Column, glass, about 2 m long and internal diameter about 2.5 mm, packed with 5% (m/m) of a phenyl-methyl silicone (preferably OV-17) on a silanised support (80/100 mesh) - such as Supelcoport or Chromosorb W-HP (Note 5).

5.3.3 Micro-syringe, 1 µl.

6 REAGENTS

6.1 Extraction and purification of the unsaponifiable matter

6.1.1 The reagents included under 5.3 of method 2.401 except items 5.3.6 and 5.3.7.

6.1.2 Ion-exchange resin, analytical quality, Amberlite MB-3 or equivalent.
Before use, treat the resin as follows: Introduce 6 - 7 g of the resin into a chromatographic column (5.1.3). Elute 100 ml of anyhdrous methanol at approximately one drop per second. After the whole of the methanol has been eluted pass a current of dry air through the column so as to eliminate any remaining methanol. Store the prepared resin in an airtight container.
Immediately prior to its use, suspend the quantity of resin required (6 g) in 10 - 15 ml of diethyl ether for 15 minutes and shake from time to time.

6.1.3 Betulin, minimum purity 99%, solution in di-isopropyl ether 0.5 mg/ml, internal standard.

6.2 Isolation of the sterols and triterpenoid glycols by means of thin-layer chromatography

6.2.1 Hexane, for chromatography.

6.2.2 Diethyl ether, analytical reagent quality.

6.2.3 Di-isopropyl ether, analytical reagent quality.

6.2.4 Developing solvent: mixture of hexane (6.2.1), diethyl ether (6.2.2), 2/1, (V/V) (Note 6).

6.2.5 Silica gel of suitable quality for TLC, for example Merck type G or equivalent.

6.2.6 Cholesterol, 10 mg/ml chromatographic standard solution in di-isopropyl ether.

6.2.7 2', 7' - dichlorofluorescein, 1 g/l solution in 95 per cent ethanol (V/V), visualising reagent (Note 7).

6.2.3 Nitrogen, at least 99% pure.

6.3 Analysis of the sterols and triterpenoid glycols by gas-liquid chromatography

6.3.1 Silanisation reagent
Mix 9 ml anhydrous pyridine (freshly distilled over sodium hydroxide), 2 ml analytical grade hexamethyldisilazane and 1 ml analytical grade trimethylchlorosilane in a small glass vial which can be kept sealed, preferably with a septum.

6.3.2 β-sitosterol, chromatographic standard, minimum purity 98%, 10 mg/ml solution in di-isopropyl ether.

7 PROCEDURE

7.1 Extraction and purification of the unsaponifiable matter

Prepare the sample according to the instructions given in method 2.001.

Extract the unsaponifiable matter from 5 g of sample, following the method 2.401 (diethyl ether method) up to and including the addition of acetone and elimination of the volatile solvent using a boiling-water bath. If the absolute concentration of erythrodiol is to be determined, the sample must be accurately weighed (i.e. to the nearest 0.01 g) and 5 ml of the betulin solution (6.1.3) should be added to the saponified sample solution just prior to its extraction with diethyl ether (Note 8).

Once the solvent has been eliminated and the residual water removed by entrainment with acetone, dissolve the unsaponifiable matter in the same flask in 20 ml of diethyl ether. Add 6 g of prepared ion-exchange resin (6.1.2) and shake the solution continuously for 20 - 30 minutes using a magnetic agitator (5.1.5).

Filter the ethereal solution through a paper and collect it in a 25 ml flask (5.1.2), in two portions, eliminating the solvent each time using an evaporator (5.1.5).

After all the solution has been filtered, wash the resin and flask, with three portions of 3 - 4 ml of the same solvent. Collect the solvent in the 25 ml flask containing the unsaponifiable matter. Evaporate, leaving the unsaponifiable matter dry (Note 9).

