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COMMISSION REGULATION (EEC) No 2568/91

of 11 July 1991

on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis

(OJ L 248, 5.9.1991, p. 1)

Amended by:

		Official Journal		
		No	page	date
►**M1**	Commission Regulation (EEC) No 3682/91 of 17 December 1991	L 349	36	18.12.1991
►**M2**	Commission Regulation (EEC) No 1429/92 of 26 May 1992	L 150	17	2.6.1992
►**M3**	Commission Regulation (EEC) No 1683/92 of 29 June 1992	L 176	27	30.6.1992
►**M4**	Commission Regulation (EEC) No 1996/92 of 15 July 1992	L 199	18	18.7.1992
►**M5**	Commission Regulation (EEC) No 3288/92 of 12 November 1992	L 327	28	13.11.1992
►**M6**	Commission Regulation (EEC) No 183/93 of 29 January 1993	L 22	58	30.1.1993
►**M7**	amended by Commission Regulation (EEC) No 826/93 of 6 April 1993	L 87	6	7.4.1993
►**M8**	Commission Regulation (EEC) No 620/93 of 17 March 1993	L 66	29	18.3.1993
►**M9**	Commission Regulation (EC) No 177/94 of 28 January 1994	L 24	33	29.1.1994
►**M10**	Commission Regulation (EC) No 2632/94 of 28 October 1994	L 280	43	29.10.1994
►**M11**	Commission Regulation (EC) No 656/95 of 28 March 1995	L 69	1	29.3.1995
►**M12**	Commission Regulation (EC) No 2527/95 of 27 October 1995	L 258	49	28.10.1995
►**M13**	Commission Regulation (EC) No 2472/97 of 11 December 1997	L 341	25	12.12.1997
►**M14**	Commission Regulation (EC) No 282/98 of 3 February 1998	L 28	5	4.2.1998
►**M15**	Commission Regulation (EC) No 2248/98 of 19 October 1998	L 282	55	20.10.1998
►**M16**	Commission Regulation (EC) No 379/1999 of 19 February 1999	L 46	15	20.2.1999
►**M17**	Commission Regulation (EC) No 455/2001 of 6 March 2001	L 65	9	7.3.2001
►**M18**	Commission Regulation (EC) No 2042/2001 of 18 October 2001	L 276	8	19.10.2001
►**M19**	Commission Regulation (EC) No 796/2002 of 6 May 2002	L 128	8	15.5.2002

Corrected by:

►**C1** Corrigendum, OJ L 347, 28.11.1992, p. 69 (2568/91)

►**C2** Corrigendum, OJ L 176, 20.7.1993, p. 26 (183/93)

►**C3** Corrigendum, OJ L 96, 28.3.1998, p. 47 (2472/97)

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COMMISSION REGULATION (EEC) No 2568/91

of 11 July 1991

on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community,

Having regard to Council Regulation No 136/66/EEC of 22 September 1966 on the establishment of a common organization of the market in oils and fats [1], as last amended by Regulation (EEC) No 3577/90 [2], and in particular Article 35a thereof,

Whereas the Annex to Regulation No 136/66/EEC contains the descriptions and definitions of olive oil and olive-residue oil marketed within each Member State, in intra-Community trade and in trade with third countries;

Whereas, for the purpose of differentiating between the various types of oil, the physical and chemical characteristics of each of them and the organoleptic characteristics of virgin oil should be defined, in order to guarantee the purity and quality of the products concerned, without prejudice to other existing provisions;

Whereas the presence of the characteristics of the different types of oil should be determined uniformly throughout the Community; whereas, to that end, Community methods of chemical analysis and organoleptic evaluation should be established; whereas the use should be permitted, for a transitional period, of other methods of analysis applied in the Member States provided that where there is a difference in the results, those obtained using the common method will be decisive;

Whereas the definition of the physical and chemical characteristics of olive oil and of the methods of analysis entails the amendment of the additional notes to Chapter 15 of the combined nomenclature;

Whereas the method of evaluating the organoleptic characteristics of virgin oil includes the setting up of panels of selected and trained tasters; whereas the period necessary for establishing such a structure should therefore be fixed; whereas in view of the difficulties that some Member States will encounter in setting up panels of tasters, the use of panels in other Member States should be authorized;

Whereas, in order to ensure that the system of levies applicable to imports of olive residues functions correctly, a single method for the determination of the oil content of these products should be laid down;

Whereas, in order not to harm trade, provision should be made for oil packaged prior to the entry into force of this Regulation to be disposed of during a limited period;

Whereas it is necessary to repeal Commission Regulation (EEC) No 1058/77 [3], as last amended by Regulation (EEC) No 1858/88 [4];

Whereas the Management Committee for Oils and Fats has not delivered an opinion within the time limit set by its chairman,

HAS ADOPTED THIS REGULATION:

Article 1

1.   Oils, the characteristics of which comply with those set out in points 1, 2 and 3 of Annex I to this Regulation, shall be deemed to be

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[1] OJ No 172, 30. 9. 1966, p. 3025/66.
[2] OJ No L 353, 17. 12. 1990, p. 23.
[3] OJ No L 128, 24. 5. 1977, p. 6.
[4] OJ No L 166, 1. 7. 1988, p. 10.

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virgin olive oil within the meaning of point 1 (a), (b) and (c) of the Annex to Regulation No 136/66/EEC.

2.   Oil, the characteristics of which comply with those set out in point 4 of Annex I to this Regulation, shall be deemed to be lampante virgin olive oil within the meaning of point 1 (d) of the Annex to Regulation No 136/66/EEC.

3.   Oil, the characteristics of which comply with those set out in point 5 of Annex I to this Regulation, shall be deemed to be refined olive oil within the meaning of point 2 of the Annex to Regulation No 136/66/EEC.

4.   Oil, the characteristics of which comply with those set out in point 6 of Annex I to this Regulation, shall be deemed to be pure olive oil within the meaning of point 3 of the Annex to Regulation No 136/66/EEC.

5.   Oil, the characteristics of which comply with those set out in point 7 of Annex I to this Regulation, shall be deemed to be olive-residue oil within the meaning of point 4 of the Annex to Regulation No 136/66/EEC.

6.   Oil, the characteristics of which comply with those set out in point 8 of Annex I to this Regulation, shall be deemed to be refined olive-residue oil within the meaning of point 5 of the Annex to Regulation No 136/66/EEC.

7.   Oil, the characteristics of which comply with those set out in point 9 of Annex I to this Regulation, shall be deemed to be olive-residue oil within the meaning of point 6 of the Annex to Regulation No 136/66/EEC.

▼**M15**

8.   However, for the ►**M18** 1998/1999 to 2002/03 marketing years ◄, oil in bulk or put up in immediate packaging with a net content of 100 kilograms or more, originating entirely in Morocco, the characteristics of which comply with those set out in Annex I(1), (2), (3) and (4) to this Regulation and, notwithstanding paragraphs 1 and 2, with a linolenic acid content of not more than 1 %, shall also be deemed to be virgin olive oil within the meaning of point 1(a), (b), (c) and (d) of the Annex to Regulation No 136/66/EEC.

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Article 2

1.   The characteristics of the oils laid down in Annex I shall be determined in accordance with the methods of analysis set out below:

— for the determination of the free fatty acids, expressed as the percentage of oleic acid, the method set out in Annex II,

— for the determination of the peroxide index, the method set out in Annex III,

▼**M19**

— for determination of the wax content, the method given in Annex IV,

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— for the determination of the sterol content, the method set out in Annex V,

— for the determination of erythrodiol and uvaol, the method set out in Annex VI,

— for the determination of the saturated fatty acids in position 2 of the triglyceride, the method set out in Annex VII,

— for the determination of the trilinolein content, the method set out in Annex VIII,

— for spectrophotometric analysis, the method set out in Annex IX,

— for the determination of the fatty acid composition, the method set out in Annex X A and X B,

— for the determination of the volatile halogenated solvents, the method set out in Annex XI,

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— for the evaluation of the organoleptic characteristics of virgin olive oil, the method set out in Annex XII,

— for proof that refining has taken place, the method set out in Annex XIII,

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— for the determination of stigmastadienes, the method set out in Annex XVII,

▼**M13**

— for determining the content of triglycerides with ECN42, the method set out in Annex XVIII,

▼**M19**

— for determination of the aliphatic alcohol content, the method given in Annex XIX.

2.   Verification by national authorities or their representatives of the organoleptic characteristics of virgin oils shall be effected by tasting panels approved by the Member States.

The organoleptic characteristics of an oil as referred to in the first subparagraph shall be deemed consonant with the category declared if a panel approved by the Member State confirms the grading.

Should the panel not confirm the category declared as regards the organoleptic characteristics, at the interested party's request the national authorities or their representatives shall have two counter-assessments carried out by other approved panels, at least one by a panel approved by the producer Member State concerned. The characteristics concerned shall be deemed consonant with the characteristics declared if at least two of the counter-assessments confirm the declared grade. If that is not the case, the interested party shall be responsible for the cost of the counter-assessments.

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3.   When the national authorities or their representatives verify the characteristics of the oil as provided for in paragraph 1, samples shall be taken in accordance with international standards EN ISO 661 on the preparation of test samples and EN ISO 5555 on sampling. However, notwithstanding point 6.8 of standard EN ISO 5555, in the case of batches of such oils in immediate packaging not exceeding 100 litres, the sample shall be taken in accordance with Annex Ia to this Regulation.

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Without prejudice to standard EN ISO 5555 and Chapter 6 of standard EN ISO 661, the samples taken shall be put in a dark place away from strong heat as quickly as possible and sent to the laboratory for analysis no later than:

— the tenth working day after they are taken, during the period from October to May, and

— the fifth working day after they are taken, during the period from June to September.

▼**M17**

4.   For the purposes of the verification provided for in paragraph 3, the analyses referred to in Annexes II, III, IX and XII and, where applicable, any counter-analyses required under national law, shall be carried out before the minimum durability date. Where sampling is done more than four months before the minimum durability date, the analyses shall be carried out no later than the fourth month after the month in which the sample was taken. No time limit shall apply to the other analyses provided for in that Regulation.

Unless the sample was taken less than one month before the minimum durability date, if the results of the analyses do not match the characteristics of the category of olive oil or olive-residue oil declared, the party concerned shall be notified no later than one month before the end of the period laid down in the first subparagraph.

▼**M19**

5.   For the purpose of determining the characteristics of olive oils by the methods provided for in paragraph 1, the analysis results shall be directly compared with the limits laid down in this Regulation.

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Article ►**M19** *3* ◄

Where it is found that the organoleptic characteristics of an oil do not correspond to its description, the Member State concerned shall, without prejudice to any other penalties, apply administrative financial penalities, to be determined in the light of the seriousness of the irregularity detected.

In assessing the irregularity, attention shall be paid in particular to natural changes in the characteristics of an oil kept under normal conditions.

At the beginning of each half-year, the Member States shall inform the Commission of the number and type of irregularities detected and the penalties applied during the previous half-year.

Article 4

▼**M19**

1.   The Member States may approve assessment panels so that national authorities or their representatives can assess and verify organoleptic characteristics.

The terms of approval shall be set by Member States and ensure that:

— the requirements of Annex XII.4 are met,

— the panel head is given training recognised for this purpose by the Member State,

— continued approval depends on performance in annual checks arranged by the Member State.

Member States shall notify to the Commission a list of approved panels and the action taken under this paragraph.

▼**M5**

2.   Where Member States encounter difficulties in setting up tasting panels in their territory, they may call on a tasting panel approved in another Member State.

3.   Each Member State draw up a list of tasting panels set up by professional or inter-branch organizations in accordance with the conditions laid down in paragraph 1 and shall ensure that those conditions are complied with.

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Article 6

1.   The oil content of oil cake and other residues resulting from the extraction of olive oil (CN codes 2306 90 11 and 2306 90 19) shall be determined using the method set out in Annex XV.

2.   The oil content referred to in paragraph 1 shall be expressed as a percentage of the weight of oil to the weight of dry matter.

Article 7

The Community provisions concerning the presence of undesirable substances, other than those referred to in Annex XI, shall apply.

Article 8

1.   Member States shall notify the Commission of the measures taken to implement this Regulation.

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2.   Member States shall send the Commission, at the beginning of each half-year, a statement of the analytical data relating to the tests carried out during the previous half-year.

The results shall be considered by the Management Committee for Oils and Fats in accordance with the procedure laid down in Article 39 of Regulation No 136/66/EEC.

Article 9

Regulation (EEC) No 1058/77 is hereby repealed.

Article 10

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

However, the method set out in Annex XII shall apply from ►**M1** 1 November 1992 ◄, *except in so far as operations relating to the intervention system are concerned*.

▼**M5**

That method shall not apply to virgin olive oil prepared for the market prior to 1 November 1992.

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2.   This Regulation shall not apply to olive oil and olive-residue oil packaged before the entry into force of this Regulation and marketed up to 31 October 1992.

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

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ANNEXES

Summary

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ANNEX I

CHARACTERISTICS OF OLIVE OIL

Category	Acidity (%) (*)	Peroxide value mEq 02/kg (*)	Halogenated solvents mg/kg (*) (1)	Waxes mg/kg (**)	Saturated fatty acids in position 2 triglyceride (%)	Stigmastadienes mg/kg (2)	Difference between HPLC and theoretical calculation of ECN42	K_{232} (*)	K_{270} (*)	K_{270} after alumina (3)	Delta-K (*)	Organoleptic assessment Median of defects (Md) (*)	Organoleptic assessment Median for 'fruity' (Mf) (*)
1. Extra virgin olive oil	≤ 1,0	≤ 20	≤ 0,20	≤ 250	≤ 1,3	≤ 0,15	≤ 0,2	≤ 2,50	≤ 0,20	≤ 0,10	≤ 0,01	Md = 0	Mf > 0
2. Virgin olive oil	≤ 2,0	≤ 20	≤ 0,20	≤ 250	≤ 1,3	≤ 0,15	≤ 0,2	≤ 2,60	≤ 0,25	≤ 0,10	≤ 0,01	Md ≤ 2,5	Mf > 0
3. Ordinary virgin olive oil	≤ 3,3	≤ 20	≤ 0,20	≤ 250	≤ 1,3	≤ 0,15	≤ 0,2	≤ 2,60	≤ 0,25	≤ 0,10	≤ 0,01	Md ≤ 6,0 (4)	—
4. Virgin lampante olive oil	> 3,3	> 20	> 0,20	≤ 300 (5)	≤ 1,3	≤ 0,50	≤ 0,3	≤ 3,70	> 0,25	≤ 0,11	—	Md > 6	—
5. Refined olive oil	≤ 0,5	≤ 5	≤ 0,20	≤ 350	≤ 1,5	—	≤ 0,3	≤ 3,40	≤ 1,20	—	≤ 0,16	—	—
6. Olive oil	≤ 1,5	≤ 15	≤ 0,20	≤ 350	≤ 1,5	—	≤ 0,3	≤ 3,30	≤ 1,00	—	≤ 0,13	—	—
7. Crude olive-pomace oil	> 0,5 (**)	—	—	>350 (6)	≤ 1,8	—	≤ 0,6	—	—	—	—	—	—
8. Refined olive-pomace oil	≤ 0,5	≤ 5	≤ 0,20	> 350	≤ 2,0	—	≤ 0,5	≤ 5,50	≤ 2,50	—	≤ 0,25	—	—
9. Olive-pomace oil	≤ 1,5	≤ 15	≤ 0,20	> 350	≤ 2,0	—	≤ 0,5	≤ 5,30	≤ 2,00	—	≤ 0,20	—	—

(1) Overal upper limit for compounds detected by electron capture detector.
For compounds detected individually the upper limit is 0,10 mg/kg.
(2) Sum of isomers that could (or could not) be separated by capillary column.
(3) For the purpose of determining the presence of refined oil, where the K_{270} exceeds the limit for the category concerned, it must be determined again after passage over alumina.
(4) Where the median for 'fruity' is 0, the median of the defects must be not more than 2,5.
(5) Oils with a wax content between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if total aliphatic alcohol is less than or equal to 350 mg/kg or if the percentage of erythrodiol and uvaol is less than or equal to 3,5.
(6) Oils with a wax content between 300 mg/kg and 350 mg/kg are considered to be crude olive-pomace oil if total aliphatic alcohol is greater than 350 mg/kg and the percentage of erythrodiol and uvaol is greater than 3,5.

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Category	Acid content						Sum of the trans-linoleic isomers (%)	Sum of the trans-linoleic and trans-linolenic isomers (%)	Cholesterol (%)	Brassicasterol (%)	Campesterol (%)	Stigmasterol (%)	Betasitosterol (%) (1)	Delta-7-Stigmastenol (%)	Total sterols (mg/kg)	Erythrodiol and uvaol (%) (**)
	Myristic (%)	Linolenic (%)	Arachidic (%)	Eicosenoic (%)	Behenic (%)	Lignoceric (%)										
1. Extra virgin olive oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
2. Virgin olive oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
3. Ordinary virgin olive oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,05	≤ 0,05	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
4. Virgin lampante olive oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,10	≤ 0,10	≤ 0,5	≤ 0,1	≤ 4,0	—	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5 (2)
5. Refined olive oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,20	≤ 0,30	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
6. Olive oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,2	≤ 0,2	≤ 0,20	≤ 0,30	≤ 0,5	≤ 0,1	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 000	≤ 4,5
7. Crude olive-pomace oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,3	≤ 0,2	≤ 0,20	≤ 0,10	≤ 0,5	≤ 0,2	≤ 4,0	—	≥ 93,0	≤ 0,5	≥ 2 500	> 4,5 (3)
8. Refined olive-pomace oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,3	≤ 0,2	≤ 0,40	≤ 0,35	≤ 0,5	≤ 0,2	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 800	> 4,5
9. Olive-pomace oil	≤ 0,05	≤ 0,9	≤ 0,6	≤ 0,4	≤ 0,3	≤ 0,2	≤ 0,40	≤ 0,35	≤ 0,5	≤ 0,2	≤ 4,0	< Camp.	≥ 93,0	≤ 0,5	≥ 1 600	> 4,5

(1) Delta-5,23-Stigmastadienol + Chlerosterol + Beta-Sitosterol + Sitostanol + Delta-5-Avenasterol + Delta-5,24-Stigmastadienol.
(2) Oils with a wax content between 300 mg/kg and 350 mg/kg are considered to be lampante olive oil if total aliphatic alcohol is less than or equal to 350 mg/kg or if the percentage of erythrodiol and uvaol is less than or equal to 3,5.
(3) Oils with a wax content between 300 mg/kg and 350 mg/kg are considered to be crude olive-pomace oil if total aliphatic alcohol is greater than 350 mg/kg and the percentage of erythrodiol and uvaol is greater than 3,5.

Notes:
(a) The results of the tests must be expressed to the same number of significant digits as that specified for each characteristic.

The last significant digit must be rounded up to the next digit if the non-significant digit that follows it is greater than 4.

(b) An oil is to be placed in a different category or declared not in conformity in terms of purity if any one of the characteristics lies outside the limit laid down.

(c) The characteristics marked with an asterisk (*), relating to the quality of the oil, mean that:
— for virgin lampante olive oil, these limits (with the exception of K$_{232}$) do not have to be respected simultaneously,
— in the case of other virgin olive oils, non-compliance with at least one of these limits shall involve a change in category, while remaining classed in one of the categories foe virgin olive oils.

(d) The characteristics marked with two asterisk (**) mean that, for all olive-pomace oils concerned, these limits do not have to be respected simultaneously.

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ANNEX Ia

Sampling of batches of olive oil or olive-residue oil in immediate packaging not exceeding 100 litres

This method of sampling applies to batches of olive oil or olive-residue oil not exceeding 125 000 litres, put up in immediate packaging not exceeding 100 litres.

If the batch in question exceeds 125 000 litres, it is to be subdivided into sub-batches of approximately 125 000 litres. The method shall then be applied to each sub-batch so constituted.

1. Content of primary samples

    Primary samples must comprise the following:

    (a) where the immediate packaging has a capacity of 6 litres or more, the oil from one immediate pack, divided into at least 6 containers of 1 litre each, of which:

    — 1 container is for the analyses referred to in Annexes II, III, IX and XII,

    — 1 container is for the other analyses, and

    — the remaining containers are for any counter-analyses;

    (b) where the immediate packaging has a capacity of 2 litres or more but less than 6 litres, the oil from 4 immediate packs, of which:

    — 1 immediate pack for the analyses referred to in Annexes II, III, IX and XII,

    — one third of another pack is for the other analyses, and

    — the remainder of the oil is for any counter-analyses;

    (c) where the immediate packaging has a capacity of 0,75 litres or more but less than 2 litres, the oil from 6 immediate packs, of which:

    — 1 immediate pack is for the analyses referred to in Annexes II, III, IX and XII,

    — another pack is for the other analyses, and

    — the remainder of the oil is for any counter-analyses;

    (d) where the immediate packaging has a capacity of less than 0,75 litres, oil from a minimum number of packs with a total capacity of more than 4,5 litres, broken down as follows:

    — the oil from the minimum number of packs whose total capacity exceeds 0,75 litres is for the analyses referred to in Annexes II, III, IX and XII,

    — the same quantity again is reserved for the other analyses, and

    — the remainder of the oil is for any counter-analyses.

2. Number of primary samples to be taken

    The minimum number of primary samples to be taken will be determined by the size of the batch in accordance with the following table:

Size of batch (litres) less than	Minimum number of primary samples
7 500	2
25 000	3
75 000	4
125 000	5

    The immediate packs selected to form a primary sample must be adjacent to each other in the batch.

    In cases of doubt, Member States shall increase the number of primary samples to be taken.

3. Analyses and results

    Where possible the oil must be kept in the original containers up to the time of analysis.

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(a) Each of the primary samples taken is to be subdivided into laboratory samples, in accordance with point 2.5 of standard EN ISO 5555, and analysed as follows:

— determination of free fatty acids, as referred to in the first indent of Article 2(1),

— determination of the peroxide value, as referred to in the second indent of Article 2(1),

— spectrophotometric analysis, as referred to in the eighth indent of Article 2(1),

— determination of the fatty acid composition, as referred to in the ninth indent of Article 2(1).

(b) Where one of the results of the analyses referred to in (a) for at least one of the primary samples taken from the same batch does not comply with the characteristics of the category of oil declared, the whole of the batch concerned must be declared not to comply.

Where all the results of the analyses referred to in (a) for each of the primary samples taken from the same batch are uniform, given the repeatability characteristics of the methods concerned, and comply with the characteristics of the category of oil declared, one of the primary samples from that batch is to be subject to further analysis.

(c) Where one of the results of the analyses referred to in the second paragraph of point (b) does not comply with the characteristics of the category of oil declared, the whole of the batch concerned is to be declared not to comply.

Where all the results of the analyses referred to in the second paragraph of point (b) comply with the characteristics of the category of oil declared, the whole batch is to be declared to comply.

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ANNEX II

DETERMINATION OF THE FREE FATTY ACIDS

1.   DETERMINATION OF ACIDITY

The determination of free fatty acids in olive oils. The content of free fatty acids is expressed as acidity calculated conventionally.

1.1.   **Principle**

A sample is dissolved in a mixture of solvents and the free fatty acids present titrated using an ethanolic solution of potassium hydroxide.

1.2.   **Reagents**

All the reagents should be of recognized analytical quality and the water used either distilled or of equivalent purity.

1.2.1.   ►__C1__ Diethyl ether ◄; 95 % ethanol (v/v), mixture of equal parts by volume.

Note: ►__C1__ Diethyl ether ◄ is highly inflammable and may form explosive peroxides. Special care should be taken in its use.

Neutralize precisely at the moment of use with the potassium hydroxide solution (1.2.2), with the addition of 0,3 ml of the phenolpthalein solution (1.2.3) per 100 ml of mixture.

Note: If it is not possible to use ►__C1__ diethyl ether ◄, a mixture of solvents containing ethanol and toluene may be used. If necessary, ethanol may be replaced by propanol-2.

1.2.2.   Potassium hydroxide, titrated ethanolic solution, c(KOH) about 0,1 mol/l or, if necessary, c(KOH) about 0,5 mol/l.

The exact concentration of the ethanolic solution of potassium hydroxide must be known and checked immediately prior to use. Use a solution prepared at least five days before use and decanted into a brown glass bottle with a rubber stopper. The solution should be colourless or straw coloured.

Note: A stable colourless solution of potassium hydroxide may be prepared as follows. Bring to the boil 1 000 ml of ethanol with 8 g of potassium hydroxide and 0,5 g of aluminium shavings and continue boiling under reflux for one hour. Distill immediately. Dissolve in the distillate the required quantity of potassium hydroxide. Leave for several days and decant the clear supernatant liquid from the precipitate of potassium carbonate.

The solution may also be prepared without distillation as follows: to 1 000 ml of ethanol add 4 ml of aluminium butylate and leave the mixture for several days. Decant the supernatant liquid and dissolve the required quantity of potassium hydroxide. The solution is ready for use.

1.2.3.   Phenolphthalein, 10 g/l solution in 95 to 96 % ethanol (v/v) or alkaline blue, (in the case of strongly coloured fats) 20 g/l solution in 95 to 96 % ethanol (v/v).

1.3.   **Apparatus**

Usual laboratory equipment including:

1.3.1.   analytical balance;

1.3.2.   250 ml conical flask;

1.3.3.   10 ml burette, graduated in 0,05 ml.

1.4.   **Procedure**

1.4.1.   Preparation of the specimen for testing

(Carry out the test on the filtered sample. Where moisture and impurities together are less than 1 %, use the specimen without further treatment; where they exceed 1 %, it should be filtered.)

1.4.2.   Taking the sample

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Take a sample depending on the presumed acid number in accordance with the following table:

Expected acid value	Mass of sample (g)	Weighing accuracy (g)
< 1	20	0,05
1 to 4	10	0,02
4 to 15	2,5	0,01
15 to 75	0,5	0,001
> 75	0,1	0,0002

Weigh the sample in the conical flask (1.3.2).

1.4.3. Determination

Dissolve the sample (1.4.2) in 50 to 150 ml of the previously neutralized mixture of diethyl ►**C1** ether ◄ and ethanol (1.2.1).

Titrate while stirring with the 0,1 mol/l solution of potassium hydroxide (1.2.2) (see Note 2) until the indicator changes (the pink colour of the phenolphtalein persists for at least 10 seconds).

Note 1. The titrated ethanolic solution of potassium hydroxide (1.2.2) may be replaced by an aqueous solution of potassium or sodium hydroxide provided that the volume of water introduced does not induce phase separation.

Note 2. If the quantity of 0,1 mol/l potassium hydroxide solution required exceeds 10 ml, use the 0,5 mol/l solution.

Note 3. If the solution becomes cloudy during titration, add enough of the solvents (1.2.1) to give a clear solution.

1.5. **Acidity: expressed as percentage of oleic acid**

Acidity as a percentage by weight is equal to:

$$V \times c \times \frac{M}{1\,000} \times \frac{100}{m} = \frac{V \times c \times M}{10 \times m}$$

where:

V = the volume of titrated potassium hydroxide solution used, in millilitres;

c = the exact concentration in moles per litre of the titrated solution of potassium hydroxide used;

M = the molar weight in grams per mole of the acid used to express the result (= 282);

m = the weight in grams of the sample.

▼**C1**

Take as the result, the arithmetic mean ►**M6** of two calculations ◄ carried out.

▼__B__

ANNEX III

DETERMINATION OF PEROXIDE VALUE

1. SCOPE

This Standard describes a method for the determination of the peroxide value of oils and fats.

2. FIELD OF APPLICATION

This Standard is applicable to animal and vegetable oils and fats.

3. DEFINITION

The peroxide value is the quantity of those substances in the sample, expressed in terms of milliequivalents of active oxygen per kilogram, which oxidize potassium iodide under the operating conditions described.

4. PRINCIPLE

Treatment of the test portion, in solution in acetic acid and chloroform, by a solution of potassium iodide. Titration of the liberated iodine with standardized sodium thiosulphate solution.

5. APPARATUS

All the equipment used shall be free from reducing or oxidizing substances.

Note: Do not grease ground surfaces.

5.1. 3 ml glass scoop.

5.2. Flasks, with ground necks and stoppers, of about 250 ml capacity, dried beforehand and filled with a pure, dry inert gas (nitrogen or, preferably, carbon dioxide).

5.3. 25- or 50-ml burette, graduated in 0,1 ml.

6. REAGENTS

6.1. Chloroform, analytical reagent quality, freed from oxygen by bubbling a current of pure, dry inert gas through it.

6.2. Glacial acetic acid, analytical reagent quality, freed from oxygen by bubbling a current of pure, ►__C1__ dry inert gas ◄ through it.

6.3. Potassium iodide, saturated aqueous solution, recently prepared, free from iodine and iodates.

6.4. Sodium thiosulphate, 0,01 or 0,002 ►__C1__ Mol/L accurately ◄ standardized aqueous solution, standardized just before use.

6.5. Starch solution, 10 g/l aqueous dispersion, recently prepared from natural soluble starch.

7. SAMPLE

Take care that the sample is taken and stored away from the light, kept cold and contained in completely filled glass containers, hermetically sealed with ground-glass or cork stoppers.

8. PROCEDURE

The test shall be carried out in diffuse daylight or in artificial light. Weigh in a glass scoop (5.1) or, failing this, in a flask (5.2), to the nearest 0,001 g, a mass of the sample in accordance with the following table, according to the expected peroxide value:

▼**B**

Expected peroxide value (meq)	Weight of test portion (g)
0 to 12	5,0 to 2,0
12 to 20	2,0 to 1,2
20 to 30	1,2 to 0,8
30 to 50	0,8 to 0,5
50 to 90	0,5 to 0,3

Unstopper a flask (5.2) and introduce the glass scoop containing the test portion. Add 10 ml of chloroform (6.1). Dissolve the test portion rapidly by stirring. Add 15 ml of acetic acid (6.2), then 1 ml of potassium iodide solution (6.3). Insert the stopper quickly, shake for one minute, and leave for exactly five minutes away from the light at a temperature from 15 to 25 ºC.

Add about 75 ml of distilled water. Titrate the liberated iodine with the sodium thiosulphate solution ►**C1** (6.4) (0,002 Mol/L solution for expected values less than 12, and 0,01 Mol/L solution ◄ for expected values above 12) shaking vigorously, using starch solution (6.5) as indicator.

Carry out two determinations on the same test sample.

Carry out simultaneously a blank test. If the result of the blank exceeds 0,05 ml of ►**C1** 0,01 mol/L sodium ◄ thiosulphate solution (6.4), replace the impure reagents.

9. EXPRESSION OF RESULTS

The peroxide value (PV), expressed in milliequivalents of active oxygen per kilogram, is given by the formula:

$$PV = \frac{V \times T \times 1\,000}{m}$$

where:

V = the number of ml of the standardized sodium thiosulphate solution (6.4) used for the test, corrected to take into account the blank test;

T = ►**C1** the exact molarity ◄ of the sodium thiosulphate solution (6.4) used;

m = the weight in g, of the test portion.

Take as the result the arithmetic mean of the two determinations carried out.

▼__M6__

ANNEX IV

DETERMINATION OF WAX CONTENT BY CAPILLARY COLUMN GAS-LIQUID CHROMATOGRAPHY

1. SCOPE

This method describes a procedure for the determination of the wax content of certain fats and oils, under the test conditions.

It may be used in particular to distinguish between olive oil obtained by pressing and that obtained by extraction (olive-pomace oil).

2. PRINCIPLE

Addition of a suitable internal standard to the fat or oil, then fractionation by chromatography on a hydrated silica gel column. Recovery of the fraction eluted first under the test conditions (whose polarity is less than that of the triglycerides), then direct analysis by capillary column gas-liquid chromatography.

3. APPARATUS

3.1. 25-ml Erlenmeyer flask.

3.2. Glass column for chromatography, 15 mm internal diameter and 30-40 cm long.

3.3. Suitable gas-liquid chromatograph with a capillary column, equipped with a system for direct introduction into the column comprising the following:

3.3.1 Thermostat-controlled oven for the columns, capable of maintaining the desired temperature to within 1 ºC.

3.3.2. Cold injector for direct introduction into the column.

3.3.3. Flame-ionization detector and converter-amplifier.

3.3.4. Recorder-integrator capable of working with the converter-amplifier (3.3.3.), rate of response below 1 second, with variable paper speed.

3.3.5. Capillary column, glass or fused silico, 10 to 15 m long 0,25 to 0,32 mm internal diameter, internally covered with SE-52 or SE-54 liquid, or equivalents, to a uniform thickness of 0.10 to 0,30 μm.

3.4. Microsyringe with facilities for on-column injection capacity 10 μl, equipped with a casehardened needle.

4. REAGENTS

4.1. Silica gel, 70/230 mesh, article 7754 Merck.

Place the gel in the oven at 500 ºC for four hours. Allow to cool, then add 2 % water. Shake well the homogenize slurry. Keep in darkness for at least 12 hours prior to use.