7.2 Isolation of the sterols and triterpendoid glycols by thin-layer chromatography (TLC)

7.2.1 Preparation of the TLC plates (if desired, commercially prepared plates may be used).

Carefully clean the surface of the plates with hexane, ethanol, and acetone so as to ensure the total removal of any fatty residue. Transfer them to the spreader (5.2.3). Weigh 30 g silical gel (6.2.5) into a conical flask (5.2.1). Add 60 ml of distilled water. Stopper the flask. Shake vigorously for one minute so as to ensure that the slurry is as homogeneous as possible.

Transfer the slurry immediately to the spreader (5.2.3). Spread the slurry on the plates (5.2.4) to a thickness of 0.25 mm. No more than 4 minutes should be taken in preparing the slurry and spreading it on the plates. Leave the plates to dry in air until the surface is seen to be dry. Transfer the plates to the rack (5.2.6) and place them in the drying oven (5.1.12) for 1 hour at 103 $\pm$ 2°C. After activation, remove the plates from the oven and store in the cabinet (5.2.7).

7.2.2 Preparation of the developing tank.

Pour developing solvent (6.2.4) into the tank (5.2.5) to a level of 10 mm. Cover the tank and leave for about 1 hour to obtain equilibrium between the liquid and vapour phases.

7.2.3 TLC of the unsaponifiable matter

Dissolve the purified and dried unsaponifiable matter, contained in the flask (5.1.2), in 5 ml of di-isopropyl ether (6.2.3). Transfer 2 ml of the unsaponifiable solution to a 5 ml flask (5.2.2). Evaporate the solvent under nitrogen (6.2.8) with the evaporator (5.1.5).

Dissolve the residue in 200 µl of di-isopropyl ether (6.2.3). Using a micro-pipette or a micro-syringe (5.2.3) deposit on a TLC plate, prepared as indicated in 7.2.1, the whole of the unsaponifiable matter solution (200 µl) in fine drops and in a uniform straight line, 2 cm from the bottom edge of the plate and 2.5 cm from the left and right edges (Note 10). Deposit 15 - 20 µl of the cholesterol solution (6.2.6) 1 cm from the right edge of the line of application of the sample solution and 1 cm from the left edge. Place the plate immediately in the developing tank (5.2.5), prepared as indicated in 7.2.2. Cover the tank and leave until the solvent front reaches 2 cm from the top edge of the plate.

Remove the plate from the tank and allow it to dry in the air for about 3 or 4 minutes.

Spray the plate with the visualising reagent (6.2.7) using the atomiser (5.2.9) and examine under UV light (5.2.13).

Using the two cholesterol spots as a reference, locate the position of the sterol band and that of the triterpenoid glycols, which should be below, and resolved from, the sterols (Note 11).

7.2.4 Recovery of the sterols and triterpenoid glycols fractions

Mark off the horizontal silica bands containing the sterols and the triterpenoid glycols. Remove them together using a spatula, collecting the powder on glazed paper. Transfer the silica immediately to the elution column (5.2.10). Top up the column with unused silica gel (6.2.5). Mount the column vertically above a 10 ml flask (5.2.2). Add di-isopropyl ether (6.2.3) in 1 ml portions, adding each portion as soon as the previous portion has penetrated the surface of the silica column. Six portions should be added amounting to a total of 6 ml.

After elution is complete, evaporate the solvent from the collected eluant using an evaporator (5.1.5) with a 35°C water bath.

7.3 Analysis of the sterols and triterpenoid glycols by gas-liquid chromatography (GLC)

7.3.1 Silanisation

Add 100 µl of silanisation reagent (6.3.1) to the flask containing the sterol and triterpenoid glycol fractions (Note 12). Stopper the flask and shake gently with a rotating movement for 1 to 2 minutes. Allow to stand for about 20 minutes; the solution is then ready for injection into the chromatograph.