4.2. n-hexane, for chromatography.

4.3. Ethyl ether, for chromatography.

4.4. n-heptane, for chromatography.

4.5. Standard solution of lauryl arachidate, at 0,1 % (m/v) in hexane (internal standard).

4.6. Carrier gas: hydrogen, pure, for gas-liquid chromatography.

4.7. Auxiliary gases:

— hydrogen, pure, for gas-liquid chromatography,

— air, pure, for gas-liquid chromatography.

5. PROCEDURE

5.1. Separation of the wax friction.

5.1.1. Preparation of the chromatographic column.

Suspend 15 g of silica gel hydrated at 2 % in anhydrous n-hexane and introduce into the column.

▼__M6__

Allow to settle spontaneously. Complete settling with the aid of an electric shaker to make the chromatographic band more homogeneous. Percolate 30-ml n-hexane to remove any impurities.

5.1.2. Column chromatography

Weigh exactly 500 mg of the sample into a 25-ml flask, and add a suitable amount of internal standard, depending on the assumed wax content, e.g. add 0,1 mg lauryl arachidate in the case of olive oil, and 0,25 to 0,5 mg in the case of olive-pomace oil.

Transfer the prepared sample to the chromatographic column, prepared according to 5.1., with the aid of two 2-ml portions of n-hexane.

Allow the solvent to flow to 1 mm above the upper level of the absorbent. Then start chromatographic elution; collect 140 ml of the n-hexane/ethyl ether mixture, at 99:1, at a flow of about 15 drops every 10 seconds (2,1 ml/minute).

Dry the resultant fraction in a rotary evaporator until almost all the solvent is eliminated. Remove the last 2 or 3 ml of solvent with the help of a weak current of nitrogen, then add 10 ml n-heptane.

5.2. Gas-liquid chromatographic analysis

5.2.1. Preliminary procedure, conditioning of column.

5.2.1.1. Fit the column to the gas-liquid chromatograph, connecting the inlet port to the on-column system and the outlet port to the detector.

Check the gas-liquid chromatography apparatus (operation of gas loops, detector and recorder efficiency, etc.).

5.2.1.2. If the columns is being used for the first time, it is advisable to condition it. Run a light flow of gas through the column, then switch on the gas-liquid chromatography apparatus. Gradually heat to a temperature at least 20 ºC above the operating temperature (note). Maintain this temperature for at least two hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder, regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as that required to perform the analysis. The base-line should be linear, with no peaks of any kind, and must have any deviation.

A negative rectilinear deviation indicates that the column connections are not correct; a positive deviation indicates that the column has not been properly conditioned.

Note: Keep the conditioning temperature at all times at least 20 ºC below the maximum temperature specified for the eluent employed.

5.2.2. Choice of operating conditions.

5.2.2.1. The operating conditions are generally as follows:

— column temperature: 80 ºC at first, rising by 30 ºC/minute to 120 ºC, then programmed to increase by 5 ºC/minute up to 340 ºC,
— detector temperature: 350 ºC,
— linear speed of carrier gas: hydrogen, 20 to 35 cm/sec,
— instrument sensitivity: 4 to 16 times the minimum attenuation,
— Recorder sensitivity: 1 to 2 mV, from bottom of scale,
— paper speed: 30 cm/hour,
— amount injected: 0,5-1 μl solution.

These conditions may be modified to suit the characteristics of the column and the gas-liquid chromatographic apparatus (in order to obtain chromatgrams meeting the following conditions: retention time of C32 internal standard must be 25 ± 2 minutes and the most representative peak of the waxes must lie between 60 and 100 % from the bottom of the scale).

5.2.2.2. Determine the peak integration parameters in such a way as to obtain a correct evaluation of the peak areas considered.

5.2.3. Performance of the analysis

5.2.3.1. Take up 1 μl of the solution with the aid of the 10 μl micry-syringe; draw back the piston until the needle is empty. Introduce the needle in the injection system and inject quickly after one to two seconds. After about five seconds, gently extract the needle.

5.2.3.2. Perform the recording until the waxes are completely eluted.

▼**M6**

The base-line must always satisfy the required conditions (5.2.1.2).

5.2.4. Peak identification

Identify the peaks from the retention times, by comparing them with mixtures of waxes with known retention times, analysed under the same conditions.

Figure 1 gives a wax chromatogram of a virgin olive oil.

5.2.5. Quantitative analysis

5.2.5.1. Determine the areas of the peaks corresponding to the internal standard and the aliphatic esters from C40 to C46 with the aid of the integrator.

5.2.5.2. Determine the wax content of each of the esters, in mg/kg of fat, according to the formula:

$$\text{ester (mg/kg)} = \frac{A_x \cdot m_s \cdot \blacktriangleright \underline{\textbf{M9}} \ 1\,000 \ \blacktriangleleft}{A_s \cdot m}$$

where:

A_x = area of the peak of each ester;

A_s = area of the lauryl arachidate peak;

m_s = mass of the lauryl arachidate added, in milligram;

m = mass of the sample taken for determination, in grams.

6. EXPRESSION OF THE RESULTS

Give the different wax contents, and the sum of those contents, in mg/kg of fat.

▼__M6__

APPENDIX

Determination of linear gas speed

Inject 1 to 3 μl methane (propane) into the gas-liquid chromatographic apparatus, after adjusting it to the normal operating conditions. Measure the time the gas takes to run through the column, from the moment it is injected until the peak emerges (t_M).

The linear speed in cm/sec. is given by the formula L/t_M where L is the length of the column, in cm, and t_M is the time measured in seconds.
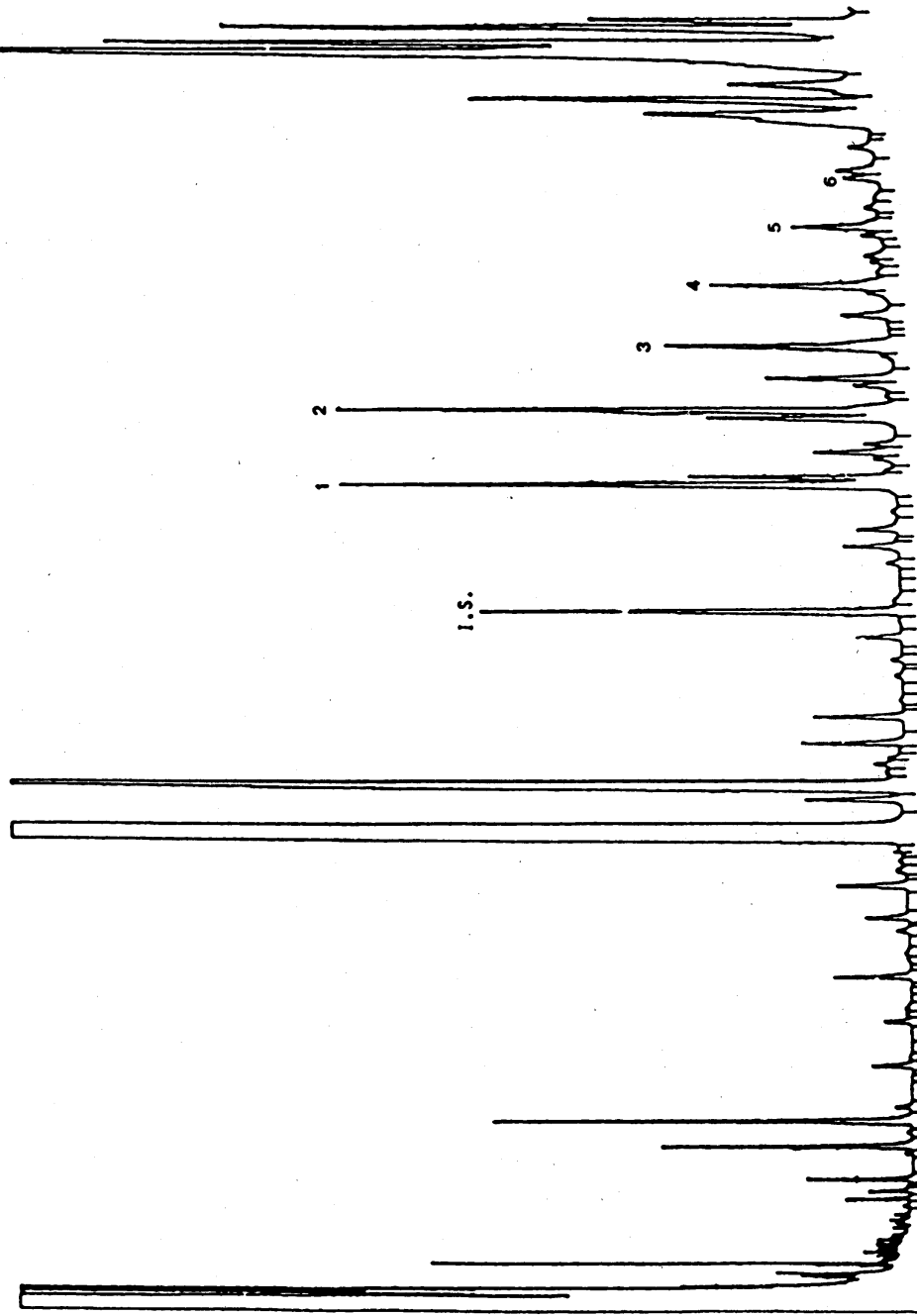
▼M6

FIGURE 1: *Wax chromatogram of a virgin olive oil.*

I.S. = Internal standard Ester C32

1 = Esters C36,
2 = Esters C38,
3 = Esters C40,
4 = Esters C42,
5 = Esters C44,
6 = Esters C46,

▼**B**

ANNEX V

DETERMINATION OF THE COMPOSITION AND CONTENT OF STEROLS BY CAPILLARY-COLUMN GAS CHROMATOGRAPHY

1. SCOPE

   The method describes a procedure for determining the individual and total sterols content of fatty substances.

2. PRINCIPLE OF THE METHOD

   The fatty substance, with added α-cholestanol as an internal standard, is saponified with potassium hydroxide in ethanolic solution and the unsaponifiables are then extracted with ►**C1** diethyl ether ◄.

   The sterol fraction is separated from the unsaponifiable extract by chromatography on a basic silica gel plate. The sterols recovered from the silica gel are transformed into trimethyl-silyl ethers and are analysed by capillary-column gas chromatography.

3. APPARATUS

   3.1. 250 ml flask fitted with a reflux condenser having ground-glass joints.

   3.2. 500 ml separating funnels.

   3.3. 250 ml flasks.

   3.4. Complete apparatus for analysis by thin-layer chromatography using 20 × 20 cm glass plates.

   3.5. Ultraviolet lamp having a wavelength of 366 or 254 nm.

   3.6. 100 μl and 500 μl microsyringes.

   3.7. A cylindrical filter funnel with a G3 porous septum (porosity 15 to 40 μm) of diameter approximately 2 cm and a depth of some 5 cm, with an attachment suitable for filtration under vacuum and a 12/21 male ground glass joint.

   3.8. 50 ml vacuum conical flask with a 12/21 ground-glass female joint which can be fitted to the filter funnel (3.7).

   3.9. A 10 ml test tube with a tapering bottom and a sealing stopper.

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

   3.10.1. a thermostatic chamber for columns capable of maintaining the desired temperature with an accuracy of ± 1 ºC;

   3.10.2. a temperature-adjustable vaporization unit with a persilanized glass vapourizing element;

   3.10.3. a flame ionization detector and converter-amplifier;

   3.10.4. an integrator-recorder suitable for use with the converter-amplifier (3.10.3) having a response time of not more than one second and a variable paper speed.

   3.11. A glass or fused-silica capillary column of length 20 to 30 m, internal diameter 0,25 to 0,32 mm, entirely coated with SE-52 or SE-54 liquid or equivalent in a uniform thickness between 0,10 and 0,30 μm.

   3.12. A 10 μl gas chromatography microsyringe with a hardened needle.

4. REAGENTS

   4.1. Potassium hydroxide, ►**C1** approximately 2 mol/L ethanolic solution. ◄ Dissolve 130 g of potassium hydroxide ►**C1** (minimum concentration 85 %) ◄ with cooling in 200 ml of distilled water and then make up to one litre with ethanol. Keep the solution in well-stoppered dark glass bottles.

   4.2. ►**C1** Diethyl ether ◄, analytical purity.

   4.3. Anhydrous sodium sulphate, analytical purity.

   4.4. Glass plates coated with silica gel, without fluorescence indicator, thickness 0,25 mm (commercially available ready for use).

▼B

4.5. Potassium hydroxide, ►C1 0,2 mol/L ◄ ethanolic solution. Dissolve 13 g of potassium hydroxide in 20 ml of distilled water and make up to one litre with ethanol.

4.6. Benzene, for chromatography. (See 5.2.2)

4.7. Acetone, for chromatography. (See 5.2.2)

4.8. Hexane, for chromatography. (See 5.2.2)

4.9. ►C1 Diethyl ether ◄, for chromatography. (See 5.2.2)

4.10. Chloroform, analytical purity. (See 5.2.2)

4.11. Reference solution for thin-layer chromatography: cholesterol or phytosterols, ►M6 2 % ◄ solution in chloroform.

4.12. 2,7-dichlorofluorescein, 0,2 % ethanolic solution. Make slightly basic by adding a few drops of ►C1 2 mol/L ◄ alcoholic potassium hydroxide solution.

4.13. Anhydrous pyridine, for chromatography.

4.14. Hexamethyl disilazane.

4.15. Trimethylchlorosilane.

4.16. Reference solutions of sterol trimethylsilyl ethers. To be prepared at the time of use from pure sterols or mixtures of sterols obtained from oils containing them.

4.17. ►C1 β-cholestanol ◄, 0,2 % solution (m/V) in chloroform (internal standard).

4.18. Carrier gas: hydrogen or helium, gas-chromatographic purity.

4.19. Auxiliary gases:

— hydrogen, gas-chromatographic purity,

— air, gas-chromatographic purity.

5. PROCEDURE

5.1. Preparation of the unsaponifiables.

5.1.1. Using the 500 μl microsyringe ►C1 introduce, into a 250 ml flask a volume of 0,2 % β-cholestanol solution in chloroform (4.17) containing an amount of cholestanol corresponding to approximately 10 % of the sterol content of the sample aliquot to be taken for the determination. ◄ For example, for 5 g of sample add 500 μl of the 0,2 % α-cholestanol solution in the case of an olive oil and 1 500 μl for ►M6 —————— ◄ olive-pomaca oil.

Evaporate to ►C1 dryness in a current ◄ of nitrogen and then weigh accurately 5 g of the dry filtered sample into the same flask.

►M6 Oils ◄ and fats containing appreciable quantities of cholesterol may show a peak having a retention time identical to cholestanol. If this occurs the sterol fraction will have to be analyzed in duplicate with and without internal standard ►M6 or betulinol will have to be used instead of cholestanol ◄.

5.1.2. Add 50 ml of ►C1 2 mol/L ◄ ethanolic potassium hydroxide solution, fit the reflux condenser and heat to gentle boiling on a water bath with continuous vigorous stirring until saponification takes place (the solution becomes clear). Continue heating for a further 20 minutes, then add 50 ml of ►C1 distilled water to the ◄ top of the condenser, detach the condenser and cool the flask to approximately 30 ºC.

5.1.3. Transfer the contents of the flask quantitatively into a 500 ml separating funnel using several rinses of distilled water, amounting in all to about 50 ml. Add approximately 80 ml of ►C1 diethyl ether ◄, shake vigorously for approximately 30 seconds and allow to settle (Note 1).

Separate off the lower aqueous phase collecting it in a second separating funnel. Perform two further extractions on the aqueous phase in the same way using 60 to 70 ml of ethyl ether on each occasion.

Note 1. Any emulsion can be destroyed by adding small quantities of ethyl or methyl alcohol by means of a spray.

5.1.4. Pool the ether extracts into a single separating funnel and wash with distilled water (50 ml at a time) until the wash water gives a neutral reaction.

▼**B**

When the wash water has been removed, dry with anhydrous sodium sulphate and ►**C1** filter through anhydrous ◄ sodium sulphate into a previously weighed 250 ml flask, washing the funnel and filter with small quantities of ►**C1** diethyl ether. ◄

5.1.5. Distil the ether down to a few ml, then bring to dryness under a slight vacuum or in a current of nitrogen, completing drying in a stove at 100 ºC for approximately a quarter of an hour, and then weigh after cooling in a desiccator.

5.2. Separation of the sterol fraction.

5.2.1. Preparation of the basic plates. Immerse the silica gel plates (4.4) completely in the ►**C1** 0,2 mol/L ◄ ethanolic potassium hydroxide solution (4.5) for 10 seconds, then allow to dry in a fume cupboard for two hours and finally place ►**C1** in an oven at ◄ 100 ºC for one hour.

Remove from the stove and keep in a calcium chloride desiccator until required for use (plates treated in this way must be used within 15 days).

Note 2. When basic silica gel plates are used to separate the sterol fraction there is no need to treat the unsaponifiables with alumina. In this way all compounds of an acid nature (fatty acids and others) are retained on the spotting line and the sterols band is clearly separated from the aliphatic and triterpene alcohols band.

5.2.2. Place a 95:5 (v/v) benzene/acetone ►**C1** mixture into the ◄ plate-developing chamber to a depth of approximately 1 cm. As an alternative a 65:35 (v/v) hexane/ethyl ether mixture may be used. Close the chamber with the appropriate cover and leave thus for approximately half an hour so that liquid-vapour equilibrium is established. Strips of filter paper dipping into the eluent may be placed on the internal surfaces of the chamber. This reduces developing time by approximately one-third and brings about more uniform and regular elution of the components.

Note 3. The developing mixture should be replaced for every test in order to achieve perfectly reproducible elution conditions.

5.2.3. Prepare an approximately 5 % solution of the unsaponifiables (5.1.5) in chloroform and, using the 100 μl microsyringe, streak a chromatographic plate ►**C1** (5.2.1) with 300 μl ◄ approximately 2 cm from one end in a streak which is as thin and as uniform as possible. In line with the streak place 2 to 3 μl of the sterol reference solution (4.11) at one end of the plate so that the sterol band can be identified after developing.

5.2.4. Place the plate in the developing chamber prepared as specified in 5.2.2. The ambient temperature should be maintained between 15 and 20 ºC. Immediately close the chamber with the cover and allow to elute until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber and evaporate the solvent in a flow of hot air or by leaving the plate for a short while under a hood.

5.2.5. Spray the plate lightly and uniformly with the 2,7-dichlorofluoroscein solution. When the plate is observed under ultraviolet light the sterol band can be identified through being aligned with the stain obtained from the reference solution. Mark the limits of the band along the edges of the fluorescence with a black pencil.

5.2.6. Using a metal spatula scrape off the silica gel in the marked area. Place the finely comminuted material removed into the filter funnel (3.7). Add 10 ml of hot chloroform, mix carefully with the metal spatula and filter under vacuum, collecting the filtrate in the conical flask (3.8) attached to the filter funnel.

Wash the residue ►**C1** in the funnel three times with diethyl ether ◄ (approximately 10 ml each time) collecting the filtrate in the same flask attached to the funnel. Evaporate the filtrate to a volume of 4 to 5 ml, transfer the residual solution to the previously weighed 10 ml test tube (3.9), evaporate to dryness by mild heating in a gentle flow of nitrogen, make up again using a few drops of acetone, evaporate again to dryness, place in a stove at 105 ºC for approximately 10 minutes and then allow to cool in a desiccator and weigh.

The residue contained in the test tube consists of the sterol fraction.

5.3. Preparation of the trimethylsilyl ethers.

▼**B**

5.3.1. Add the silylation reagent, consisting of a 9:3:1 (v/v/v) mixture of pyridine/hexamethyl disilazane/trimethyl chlorosilane (Note 4) in the ratio of 50 μl for every milligram of sterols to the test tube containing the sterol fraction, avoiding any uptake of moisture (Note 5).

> *Note 4.* Solutions which are ready for use are available commercially. Other silanizing reagents such as, for example, bis-trimethyl-silyl, trifluor acetamide + 1 % trimethyl chlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available.

5.3.2. Stopper the test tube, shake carefully (without overturning) until the sterols are completely dissolved. Stand for at least 15 minutes at ambient temperature and then centrifuge for a few minutes. The clear solution is ready for gas chromatographic analysis.

> *Note 5.* The slight opalescence which may form is normal and does not cause any interference. The formation of a white floc or the appearance of a pink colour are indicative of the presence of moisture or deterioration of the reagent. If these occur the test must be repeated.

5.4. Gas chromatographic analysis.

5.4.1. Preliminary operations, column packing.

5.4.1.1. Fit the column in the gas chromatograph, attaching the inlet end to the evaporator connected to the splitting system and the outlet end to the detector.

Carry out general checks on the gas chromatograph unit (leaks from the gas circuits, detector efficiency, efficiency of the splitting system and recording system, etc.).

5.4.1.2. If the column is being used for the first time it is recommended that it should be subjected to conditioning. Pass a gentle flow of gas through the column and then switch on the gas chromatography unit and begin gradual heating up to a temperature of at least 20 ºC above the operating temperature (Note 6). Hold this temperature for at least two hours, then place the entire unit in operating mode (adjustment of gas flows and splitting, ignition of the flame, connection with the electronic recorder, adjustment of the column chamber, detector and injector temperature, etc.) and then record the signal with a sensitivity at least two times greater than that intended for the analysis. The course of the base line must be linear, without peaks of any kind, and must not drift.

A negative straight-line drift indicates leakage from the column connections; a positive drift indicates inadequate conditioning of the column.

> *Note 6.* The conditioning temperature must always be at least 20 ºC less than the maximum temperature specified for the stationary phase used.

5.4.2. Choice of operating conditions.

5.4.2.1. The guideline operating conditions are as follows:

— column temperature: 260 ± 5 ºC,
— evaporator temperature: 280 ºC,
— detector temperature: 290 ºC,
— linear velocity of the carrier gas: helium 20 to 35 cm/s, hydrogen 30 to 50 cm/s,
— splitting ratio: from 1:50 to 1:100,
— instrument sensitivity: from 4 to 16 times the minimum attenuation,
— recording sensitivity: 1 to 2 mV f.s.,
— paper speed: 30 to 60 cm/hour,
— amount of substance injected: 0,5 to 1 μl of TMSE solution.

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 retention time for β-sitosterol should be 20 ± 5 minutes,
— the campesterol peak should be: for olive oil (mean content 3 %) 15 ± 5 % of full scale; for soya oil (mean content 20 %) 80 ± 10 % of full scale,
— all the sterols present must be separated. In addition to being separated the peaks must also be completely resolved, i.e. the peak trace should return to the base line before leaving for the next peak. Incomplete resolution is however tolerated provided that the peak at TRR 1,02 can be quantified using the perpendicular.

▼**B**

5.4.3. Analytical procedure.

5.4.3.1. Using the 10 μl microsyringe take 1 μl of hexane, draw in 0,5 μl of air and then 0,5 to 1 μl of the sample solution. Raise the plunger of the syringe further so the needle is emptied. Push the needle through the membrane of the injection unit and after one to two seconds inject rapidly, then slowly remove the needle after some five seconds.

5.4.3.2. Continue recording until the TMSE of the sterols present are completely eluted

The base line must continue to meet the requirements (5.4.1.2).

5.4.4. Peak identification.

Identify individual peaks on the basis of retention times and by comparison with mixtures of sterol TMSE analysed under the same conditions.

The sterols are eluted in the following order: cholesterol, brassicasterol, 24-methylene cholesterol, campesterol, campestanol, stigmasterol, Δ 7-campesterol, Δ 5,23-stigmastadienol, ►**C1** chlerosterol ◄, β-sistosterol, sitostanol, Δ 5-avenasterol, Δ 5,24-stigmastadienol ►**C1** , Δ 7-stigmasterol, ◄ Δ 7-avenasterol.

The retention times for sitosterol for SE-52 and SE-54 columns are shown in Table 1.

Figures 1 and 2 illustrate typical chromatograms for some oils.

5.4.5. Quantitative evaluation.

5.4.5.1. Calculate the areas of the ►**C1** β-cholestanol ◄ and the sterol peaks using the integrator. Ignore peaks for any compounds which are not included among those listed in Table 1. The response coefficient for ►**C1** β-cholestanol ◄ is to be equal to 1.

5.4.5.2. Calculate the concentration of each individual sterol in mg/100 g of fatty material as follows:

$$\text{sterol x} = \frac{A_x \cdot m_s \cdot 100}{A_s \cdot m}$$

where:

A_x = peak area for sterol x ►**M6** ——————— ◄;

A_s = area of the ►**C1** β-cholestanol ◄ peak ►**M6** ——————— ◄;

m_s = mass of ►**C1** β-cholestanol ◄ added, im milligrams;

m = mass of the sample used for determination, in grams.

6. EXPRESSION OF THE RESULTS

6.1 Record individual sterol concentrations as mg/100 g of fatty material and their sum as 'total sterols'.

6.2 Calculate the percentage of each individual sterol from the ratio of the relevant peak area to the total peak area for sterols.

$$\% \text{ of sterol x} = \frac{A_x}{\Sigma A} \cdot 100$$

where:

A_x = peak area for x;

ΣA = total peak area for sterols.

▼__B__

APPENDIX

Determination of the linear velocity of the gas

With the gas chromatograph set to normal operating conditions inject 1 to 3 μl of methane (or propane) and measure the time taken by the gas to pass through the column from the time of injection to the time at which the peak appears (t_M).

The linear velocity in cm/s is given by L/t_M, where L is the length of the column in centimetres and t_M is the measured time in seconds.

Table I

Relative retention times for sterols

Pe-ak	Identification		Relative retention time	
			SE 54 column	SE 52 column
1	cholesterol	Δ-5-cholesten-3β-ol	0,67	0,63
2	cholestanol	5α-cholestan-3β-ol	0,68	0,64
3	brassicasterol	[24S]-24-methyl-Δ-5,22-cholestadien-3β-ol	0,73	0,71
4	24-methylene-cholesterol	24-methylene-Δ-5,24-cholesten-3β-ol	0,82	0,80
5	campesterol	[24R]-24-methyl-Δ-5-cholesten-3β-ol	0,83	0,81
6	campestanol	[24R]-24-methyl-cholestan-3β-ol	0,85	0,82
7	stigmasterol	[24R]-24-ethyl-Δ-5,22-cholestadien-3β-ol	0,88	0,87
8	Δ-7-campesterol	[24R]-24-methyl-Δ-7-cholesten-3β-ol	0,93	0,92
9	Δ-5,23-stigmastadienol	[24R,S]-24-ethyl-Δ-5,23-cholestadien-3β-ol	0,95	0,95
10	chlerosterol	[24S]-24-ethyl-Δ-5,25-cholastadien-3β-ol	0,96	0,96
11	β-sitosterol	[24R]-24-ethyl-Δ-5-cholestan-3β-ol	1,00	1,00
12	sitostanol	24-ethyl-cholestan-3β-ol	1,02	1,02
13	Δ-5-avenasterol	[24Z]-24-ethylidene-5-cholesten-3β-ol	1,03	1,03
14	Δ-5,24-stigmastadienol	[24R,S]-24-ethyl-Δ-5,24-cholestadien-3β-ol	1,08	1,08
15	Δ-7-stigmastenol	[24R,S]-24-Ethyl-Δ-7,24-cholestadien-3β-ol	1,12	1,12
16	Δ-7-avenasterol	[24Z]-24-ethyliden-Δ-7-cholesten-3β-ol	1,16	1,16

▼B

Figure 1

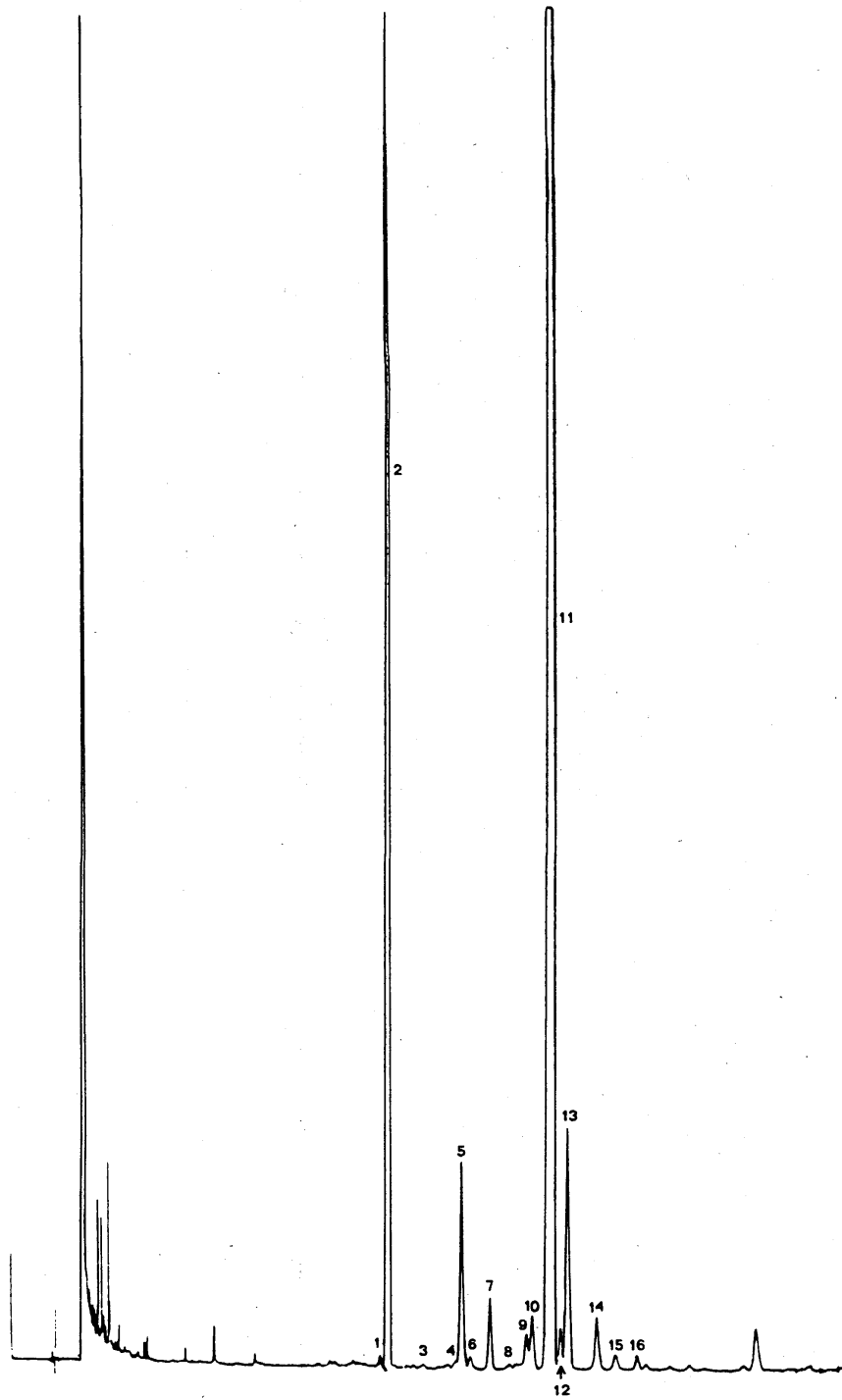*Gas chromatogram of the sterol fraction of an unrefined olive oil*
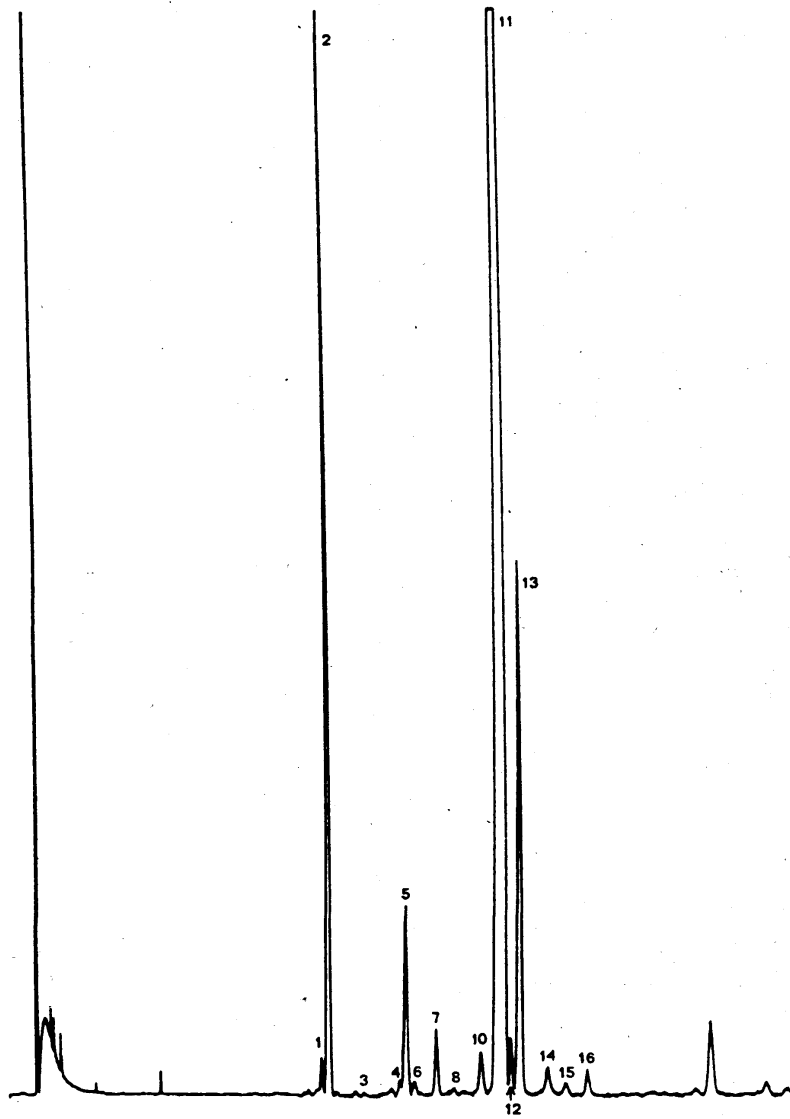
Figure 2

Gas chromatogram of the sterol fraction of a refined olive oil

▼**B**

ANNEX VI

DETERMINATION OF ERYTHRODIOL AND UVAOL

INTRODUCTION

Erythrodiol (commonly understood as the glycols erythrodiol and uvaol together) is a constituent of the unsaponifiable fraction, characteristic of some types of fatty substances. It is found at considerably higher concentrations in solvent-extracted olive oil than in other oils, such as pressed olive oil and grape pip oil, which also contain it, and so its presence may demonstrate the presence of solvent-extract olive oil.