7.3.2 GLC conditions

The GLC conditions usually producing optimum values given below, are for guidance purposes only and should be modified, as required, until a satisfactory performance is obtained (Note 13):

Column temperature.....240°C - 250°C
Injector and detector temperature.....290°C
Carrier gas flow (N_2).....30 - 50 ml/min

7.3.3 Injection of the sample

Inject into the chromatograph (5.3.1), by means of a micro-syringe (5.3.3), 0.5 - 0.6 µl of the solution silanised as indicated in 7.3.1, using the sensitivity required so that the β-sitosterol peak almost reaches full-scale on the recorder chart; it is preferable that all of the chromatographic recording is carried out at the same sensitivity. In any case, the sensitivity chosen must be such that accurate determinations of the erythrodiol and β-sitosterol peak areas can be made.

7.3.4 Identification of the peaks

The peak corresponding to the β-sitosterol is easily identified as it represents the major part of the sterol fraction. Should there be any doubt, the identification can be confirmed by injecting 0.5 - 0.6 µl of the standard β-sitosterol solution (6.3.2), silanised as in 7.3.1.

The erythrodiol peak appears after the β-sitosterol peak, with a retention time in relation to the latter of 1.3 to 1.5 under the conditions described (Note 3).

Peaks of other triterpenoid glycols may appear on the chromatogram, but in quantities much smaller than for the erythrodiol, at least in the oils mentioned in Note 1.

8 EXPRESSION OF RESULTS

8.1 Calculation of the quantity of erythrodiol relative to the -sitosterol content of the oil (Note 5).

The peak areas of the β-sitosterol and the erythrodiol are determined. Should they have been recorded at different sensitivites, they must both be referred to a similar sensitivity. The erythrodiol content of the oil, expressed as a percentage relative to β-sitosterol, is given by the formula:

$$\frac{A_E}{A_S} \times 100$$

where:

A_E is the peak area corresponding to the erythrodiol

A_S is the peak area corresponding to the β-sitosterol

8.2 Calculation of the quantity of erythrodiol as a percentage by mass of the oil (absolute quantity using an internal standard).

The peak areas for erythrodiol and the internal standard are determined as above (Note 5).

The erythrodiol content of the oil, expressed as a percentage by mass of the oil (absolute concentration), is given by the formula:

$$\frac{A_E \times m_i \times K_{Ei}}{A_i \times m_s \times 10}$$

where

A_E is the peak area corresponding to erythrodiol

A_i is the peak area corresponding to the internal standard

m_i is the mass in mg of the internal standard used

m_s is the mass in g of the oil sample taken

K_{Ei} is the response factor of the erythrodiol relative to the internal standard (Note 16)

8.3 Repeatability

The difference between the results of two determinations carried out on the same day by the same analyst using the same apparatus for the same test material should not exceed 1% absolute.

9 NOTES

1. Certain vegetable oils, for example, grapeseed oil, olive oil, olive residue oil, contain triterpenoid glycols in their unsaponifiable fraction. The major component of this group is erythrodiol, also called homo-olestranol, which is the glycol derived from oleanolic acid by reducing the carboxyl group to alcohol.

2. The evaporation operations can be done more quickly and easily using a vacuum rotary evaporator.

3. It is advisable, so as to simplify series analyses, to use automatic apparatus for depositing the solution to be chromatographed on thin-layer plates.

4. The use of this type of column is not essential; it can be replaced by a 250 mm glass tube, 7 - 8 mm internal diameter, which tapers off at the end so as to retain a 20 - 30 mm glass wool plug.

5. Both the type of stationary phase and the support mentioned are for the purposes of guidance only since they have been shown to produce satisfactory results, However, they may be replaced by other packings which can give similar or even better results, as long as the column meets the efficiency criteria given in 7.3.2. When SE 30 is used as the stationary phase, β-sitosterol and δ5 avenasterol are not separated. In this case, the erythrodiol content, relative to the β-sitosterol content, is expressed as a quantity relative to an "apparent" β-sitosterol content (i.e. β-sitosterol + δ5 avenasterol).