1. SCOPE

The method describes a procedure for detecting erythrodiol in fatty substances.

2. PRINCIPLE OF THE METHOD

The fatty substance is saponified with potassium ►**C1** hydroxide in ethanolic solution. The unsaponifiable fraction is then extracted with diethyl ether ◄ and purified by passage over a column of alumina.

The unsaponifiables are subjected to thin-layer chromatography on a silica gel plate until the bands corresponding to the sterol and erythrodiol fractions are separated. The sterols and the erythrodiol recovered from the plate are transformed into trimethylsilyl ethers and the mixture is analysed by gas chromatography.

The result is expressed as the percentage of erythrodiol in the mixture of erythrodiol and sterols.

3. APPARATUS

3.1. The apparatus described in Annex V (determination of the content of sterols).

4. REAGENTS

4.1. The reagents described in Annex V (determination of the content of sterols).

4.2. Reference solution of erythrodiol, 0,5 % solution in chloroform.

5. PROCEDURE

5.1. **Preparation of the unsaponifiables.**

As described at paragraph 5.1.2 of Annex V.

5.2. **Separation of erythrodiol and the sterols.**

5.2.1. See paragraph 5.2.1 of Annex V.

5.2.2. See paragraph 5.2.2 of Annex V.

5.2.3. Prepare a 5 % solution of the unsaponifiables in chloroform.

Using the 0,1 ml microsyringe, streak a chromatographic plate with 0,3 ml of solution approximately 1,5 cm from the lower edge in a streak which is as thin and uniform as possible.

At one end of the plate place a few microlitres of the solutions of cholesterol and erythrodiol to serve as a reference.

5.2.4. Place the plate in the developing chamber prepared as specified in 5.2.1. The ambient temperature should be about 20 ºC. Immediately close the chamber with the cover and allow to elute until the solvent front reaches approximately 1 cm from the upper edge of the plate. Remove the plate from the developing chamber and evaporate the solvent in a flow of hot air.

5.2.5. Spray the plate lightly and uniformly with the alcoholic 2,7-dichlorofluoroscein solution. When the plate is observed under ultraviolet light the sterol and erythrodiol bands can be identified through being aligned with the references. Mark with a spot just outside the edges of the fluorescence.

▼**B**

5.2.6. Using a metal spatula scrape off the silica gel in the marked areas. Place the material from the plate in a 50 ml flask. Add 15 ml of hot chloroform, shake well and filter through a funnel with a sintered glass disc so that the silica gel is transferred to the filter. Wash three times with hot chloroform (10 ml each time) collecting the filtrate in a 100 ml flask. Evaporate the filtrate to a volume of 4 to 5 ml, transfer to a calibrated 10 ml conical-bottomed centrifuge tube, dry by gently heating in a current of nitrogen and weigh.

5.3. **Preparation of the trimethylsily esters**

As described in paragraph 5.3 of Annex V.

5.4. **Gas chromatographic analysis**

As described in paragraph 5.4 of the above method. The operating conditions of the gas chromatograph in analysis must be such as to perform the sterol analysis and separate the TMSE from the erythrodiol and uvaol.

Once the sample has been injected, continue recording until the sterols present, the erythrodiol and the uvaol have been eluted. Then identify the peaks (the retention times for erythrodiol and uvaol relative to β-sitosterol are about 1,45 and 1,55 respectively) and calculate the areas as for the sterols.

6. EXPRESSION OF THE RESULTS

$$\text{Erythrodiol \%} = \frac{A_1 + A_2}{A_1 + A_2 + \Sigma\, A_{\text{sterols}}} \times 100$$

where:

A_1 = peak area for erythrodiol ►**M6** ──────── ◄;

A_2 = peak area for uvaol ►**M6** ──────── ◄;

$\Sigma\, A_{\text{sterols}}$ = total peak area for sterols ►**M6** ──────── ◄.

The result is expressed to one decimal place.

▼__B__

ANNEX VII

DETERMINATION OF FATTY ACIDS IN THE 2-POSITION IN THE TRIGLYCERIDES OF OILS AND FATS

1. SCOPE

   This Standard describes a method for the determination of the composition of that fraction of the fatty acids of an oil or fat which is esterified at the 2-position (or internal position) of the glycerol.

2. FIELD OF APPLICATION

   This Standard is applicable to oils and fats having a melting point below 45 ºC, owing to the peculiarities of the action of pancreatic lipase.

   It is not applicable unreservedly to oils and fats containing substantial amounts of: fatty acids with 12 or fewer carbon atoms (coconut and palm-kernel oils, butterfat), or highly unsaturated fatty acids (with more than four double bounds) containing 20 or more carbon atoms (fish and marine animals oils), or fatty acids containing oxygenated groups, other than the acid group.

3. PRINCIPLE

   Possible neutralization of acid oils and fats in a solvent. Purification by passing onto an alumina column. Partial hydrolysis of triglycerides by pancreatic lipase during a determined time. Separation of the formed monoglycerides by thin-layer chromatography and methanolysis of these monoglycerides. Analysis of these methyl esters by gas-liquid chromatography.

4. APPARATUS

4.1. 100 ml round-bottomed flask.

4.2. 25 ml round bottomed flask, with ground joint.

4.3. 1 m-long air condenser, to fit the flask 4.2.

4.4. 250 ml conical flask.

4.5. 50 ml beaker.

4.6. 500 ml separating funnel.

4.7. Chromatographic glass column, 13 mm internal diameter, 400 mm in length, fitted with a fritted glass disc and a tap.

4.8. 10 ml centrifuge tube, with ground glass stopper.

4.9. 5 ml burette, graduated in 0,05 ml.

4.10. 1 ml hypodermic syringe, fitted with a thin needle.

4.11. Microsyringe, to deliver drops of 3 to 4 μl.

4.12. Spreader for thin-layer chromatography.

4.13. Glass plates for thin-layer chromatography, 20 × 20 cm.

4.14. Glass developing tank for thin-layer chromatography, with ground-glass lid, suitable for the 20 × 20 plates.

4.15. Spray for thin-layer chromatography.

4.16. Oven regulated at 103 ± 2 ºC.

4.17. Thermostat regulatable between 30 and 45 ºC to within 0,5 ºC.

4.18. Rotary evaporator.

4.19. Vibrating electric shaker, allowing vigorous agitation of the centrifuge tube.

4.20. Ultraviolet lamp for the examination of the thin-layer plates.

For the control of the lipase activity:

4.21. pH meter.

4.22. Spiral stirer.

▼__B__

4.23. 5 ml burette.

4.24. Stop-watch.

For the possible preparation of the lipase:

4.25. Laboratory stirrer, suitable for the dispersion and mixture of heterogeneous materials.

5. REAGENTS

5.1. *n*-hexane, or, failing this, light petroleum (bp 30 to 50 ºC), chromatographic quality.

5.2. 2-propanol, or ethanol, 95 % (v/v), analytical reagent quality.

5.3. 2-propanol, or ethanol, 1/1 aqueous solution.

5.4. Diethyl ether, free from peroxides.

5.5. Acetone.

5.6. Formic acid, at least 98 % (m/m).

5.7. Developing solvent: mixture of *n*-hexane (5.1), diethyl ether (5.4) and formic acid (5.6) in proportions 70/30/1 (v/v/v).

5.8. Activated alumina for chromatography, neutral, grade Brockmann I.

5.9. Silica powder, with binder, of suitable quality for thin-layer chromatography.

5.10. Pancreatic lipase of suitable quality (Notes 1 and 2).

5.11. Sodium hydroxide, 120 g/l aqueous solution.

5.12. Hydrochloric acid, aqueous ►__C1__ solution 6 Mol/l. ◄

5.13. Calcium chloride ($CaCl_2$), 220 g/l aqueous solution.

5.14. Sodium cholate (enzymatic quality), 1 g/l aqueous solution.

5.15. Buffer solution: 1 M aqueous solution of *tris*-hydroxymethylaminomethane bring to pH 8 by addition of hydrochloric acid (5.12) (check by potentiometer).

5.16. Phenolphthalein, 10 g/l solution in 95 % (v/v) ethanol.

5.17. 2′,7′ -dichlorofluorescein, 2 g/l solution in 95 % (v/v) ethanol, rendered slightly alkaline by the addition of one drop of 1 N sodium hydroxide solution per 100 ml.

For the control of the lipase activity:

5.18. Neutralized oil.

5.19. Sodium ►__C1__ hydroxide, 0,1 M ◄ aqueous solution.

5.20. Sodium cholate (enzymatic quality), 200 g/l aqueous solution.

5.21. Gum arabic, 100 g/l aqueous solution.

6. PREPARATION OF THE SAMPLE

If the sample has an acidity below 3 %, determined according to Annex II, purify directly over alumina according to 6.2.

If the sample has an acidity over 3 %, determined according to Annex II, neutralize by alkali in the presence of a solvent according to 6.1, then pass over alumina according to 6.2.

6.1. Neutralization by alkali in the presence of solvent

Into a separating funnel (4.6) introduce about 10 g of the crude oil and add 100 ml of hexane (5.1), 50 ml of 2-propanol (5.2), a few drops of phenolphthalein solution (5.16), and an amount of the sodium hydroxide solution (5.11) corresponding to the free acidity of the oil plus 0,3 % excess. Shake vigorously for one minute, add 50 ml of distilled water, shake again and leave to settle.

After separation, remove the bottom soap layer. Also remove any intermediate layers (mucilage, insoluble matter). Wash the hexane solution of the neutralized oil with successive 25 to 30 ml portions of the 2-propanol solution (5.3) until the pink colour of the phenolphthalein disappears.

▼**B**

Remove most of the hexane by distillation under vacuum in the rotary evaporator (4.18), dry the oil at 30 to 40 ºC under vaccum with the help of a current of pure nitrogen until the hexane has been removed completely.

6.2. Purification through alumina

Prepare a suspension of 15 g of activated alumina (5.8) in 50 ml hexane (5.1) and pour it, while stirring, onto the chromatographic column (4.7). Let the alumina settle evenly, and allow the solvent level to fall to within 1 to 2 mm above the absorbent. Carefully pour onto the column a solution of 5 g of oil in 25 ml of hexane (5.1); collect the whole of the effluent from the column in a round-bottomed flask (4.1).

7. Preparation of the chromatographic plates

Thoroughly clean the glass plates (4.13) with ethanol, light petroleum and acetone to eliminate any trace of fatty matter.

In a conical flask (4.4) place 30 g of silica powder (5.9). Add 60 ml of distilled water. Stopper and shake vigorously for one minute. Transfer the slurry immediately to the spreader (4.12) and coat the clean plates with a layer 0,25 mm thick.

Dry the plates in the air for 15 minutes and then for an hour in the oven (4.16) at 103 ± 2 ºC. Cool the plates in a desiccator to room temperature before use.

Prepared plates are available in commerce.

8. PROCEDURE

8.1. Hydrolysis with pancreatic lipase.

Into the centrifuge tube (4.8) weigh about 0,1 g of the prepared sample, if the sample is a liquid oil proceed directly as below.

Add 20 mg of lipase (5.10) and 2 ml of the buffer solution (5.15). Shake well, but carefully, and then add 0,5 ml of the sodium cholate solution (5.14) and 0,2 ml of the calcium chloride solution (5.13). Close the tube with the ground stopper, shake cautiously (avoid wetting the stopper) and put the tube immediately in the thermostat (4.17) maintained at 40 ± 0,5 ºC and shake by hand for exactly one minute.

Remove the tube from the thermostat, and agitate vigorously by means of the electric shaker (4.19) for exactly two minutes.

Cool immediately in running water; add 1 ml of hydrochloric acid (5.12) and 1 ml of diethyl ether (5.4). Stopper and mix vigorously by means of the electric shaker. Allow to stand and remove the organic layer by means of the syringe (4.10), if necessary after centrifuging.

8.2. Separation of the monoglycerides by thin-layer chromatography

Apply the extract to the chromatographic plate with the microsyringe (4.11), about 1,5 cm from the bottom edge, in a thin, uniform line, as narrow as possible. Set the plate in the well-saturated developing tank (4.14) and develop with the developing solvent (5.7) at about 20 ºC, up to about 1 cm from the top edge of the plate.

Dry the plate in the air at the temperature of the tank, and spray it with the 2′,7′ -dichlorofluorescein solution (5.17). Identify the monoglyceride band (R_f about 0,035) under ultraviolet light (4.20).

8.3. Analysis of the monoglycerides by gas-liquid chromatography

Remove the band obtained in 8.2 with the help of a spatula (avoid removing components remaining on the base line) and transfer in the methylation flask (4.2).

Treat the collected silica directly by the methods described in Annex X B alternative so as to convert the monoglycerides into methyl esters, and then examine the esters by gas chromatography as described in Annex X A.

9. EXPRESSION OF RESULTS

Calculate the fatty acid composition in the 2-position to one decimal place (Note 3).

10. NOTES

Note 1: Checking the activity of the lipase

▼B

Prepare an oil emulsion by shaking a mixture of 165 ml of the solution of gum arabic (5.21), 15 g of crushed ice and 20 ml of a neutralized oil (5.18) in a suitable agitator.

In a beaker (4.5) place 10 ml of this emulsion, followed successively by 0,3 ml of the sodium cholate solution (5.20) and and 20 ml of distilled water.

Put the beaker into a thermostat maintained at 37 ± 0,5 ºC (Note 4); insert the electrodes of a pH meter (4.21) and a spiral stirrer (4.22).

By means of a burette (4.23) add dropwise the sodium hydroxide solution (5.19) until the pH reaches 8,5.

Add sufficient of an aqueous suspension of the lipase (see below). ►C1 As soon as ◄ the pH meter indicates a pH of 8,3, start the stop-watch (4.24) and drip in the sodium hydroxide solution (5.19) at such a rate as to maintain the pH at 8,3. Read off the volume of alkali solution consumed every minute.

Record the observations in the form of a graph, using the time readings as abscissae and the ml of alkali solution required to maintain the pH constant as ordinates. A linear graph should be obtained.

The lipase suspension mentioned above is a 1 per thousand (m/m) suspension in water. For each test sufficient of this suspension should be used so that about 1 ml of the alkali solution is consumed in four to five minutes. Usually about 1 to 5 mg of the powder is required.

The lipase unit is defined as the amount of enzyme which will liberate 10 μ-equivalents of acid per minute. Then the activity A of the powder used, measured in lipase units per mg, is given by the formula:

$$A = \frac{V \times 10}{m}$$

where V is the number of the sodium hydroxide solution (5.19) consumed per minute, calculated from the graph, m is the mass in mg, of the test portion of the powder.

Note 2: Preparation of the lipase

Lipases having satisfactory lipase activity are available commercially. But it is also possible to prepare them in the laboratory as follows:

Chill 5 kg of fresh pig pancreas to 0 ºC; remove the surrounding solid fat and connective tissue and triturate in a blender so as to obtain a pasty fluid. Stir this paste with the stirrer (4.25) for four to six hours with 2,5 l of anhydrous acetone and centrifuge. Extract the residue thrice more with the same volume of acetone, then twice with 1/1 (V/V) mixture of acetone and diethyl ether, and twice with diethyl ether.

Dry the residue *in vacuo* for 48 hours to obtain a stable powder, which should be stored in a refrigerator.

Note 3: In every case it is advisable to determine the composition of the total fatty acids of the same sample, since the comparison with that of the acids in the 2-position will help in the interpretation of the figures obtained.

Note 4: The temperature of hydrolysis is set at 37 ºC, as a liquid oil is being used. However, it is set at 40 ºC for the test sample, so as to allow the examination of fats with melting points up to 45 ºC.

▼**B**

ANNEX VIII

▼**C1**

DETERMINATION OF TRILINOLEIN CONTENT

▼**B**

1. SCOPE

    Determination of composition of triglyceride in olive oils ►**C1** in terms of their equivalent ◄ carbon number by high-performance liquid chromatography.

    ►**C1** The present method ◄ describes a method of separation and quantitative determination of the triglyceride composition of vegetable oils in terms of their molecular weight and degree of unsaturation as a function of their equivalent carbon number (see Note 1).

2. FIELD OF APPLICATION

    ►**C1** This method is ◄ applicable to all vegetable oils containing triglycerides of long-chain fatty acids. The method is especially appliable to the detection of the presence of small quantities of semi-drying oils (rich in linoleic acid) in vegetable oils containing oleic acid as the predominant unsaturated fatty acid, such as olive oil.

3. PRINCIPLE

    Separation of triglycerides according to their equivalent carbon number by high-performance liquid chromatography (reversed phase polarity) and interpretation of the chromatograms.

4. APPARATUS

4.1. High-performance liquid chromatograph, allowing thermostatic control of column temperature.

4.2. Injection unit for 10 μl delivery.

4.3. Detector: differential refractometer. The full-scale sensitivity should be at least 10^{-4} unit of refractive index.

4.4. Column: stainless steel tube 250 mm in length and of internal diameter 4,5 mm, packed with 5 μm diameter particles of silica with 22 to 23 % carbon in the form of octadecylsilane (Note 2).

4.5. Recorder and/or integrator.

5. REAGENTS

    The reagents should be of analytical purity. Elution solvents should be degassed, and may be recycled several times without effect on the separations.

5.1. Chloroform.

5.2. Acetone.

5.3. Acetonitrile.

5.4. Elution solvent: acetonitrile + acetone (proportions to be adjusted to obtain the desired separation; begin with 50:50 mixture).

5.5. Solubilization solvent: acetone or 1:1 acetone-chloroform mixture.

5.6. Reference triglycerides: either commercial triglycerides (tripalmitin, triolein, etc.) may be used and the retention times thence plotted in accordance with the equivalent carbon number, or alternatively a reference chromatogram obtained from soya oil (see Notes 3 and 4 and Figures 1 and 2).

6. PREPARATION OF SAMPLES

    A 5 % solution of the samples to be analysed is prepared by weighing 0,5 ± 0,001 g of the sample into a 10 ml graduated flask and making up to 10 ml with the solubilization solvent (5.5).

▼**B**

7. PROCEDURE

7.1. Set up the chromatographic system. Pump elution solvent (5.4) at a rate of 1,5 ml/mm to purge the entire system. Wait until a stable base line is obtained.

Inject 10 μl of the sample prepared as in 6.

8. CALCULATION AND EXPRESSION OF RESULTS

Use the internal standardization method, i.e. assume that the sum of the areas of the peaks corresponding to the various triglycerides is equal to 100 %. Calculate the relative percentage of each triglyceride using the formula:

$$\% \text{ triglyceride} = \frac{\text{area of peak}}{\text{sum of peak areas}} \times 100$$

The result to be given to one decimal place.

Note 1. The elution order can be determined by calculating the equivalent carbon numbers, often defined by the relation $\text{ECN} = \text{CN} - 2n$, where CN is the carbon number and n is the number of double bonds; it can be calculated much more precisely by taking into account the origin of the double bond. If n_o, n_l and n_{ln} are the numbers of double bonds attributable to oleic, linoleic and linolenic acids respectively, the equivalent carbon number can be calculated by means of a relation of the formula:

$$\text{ECN} = \text{CN} - d_o n_o - d_l n_l - d_{ln} n_{ln}$$

where the coefficients d_o, d_l and d_{ln} can be calculated by means of the reference triglycerides. Under the conditions specified in this method the relation obtained will be close to:

$$\text{ECN} = \text{CN} - [2,60 \ n_o] - [2,35 \ n_l] - [2,17 \ n_{ln}]$$

Note 2. Examples: Lichrosorb (Merck) RP18 Art 50333;

Lichrosphere or equivalent (Merck) 100 CH18 Art 50377.

Note 3. With several reference triglycerides it is also possible to calculate the resolution with respect to triolein,

$$\alpha = \text{RT}'/\text{RT}'_{olein}$$

by use of the reduced retention time $\text{RT}' = \text{RT} - \text{RT}_{solvent}$.

The graph of log α against f (number of double bonds) enables the retention values to be determined for all the triglycerides of fatty acids contained in the reference triglycerides — see Figure 2.

Note 4. The efficiency of the column should permit clear separation of the peak of trilinolein from the peaks of the triglycerides with an adjacent RT.

▼**M11**

Note 5: In order to permit clear separation of the trilinolein peak from adjacent peaks or from any interfering substances, lampante virgin oil and crude olive-residue oil should be purified beforehand in accordance with the following method:

Absorb 200 μl of undiluted oil in a silica column for liquid-solid extraction (type SEP PAC silica cartridge-waters part. No 51900).

Elute the triglycerides with 20 ml anhydrous hexane for HPLC for not more than 20 seconds.

Dry the eluted product in a nitrogen flow and dissolve in isopropanol or actone (5 ml). Inject 10 to 20 μl into HPLC. The fatty acid composition of the oil must be checked to ensure that is the same before and after purification, within the accuracy range of the analysis method adopted.;
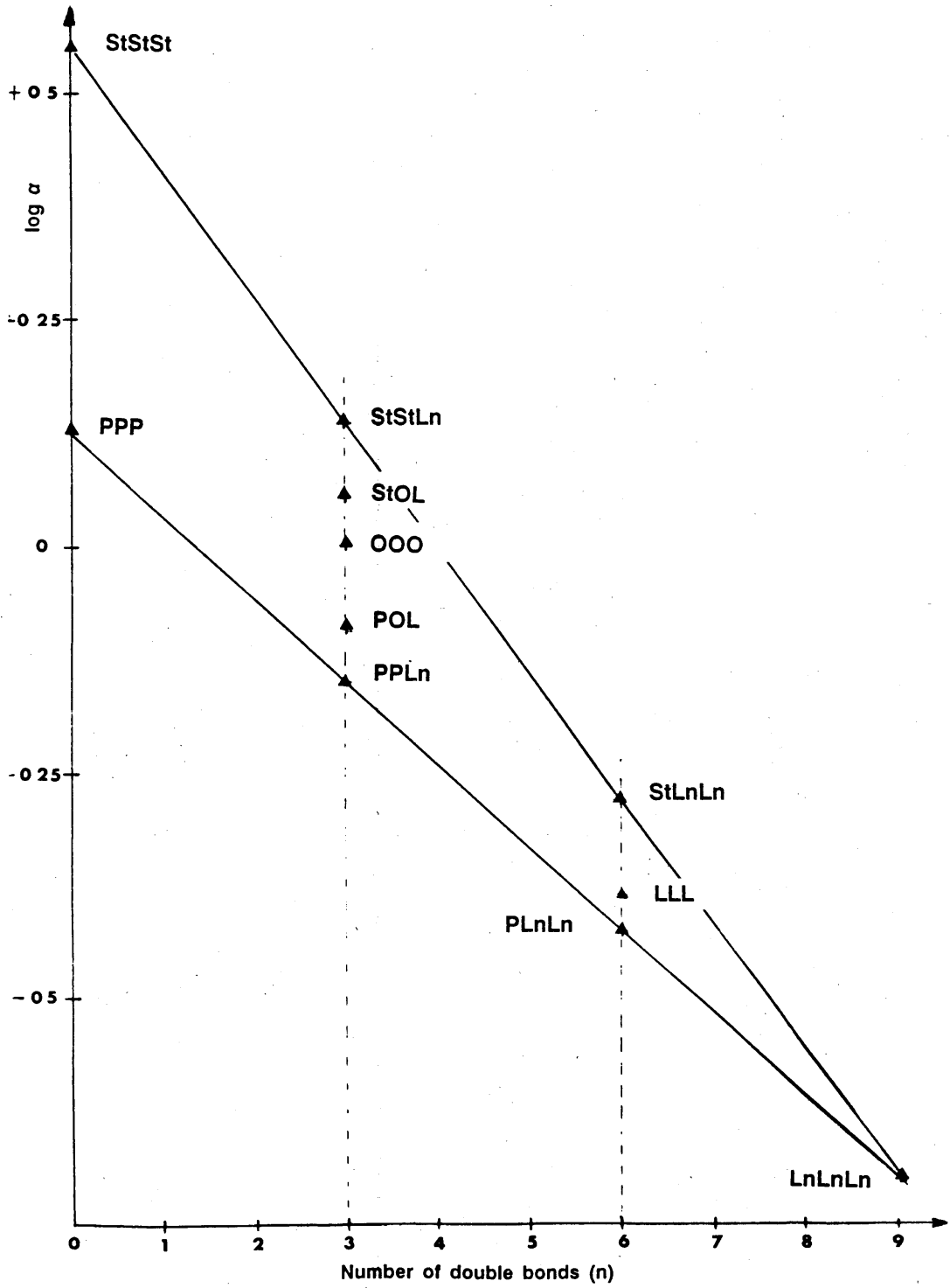
▼B

Figure 1

Chromatogram of a sample of soya oil
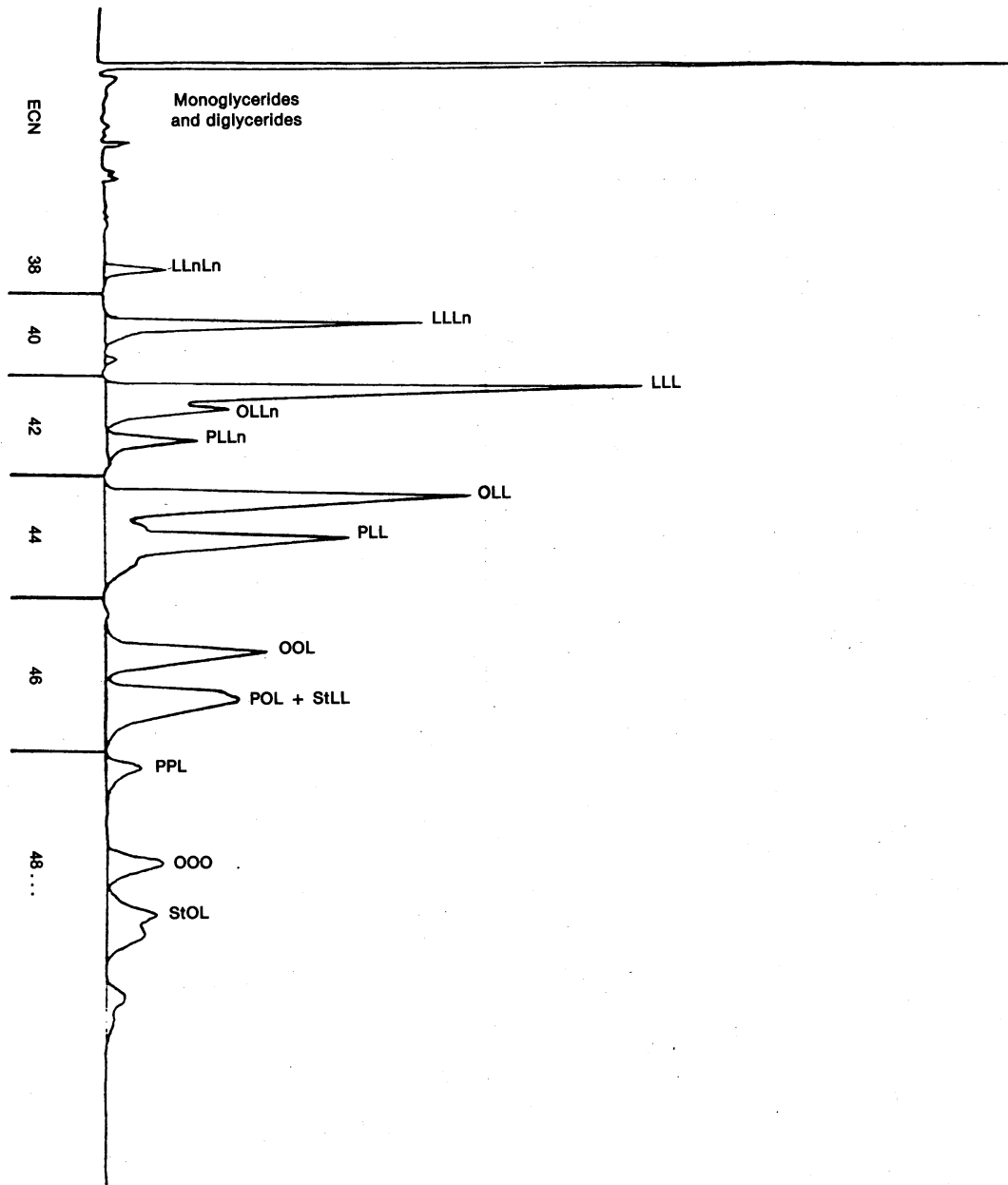
P = palmitic acid

L = linoleic acid

St = stearic acid

ln = linolenic acid

O = oleic acid

▼<u>B</u>

Figure 2

Graph of log α against f (number of double bonds)

La = lauric acid

St = stearic acid

Ln = linoleic acid

My = myristic acid

O   = oleic acid

P   = palmitic acid

L   = linoleic acid

▼**B**

ANNEX IX

SPECTROPHOTOMETRIC INVESTIGATION IN THE ULTRAVIOLET

FOREWORD

Spectrophotometric examination in the ultraviolet can provide information on the quality of a fat, its state of preservation and changes brought about in it by technological processes.

The absorption at the wavelengths specified in the method is due to the presence of conjugated diene and triene systems. These absorptions are expressed as specific extinctions $E^{1\%}_{1\,cm}$ (the extinction of 1 % solution of the fat in the specified solvent, in a thickness of 1 cm) conventionally indicated by K (also referred to as 'extinction coefficient').

1. SCOPE

The method describes the procedure for performing a spectrophotometric examination ►**C1** of olive oil in ◄ the ultraviolet.

2. PRINCIPLE OF THE METHOD

The fat in question is dissolved in the required solvent and the extinction of the solution is then determined at the specified wavelengths with reference to pure solvent. Specific extinctions are calculated from the spectrophotometer readings.

3. EQUIPMENT

3.1. A spectrophotometer for measuring extinction in the ultraviolet between 220 and 360 nm, with the possibility of reading individual nanometric units.

3.2. Rectangular quartz cuvettes, with covers, having an optical length of 1 cm. When filled with water or other suitable solvent the cuvettes should not show differences between them of more than 0,01 extinction units.

3.3. 25 ml graduated flasks.

▼**M6**

3.4. Chromatography column having an upper part 270 mm in length and a diameter of 35 mm and a lower part 270 mm in length and a diameter of approximately 10mm.

▼**B**

4. REAGENTS

4.1. Spectrophotometrically pure iso-octane (2,2,4-trimethylpentane). With reference to distilled water this should have a transmittance of not less than 60 % at 220 nm and not less than 95 % at 250 nm, or

— spectrophotometrically pure cyclohexane: with reference to distilled water this should have a transmittance of not less than 40 % at 220 nm and not less than 95 % at 250 nm.

▼**M6**

▼**B**

4.2. Basic alumina for column chromatography prepared and checked as described in Appendix I.

4.3. n-hexane, for chromatography.

5. PROCEDURE

5.1. The sample in question must be perfectly homogeneous and without suspected impurities. Oils which are liquid at ambient temperature are to be filtered through paper at a temperature of approximately 30 ºC, hard fats are to be homogenized and filtered at a temperature of not more than 10 ºC above the melting point.