When SE 30 or OV 17 are used as the stationary phase in a packed column, erythrodiol is not separated from any δ7 avenasterol that may be present. In this case it is an "apparent" erythrodiol content (i.e. erythrodiol + δ7 avenasterol) which is determined.

However, if an OV 17 capillary column is used erythrodiol can be readily separated from δ7 avenasterol, and β-sitosterol can be separated from δ5 avenasterol.

6. Other solvents producing similar results may be used instead of the hexane–diethyl ether mixture, as long as a clear separation of the sterol and triterpenoid glycol fractions is obtained. The following have also been recommended: chloroform alone; benzene–acetone (95/5) (V/V); n-heptane–acetone (85/15) (V/V).

7. Any other dye may be used which does not react with the sterols, for example, Rhodamine 6 G.

8. Should the ethereal solution of the unsaponifiable matter contain a significant amount of water, which is often the case, it is advisable to dry it with sodium sulphate. In order to do this, 8 - 10 g of anhydrous sodium sulphate are added to the solution contained in the decanting flask. This is then shaken and left for 30 - 45 minutes. The solution is then filtered immediately and collected in a second 250 ml flask. The decanting flask and the filter are washed with three 5 ml portions of diethyl ether.

9. Should another procedure be used instead of the rotary evaporator for eliminating the solvent, the temperature should not be allowed to rise above 40°C.

10. It will not be possible to deposit the whole of the unsaponifiable matter in one line application; accordingly after each line application the solvent should be allowed to evaporate before depositing a further line of unsaponifiable matter solution.

11. If sufficiently pure erythrodiol is available for use as a standard, 1% of the erythrodiol may be added to the reference cholesterol solution; the spot obtained on the chromatogram can then act as a guide for locating the postion of the erythrodiol.

However, it is not essential to do this since the position of the erythrodiol can be easily located by reference to the sterols.

12. Make sure that the syringes and all apparatus are perfectly dry. To ensure this, rinse immediately before use with anhydrous acetone, drain and evaporate the remainder in a current of dry air.

13. No precise working conditions can be given since these depend basically upon the performance of the column, the characteristics of the apparatus etc.

The performance can be regarded as being satisfactory if a symmetrical peak is obtained for β-sitosterol, without "tailing" and if the resolution of the stigmasterol-campesterol peaks is a minimum of 0.8 (Notes 14 and 15).

If these minimum conditions are not met, even by changing the working conditions within acceptable limits, it will be necessary to replace the column.

14. The resolution is given by the formula:

$$R_s = \frac{2(d_2 - d_1)}{b_2 + b_1}$$

where:

b_1 and b_2 are the widths, in mm, of the peaks for campesterol and stigmasterol

respectively, measured between the points of intersection of the tangent at the inflexion points of the curve with the base line.

d_1 and d_2 are the retention distances, in mm, from the solvent front to the maximum of the peaks for campesterol and stigmasterol respectively.

15. In order to determine if the requirements concerning the resolution of the stigmasterol and the campesterol have been met, it is not necessary to have standards of these sterols since it is easy to identify them on the chromatogram obtained from the sterol fraction. Under the operating conditions described, campesterol and stigmasterol have retention times, in relation to β-sitosterol, of approximately 0.80 and 0.82 respectively.

16. When betulin is used as the internal standard, the response factor is practically equal to unity for the concentrations indicated in the method. The response factor of erythrodiol with respect to the internal standard used (K_{Ei}) may be determined by chromatographing a reference mixture containing known amounts of erythrodiol and the internal standard under the conditions adopted for the analyses, using the formula:

$$K_{Ei} = \frac{A_E \times m_i}{A_i \times m_E}$$

where

A_E is the peak area of the erythrodiol standard

A_i is the peak area of the internal standard

m_i is the mass of the internal standard

m_E is the mass of the erythrodiol standard

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