5.2. Weigh accurately approximately 0,25 g of the sample so prepared into a 25 ml graduated flask, make up to the mark with the solvent specified and homogenize. The resulting solution must be perfectly clear. If opalescence or turbidity is present filter quickly through paper.

▼__B__

5.3. Fill a cuvette with the solution obtained and measure the extinctions at an appropriate wavelength between 232 and 276 nm, using the solvent used as a reference.

The extinction values recorded must lie within the range 0,1 to 0,8. If not the measurements must be repeated using more concentrated or more dilute solutions as appropriate.

5.4. When a determination of specific extinction is required after passage over alumina, proceed as follows. Place 30 g of basic alumina in suspension in hexane in the chromatography column. After the adsorbent has settled remove the excess hexane down to approximately 1 cm above the top of the alumina.

Dissolve 10 g of the fat, homogenized and filtered as described in 5.1, in 100 ml of hexane and pour the solution into the column. Collect the eluate and evaporate off all the solvent under vacuum at a temperature below 25 ºC.

Proceed immediately as specified in 5.2 using the fat so obtained.

6. EXPRESSION OF THE RESULTS

6.1. Record the specific extinctions (extinction coefficients) at the various wavelengths calculated as follows:

$$K_\lambda = \frac{E_\lambda}{c \cdot s}$$

where:

K_λ = specific extinction at wavelength λ;

E_λ = extinction measured at wavelength λ;

c = concentration of the solution in g/100 ml;

s = thickness of the cuvette in cm.

The results are to be expressed to two decimal places.

6.2. Spectrophotometric analysis of olive oil in accordance with the official method in the EEC regulations specifies determination of the specific extinction in iso-octane solution at wavelengths of 232 and 270 nm and the determination K, which is given by:

$$\Delta K = K_m - \frac{K_{m-4} + K_{m+4}}{2}$$

where K_m is the specific extinction at wavelength m, the wavelength for maximum absorption around 270 nm.

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APPENDIX I

Preparation of the alumina and testing its activity

A.1.1. Preperation of the alumina

Place alumina which has been previously desiccated in a furnace at 380 to 400 ºC for three hours into a hermetically sealed container, add distilled water in the ratio of 5 ml per 100 g of alumina, immediately close the container, shake repeatedly, and then allow to rest for at least 12 hours before use.

A.1.2. Checking the activity of the alumina

Prepare a chromatographic column with 30 g of alumina. Working as described in paragraph 5.4 pass a mixture consisting of:

— 95 % virgin olive oil having a specific extinction of less than 0,18 at 268 nm,

— 5 % ground-nut oil treated with earth in the refining process, having a specific extinction of not less than 4 at 268 nm

through the column.

If after passage through the column the mixture has a specific extinction of more than 0,11 at 268 nm the alumina is acceptable, if not the level of dehydration must be increased.

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APPENDIX II

Calibration of the spectrophotometer

A.2.   The equipment must be checked at intervals (at least every six months) for both wavelength response and the accuracy of the response.

A.2.1.   The wavelength may be checked using a mercury vapour lamp or by means of suitable filters.

A.2.2.   In order to check the response of the photocell and the photomultiplier proceed as follows: weigh 0,2000 g of pure potassium chromate for spectrophotometry and dissolve in 0,05 N potassium hydroxide solution in a 1 000 ml graduated flask and make up to the mark. Take precisely 25 ml of the solution obtained, transfer to a 500 ml graduated flask and dilute up to the mark using the same potassium hydroxide solution.

Measure the extinction of the solution so obtained at 275 nm, using the potassium hydroxide solution as a reference. The extinction measured using a 1 cm cuvette should be 0,200 ± 0,005.

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ANNEX X A

ANALYSIS BY GAS CHROMATOGRAPHY OF METHYL ESTERS OF FATTY ACIDS

1. SCOPE

This method gives general guidance for the application of gas chromatography, using packed or capillary columns, to determine the qualitative and quantitative composition of a mixture of fatty acid methyl esters obtained in accordance with the method specified in Annex X B.

The method is not applicable to polymerized fatty acids.

2. REAGENTS

2.1. **Carrier gas**

Inert gas (nitrogen, helium, argon, hydrogen, etc.), thoroughly dried and with an oxygen content of less than 10 mg/kg.

Note 1. Hydrogen, which is used as a carrier gas only with capillary columns, can double the speed of analysis but is hazardous. Safety devices are available.

2.2. **Auxiliary gases**

2.2.1. Hydrogen (purity ≥ 99,9 %), feree from organic impurities.

2.2.2. Air or oxygen, free from organic impurities.

2.3. **Reference standard**

A mixture of methyl esters of pure fatty acids, or the methyl esters of a fat of known composition, preferably similar to that of the fatty matter to be analyzed.

Care shall be taken to prevent the oxidation of polyunsaturated fatty acids.

3. APPARATUS

The instructions given relate to the usual equipment used for gas chromatography, employing packed and/or capillary columns and a flame-ionization detector. Any apparatus giving the efficiency and resolution specified in 4.1.2 is suitable.

3.1. **Gas chromatograph**

The gas chromatograph shall comprise the following elements.

3.1.1. Injection system

Use an injection system either:

(a) with packed columns, having the least deadspace possible (in this case the injection system shall be capable of being heated to a temperature 20 to 50 °C higher than that of the column); or

(b) with capillary columns, in which case the injection system shall be specially designed for use with such columns. It may be of the split type or it may be of the splitless on column injector type.

Note 2. In the absence of fatty acids with less than 16 carbon atoms, a moving needle injector may be used.

3.1.2. Oven

The oven shall be capable of heating the column to a temperature of at least 260 °C and of maintaining the desired temperature to within 1 °C with a packed column and within 0,1 °C with a capillary column. The last requirement is particularly important when a fused silica tube is used.

The use of temperature-programmed heating is recommended in all cases, and in particular for fatty acids with less than 16 carbon atoms.

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3.1.3. Packed column

3.1.3.1. Column, constructed of a material inert to the substances to be analyzed (i.e. glass or stainless steel) having the following dimensions:

(a) length: 1 to 3 m. A relatively short column should be used when long-chain fatty acids (above C_{20}) are present. When analyzing acids with 4 or 6 carbon atoms, it is recommended that a column 2 m in length is used;

(b) internal diameter: 2 to 4 mm.

Note 3. If polyunsaturated components with more than three double bonds are present, they may be decomposed in a stainless steel column.

Note 4. A system with packed twin columns may be used.

3.1.3.2. Packing, comprising the following elements:

(a) *support:* acid-washed and silanized diatomaceous earth, or other suitable inert support with a narrow range of grain size (25 μm range between the limits 125 to 200 μm) the average grain size being related to the internal diameter and length of the column;

(b) *stationary phase:* polyester type of polar liquid (e.g. diethylene glycol polysuccinate, butanediol polysuccinate, ethyleneglycol poly-adipate, etc.) cyanosilicones or any other liquid permitting the chromatographic separation required (see clause 4). The stationary phase should amount to 5 to 20 % (m/m) of the packing. A non-polar stationary phase can be used for certain separations.

3.1.3.3. Conditioning of the column

With the column disconnected, if possible, from the detector, gradually heat the oven to 185 ºC and pass a current of inert gas through the freshly prepared column at a rate of 20 to 60 ml/min for at least 16 hours at this temperature, and for a further 2 hours at 195 ºC.

3.1.4. Capillary column

3.1.4.1. Tube, made of a material inert to the substances to be analysed (usually glass or fused silica). The internal diameter shall be between 0,2 and 0,8 mm. The internal surface shall undergo an appropriate treatment (e.g. surface preparation, inactivation) before receiving the stationary phase coating. A length of 25 mm is sufficient in most cases.

3.1.4.2. Stationary phase, usually of the type polyglycol (poly(ethylene glycol) 20 000), polyester (butanediol polysuccinate) or polar polysiloxane (cyanosilicones). Bonded (cross-linked) columns are suitable.

Note 5. There is a risk of polar polysiloxanes giving rise to difficulties in the identification and separation of linolenic acid and C_{20} acids.

The coatings shall be thin, i.e, 0,1 to 0,2 μm.

3.1.4.3. Assembly and conditioning of the column

Observe the normal precautions for assembling capillary columns, i.e. arrangement of the column in the oven (support), choice and assembly of joints (leak tightness), positioning of the ends of the column in the injector and the detector (reduction of dead-spaces). Place the column under a flow of carrier gas (e.g. 0,3 bar (30 kPa) for a column of length 25 mm and internal diameter 0,3 mm).

Condition the column by temperature programming of the oven at 3 ºC/min from ambient temperature to a temperature 10 ºC below the decomposure limit of the stationary phase. Maintain the oven at this temperature for one hour until stabilization of the baseline. Return it to 180 ºC to work under isothermal conditions.

Note 6. Suitably pre-conditioned columns are available commercially.

3.1.5. Detector, preferably capable of being heated to a temperature above that of the column.

3.2. **Syringe**

The syringe shall have a maximum capacity of 10 μl, and be graduated in 0,1 μl divisions.

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3.3. **Recorder**

If the recorder curve is to be used to calculate the composition of the mixture analysed, an electronic recorder of high precision, compatible with the apparatus used, is required. The recorder shall have the following characteristics:

(a) rate of response, below 1,5 s, preferably 1 s (the rate of response is the time taken for the recording pen to pass from 0 to 90 % following the sudden introduction of a 100 % signal);

(b) width of the paper, 20 cm minimum;

(c) paper speed, adjustable to values between 0,4 and 2,5 cm/min.

3.4. **Integrator**

Rapid and accurate calculation can be performed with the help of an electronic integrator. This shall give a linear response with adequate sensitivity, and the correction for deviation of the base-line shall be satisfactory.

4. PROCEDURE

The operations described in 4.1 to 4.3 relate to the use of a flame-ionization detector.

As an alternative a gas chromatograph employing a catharometer detector (working on the principle of thermal conductivity changes) may be used. The operating conditions are then modified as described in clause 6.

4.1. **Test conditions**

4.1.1. Selection of optimum operating conditions

4.1.1.1. Packed column

In the selection of the test conditions, the following variables should be taken into account:

(a) the length and diameter of the column;

(b) the nature and amount of the stationary phase;

(c) the temperature of the column;

(d) the carrier gas flow;

(e) the resolution required;

(f) the size of the test portion, selected in such a way that the assembly of the detector and electrometer gives a linear response;

(g) the duration of analysis.

In general, the values given in Table 1 and Table 2 will lead to the desired results, i.e. at least 2 000 theoretical Iplates per metre of column length for methyl stearate and its elution within about 15 minutes.

Where the apparatus allows it, the injector should be at a temperature of about 200 ºC and the detector at a temperature equal to or higher than that of the column.

As a rule, the ratio of the flow-rate of the hydrogen supplied to the flame-ionization detector to that of the carrier gas varies from 1:2 to 1:1 depending on the diameter of the column. The flow of oxygen is about 5 to 10 times that of the hydrogen.

Table 1

Internal diameter of column mm	Carrier gas flow ml/min
2	15 to 25
3	20 to 40
4	40 to 60

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Table 2

Concentration of stationary phase % (m/m)	Column temperature ºC
5	175
10	180
15	185
20	185

4.1.1.2. Capillary column

The properties of efficiency and permeability of capillary columns mean that the separation between constituents and the duration of the analysis are largely dependent on the flow-rate of the carrier-gas in the column. It will therefore be necessary to optimize the operating conditions by acting on this parameter (or more simply on the headloss of the column), according to whether one wishes to improve the separations or to make a rapid analysis.

4.1.2. Determination of the number of theoretical plates (efficiency) and resolution (See Figure 1)

Carry out the analysis of a mixture of methyl stearate and methyl oleate in about equivalent proportions (for example, methyl esters from cocoa butter).

Choose the temperature of the column and the carrier gas flow so that the maximum of the methyl stearate peak is recorded about 15 minutes after the solvent peak. Use a sufficient quantity of the mixture of methyl esters that the methyl stearate peak occupies about three-quarters of the full scale.

Calculate the number of theoretical plates, n (efficiency), using the formula:

$$n = 16 \left[\frac{dr_1}{\omega_1} \right]^2$$

and the resolution, R, using the formula:

$$R = \frac{2\Delta}{\omega_1 + \omega_2}$$

where:

dr_1 is the retention distance, in millimetres, from the start of the chromatogram to the maximum of the peak for methyl stearate;

ω_1 and ω_2 are the widths, in millimetres, of the peaks for methyl stearate and methyl oleate respectively, measured between the points of intersection of the tangents at the points of inflection of the curve with the base-line;

Δ is the distance, in millimetres, between the peak maxima for methyl stearate and methyl oleate;

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and the resolution index, lr, using the formula
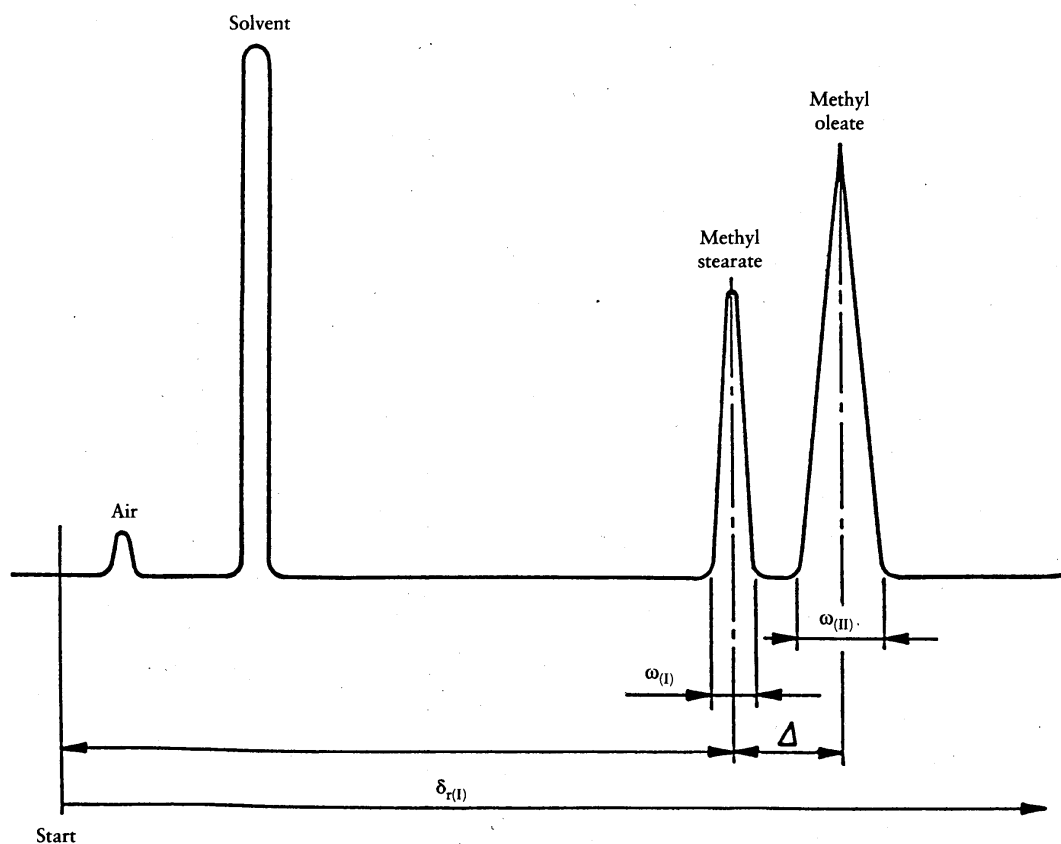
$$\frac{a}{b}$$

where:

a = the height of the smallest peak, measured from the base line;

b = the height of the lowest point of the valley between the two adjacent peaks, measured from the base line.

▼**B**

Figure 1

Chromatogram for determining the number of theoretical plates (efficiency) and resolution

The operating conditions to be selected are those which will afford at least 2 000 theoretical plates per metre of column length for methyl stearate and a resolution of at least 1,25.

4.2. **Test portion**

Using the syringe (3.2) take 0,1 to 2 μl of the solution of methyl esters prepared according to Annex X B and inject them into the column.

In the case of esters not in solution, prepare a solution of approximately 100 mg/ml in heptane of chromatographic quality, and inject 0,1 to 1 ml of this solution.

If the analysis is for constituents present only in trace amounts, the size of the test portion may be increased (up to 10-fold).

4.3. **Analysis**

Generally, the operating conditions shall be those defined in 4.1.1.

Nevertheless, it is possible to work with a lower column temperature when the determination of fatty acids with fewer than 12 carbon atoms is required, or at a higher temperature when determining fatty acids with more than 20 carbon atoms. On occasion, it is possible to employ temperature programming in both these cases. For example, if the sample contains the methyl esters of fatty acids with fewer than 12 carbon atoms, inject the sample at 100 °C (or at 50 to 60 °C if butyric acid is present) and immediately raise the temperature at a rate of 4 to 8 °C/min to the optimum. In certain cases, the two procedures can be combined.

After the programmed heating, continue the elution at a constant temperature until all the components have been eluted. If the instrument does not have programmed heating, use it at two fixed temperatures between 100 and 195 °C.

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If necessary, it is recommended that an analysis be carried out on two fixed phases with different polarities to verify the absence of masked peaks, for example in the case of the simultaneous presence of $C_{18:3}$ and $C_{20:0}$, or $C_{18:3}$ and $C_{18:2}$ conjugated.

4.4. **Preparation of the reference chromatogram and reference graphs**

Analyze the reference standard mixture (2.3) using the same operating conditions as those employed for the sample, and measure the retention times or retention distances for the constituent fatty acids. Construct on semi-logarithmic paper, for any degree of unsaturation, the graphs showing the logarithm of retention time or distance as a function of the number of carbon atoms. In isothermal conditions, the graphs for straight-chain acids of the same degree of unsaturation should be straight lines. These lines should be approximately parallel.

It is necessary to avoid conditions such that 'masked peaks' exist, i.e. where the resolution is insufficient to separate two constituents.

5. EXPRESSION OF RESULTS

5.1. **Qualitative analysis**

Identify the methyl ester peaks for the sample from the graphs prepared in 4.4, if necessary by interpolation.

5.2. **Quantitative analysis**

5.2.1. Determination of the composition

Apart from exceptional cases, use the internal normalization method, i.e. assume that the whole of the components of the sample are represented on the chromatogram, so that the total of the areas under the peaks represents 100 % of the constituents (total elution).

If the equipment includes an integrator, use the figures obtained therefrom. If not, determine the area under each peak by multiplying the height of the peak by its width at mid-height, and where necessary take into account the various attenuations used during the recording.

5.2.2. Method of calculation

5.2.2.1. General case

Calculate the content of a given component *i,* expressed as a percentage by mass of methyl esters, by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all the peaks, using the following formula:

$$\frac{A_i}{\Sigma A} \times 100$$

where:

A_i is the area under the peak corresponding to component i;

ΣA is the sum of the areas under all the peaks.

Give the result to one decimal place.

Note 7: In this general case, the result of the calculation based on relative areas is considered to represent a percentage by mass. For the cases in which this assumption is not allowed, see 5.2.2.2.

5.2.2.2. Use of correction factors

In certain cases, for example in the presence of fatty acids with fewer than eight carbon atoms or of acids with secondary groups, when using thermal conductivity detectors or where the highest degree of accuracy is particularly required, correction factors should be used to convert the percentages of peak areas into mass percentages of the components.

Determine the correction factors with the help of a chromatogram derived from the analysis of a reference mixture of methyl esters of known composition, carried out under operating conditions identical with those used for the sample.

For this reference mixture, the percentage by mass of component *i* is given by the formula:

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$$\frac{m_i}{\Sigma m} \times 100$$

where:

m_i is the mass of component i in the reference mixture;

Σm is the total of the masses of the various components of the reference mixture.

From the chromatogram of the reference mixture (4.4) calculate the percentage (area/area) for component *i* as follows:

$$\frac{A_i}{\Sigma A} \times 100$$

where:

A_i is the area under the peak corresponding to component *i;*

ΣA is the sum of the areas under all the peaks.

The correction factor is then calculated as:

$$K_i = \frac{m_i \times \Sigma A}{A_i \times \Sigma m}$$

Commonly, the correction factors are expressed relative to K_{C16}, so that the relative factors become:

$$K_i' = \frac{K_i}{K_{C16}}$$

For the sample, the content of each component *i*, expressed as a percentage by mass of methyl esters, is:

$$\frac{K_i' \times A_i}{\Sigma\left(K_i' \times A_i\right)} \times 100$$

Give the results to one decimal place.

5.2.2.3. Use of an internal standard

In certain analyses (for example where not all of the fatty acids are quantified, such as when acids with four and six carbons are present alongside acids with 16 and 18 carbons, or when it is necessary to determine the absolute amount of a fatty acid in a sample) it is necessary to use an Internal Standard. Fatty acids with five, 15 or 17 carbons are frequently used. The correction factor (if any) for the Internal Standard should be determined.

The percentage by mass of component *i,* expressed as methyl esters, is then given by the formula:

$$\frac{m_s \times K_i' \times A_i}{m \times K_s' \times A_s} \times 100$$

where:

A_i is the area under the peak corresponding to component *i;*

A_s is the area under the peak corresponding to the Internal Standard;

K_i' is the correction factor for component *i* (relative to K_{C1});

K_s' is the correction factor for the Internal Standard (relative to K_{C16});

m is the mass, in milligrams, of the test portion;

m_s is the mass, in milligrams, of the Internal Standard.

Give the results to one decimal place.

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6. SPECIAL CASE — DETERMINATION OF TRANS-ISOMERS

It is possible to determine the content of trans-isomers in fatty acids with a number of carbon atoms between 10 and 24 by separating the methyl esters using gas chromatography capillary columns having a specific polarity.

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6.1. A capillary column made of silica having an internal diameter of between 0,25 mm and 0,32 mm and a length of 50 m, coated with cyanopropisilicon, the thickness of the coating being between 0,1 and 0,3 μm (type SP 2380, C.P. sil 88, silor 10 and similar types).

6.2. The methyl esters are prepared using the procedure set out in Annex X B. As a precaution, fatty substances having a free acidity over 3 % must be neutralized in accordance with 6.1 of Annex VII.

6.3. The operating conditions for gas chromatography are overall as follows:

— column temperature set between 150 ºC and 230 ºC (for example 165 ºC for 15 minutes then increasing by 5 ºC a minute to 200 ºC);

— injector temperature: 250 ºC if the splitting system is used or the initial temperature of the column if the on-column system is used;

— detector temperature: 260 ºC;

— flow rate of the carrier gas (helium and hydrogen): 1,2 ml a minute.

The quantity injected must be such that in the conditions of sensitivity employed the height of the peak corresponding to the methyl ester of the arachidic acid is equal to or greater than 20 % of the bottom of the scale.

6.4. Identification of the various methyl esters is effected on the basis of the retention times which are compared with those for the reference mixtures (as indicated at point 2.3).

The esters of trans fatty acids are eluted before the corresponding cis-isomers. An example of a chromatogram is given in figure 2.

Figure 2:

Gas chromatogram of the trans-isomers of fatty acid using capillary column.
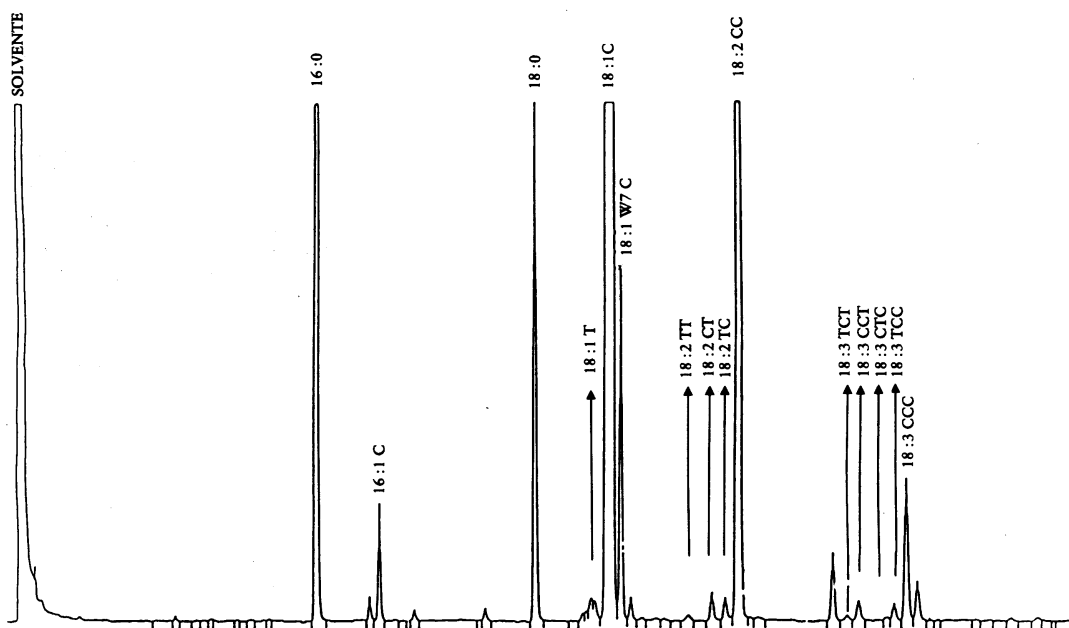
6.5. The efficiency of the column determined in accordance with point 4.1.2 must be such as to allow separation of certain critical couples, for example the couple formed by the massif of the transisoleic acids and the oleic acid peak, trans C18:1/cis C18:1, with a resolution index greater than 2.

6.6. The percentage of the various trans fatty acids is calculated on the basis of the relationship between the surface of the relevant peak and the sum of the surfaces of all the peaks present.

The percentages of:

— the trans octadecenoic acids (T 18: 1) indicated in Annex I to this Regulation as the sum of the transoleic isomers;

— the cis-trans and trans-cis octadecadienoic acids [(CT/TC) 18: 2] indicated in Annex I to this Regulation as the sum of the translinoleic isomers;

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— the trans-cis-trans, cis-cis-trans, cis-trans-cis, trans-cis-cis, octadeca-
trienoic acids [(TCT + CCT + CTC + TCC) 18: 3], indicated in
Annex I of this Regulation as the sum of the translinolenic isomers

are taken into account.

Note 8: Taking into account the particular characteristics of this
method, please give the results with 2 decimals.

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►__M2__ 7. ◄ SPECIAL CASE — USE OF A CATHAROMETER DETECTOR
(WORKING ON THE PRINCIPLE OF THERMAL CONDUCTIVITY
CHANGES)

A gas chromatograph employing a detector working on the principle of
thermal conductivity changes (a catharometer) may also be used for the
determination of the qualitative and quantitative composition of a
mixture of fatty acid methyl esters. If it is used, the conditions specified
in clause 3 and clause 4 should be modified as shown in Table 3.

For quantitative analysis, use the correction factors defined in 5.2.2.2.

Table 3

Variable	Value/condition
Column	Length: 2 to 4 m Internal diameter: 4 mm
Support	Grain size between 160 and 200 μm
Concentration of stationary phase	15 to 25 % (m/m)
Carrier gas	Helium or, failing this, hydrogen, with as low an oxygen content as possible
Auxiliary gases	None
Injector temperature	From 40 to 60 ºC above that of the column
Column temperature	180 to 200 ºC
Flow of carrier gas	Usually between 60 and 80 ml/min
Size of test portion injected	Usually between 0,5 and 2 μl

►__M2__ 8. ◄ TEST REPORT

The test report shall specify the methods used for the preparation of the
methyl esters and for the gas chromatographic analysis, and the results
obtained. It shall also mention all operating details not specified in this
International Standard, or regarded as optional, together with details of
any incidents which may have influenced the results.

The test report shall include all information necessary for the complete
identification of the sample.

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ANNEX X B

PREPARATION OF THE FATTY ACID METHYL ESTERS FROM OLIVE OIL AND OLIVE-POMACE OIL

The following two methods are recommended for preparing the fatty acid methyl esters from olive oils and olive-pomace oils:

Method A: Trans-esterification with cold methanolic solution of potassium hydroxide

Method B: Methylation by heating with sodium methylate in methanol followed by esterification in acid medium.

Each method will be applied according to the analytical parameter to be determined and the oil category as indicated below:

(a) determination of difference between actual and theoretical content of triglycerides with ECN42 (ΔECN42):

— method A will be applied to samples of all the oil categories after purification of the oil by passing it through a silica gel column;

(b) determination of the fatty acid composition:

— method A will be applied directly to samples of the following oil categories:

— virgin olive oils with an acidity of less than 3,3 %,

— refined olive oil,

— olive oil (blend of virgin olive oils and refined olive oil),

— refined olive-pomace oil,

— olive-pomace oil (blend of virgin olive oils and refined olive-pomace oil);

— method B will be applied directly to samples of the following oil categories:

— virgin olive oil with an acidity of more than 3,3 %,

— crude olive-pomace oil;

(c) determination of trans-isomers of fatty acids:

— method A will be applied directly to samples of the following oil categories:

— virgin olive oils with an acidity of less than 3,3 %,

— refined olive oil,

— olive oil (blend of virgin olive oils and refined olive oil),

— refined olive-pomace oil,

— olive-pomace oil (blend of virgin olive oils and refined olive-pomace oil);

— method A will be applied to the following categories of oils after purification of the oil by passing it through a silica gel column:

— virgin olive oil with an acidity of more than 3,3 %,

— crude olive-pomace oil.

PURIFICATION OF OIL SAMPLES

When necessary, the samples will be purified by passing the oil through a silica gel column, eluting with hexane/diethyl ether (87:13, v/v) as described in IUPAC method 2.507.

Alternatively, solid-phase extraction on silica gel phase cartridges can be used. A silica gel cartridge (1 g, 6 ml) is placed in a vacuum elution apparatus and washed with 6 ml of hexane. The vacuum is released to prevent the column from becoming dry and then a solution of the oil (0,12 g approximately) in 0,5 ml of hexane is loaded into the column and vacuum is applied. The solution is pulled down and then eluted with 10 ml of hexane/diethyl ether (87:13 v/v) under vacuum. The combined eluates are homogenised and divided in two similar volumes. An aliquot is evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. The pomace is dissolved in 1 ml of heptane and the solution is ready for fatty acid analysis by GC. The second aliquot is evaporated and the pomace is dissolved in 1 ml of acetone for triglyceride analysis by HPLC, if necessary.

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METHODS FOR PREPARING THE FATTY ACID METHYL ESTERS

1.   *Method A: Trans-esterification with cold methanolic solution of potassium hydroxide*

1.1.   **Purpose**

This rapid method is applicable to olive oils and olive-pomace oils with a free fatty acid content of less than 3,3 %. Free fatty acids are not esterified by potassium hydroxide. Fatty acid ethyl esters are trans-esterified at a lower rate than glyceridic esters and may be only partially methylated.

1.2.   **Principle**

Methyl esters are formed by trans-esterification with methanolic potassium hydroxide as an intermediate stage before saponification takes place (title 5 in ISO-5509:2000, title 5 in IUPAC method 2.301).

1.3.   **Reagents**

Methanol containing not more than 0,5 % (m/m) water.

Heptane, chromatographic quality.

Potassium hydroxide, approximately 2 N methanolic solution: dissolve 11,2 g of potassium hydroxide in 100 ml of methanol.

1.4.   **Apparatus**

Screw-top test tubes (5 ml volume) with cap fitted with a PTFE joint.

Graduated or automatic pipettes, 2 ml and 0,2 ml

1.5.   **Procedure**

In a 5 ml screw-top test tube weigh approximately 0,1 g of the oil sample. Add 2 ml of heptane, and shake. Add 0,2 ml of 2 N methanolic potassium hydroxide solution, put on the cap fitted with a PTFE joint, tighten the cap, and shake vigorously for 30 seconds. Leave to stratify until the upper solution becomes clear. Decant the upper layer containing the methyl esters. The heptane solution is suitable for injection into the gas chromatograph. It is advisable to keep the solution in the refrigerator until gas chromatographic analysis. Storage of the solution for more than 12 hours is not recommended.

2.   *Method B: Methylation by heating with sodium methylate in methanol followed by esterification in acid medium*

2.1.   **Purpose**

This method is applicable to olive oils and olive-pomace oils with a free fatty acid content of more than 3,3 %.

2.2.   **Principle**

Neutralisation of the free fatty acids and alkaline methanolysis of the glycerides, followed by esterification of the fatty acids in acid medium (title 4.2. in IUPAC method 2.301).

2.3.   **Reagents**

— heptane, chromatographic quality,
— methanol containing not more than 0,05 % (m/m) water,
— sodium methylate, 0,2 N methanolic solution: dissolve 5 g of sodium in 1 000 ml of methanol (this may be prepared from commercial solutions),
— phenolphthalein, 0,2 % methanolic solution,
— sulphuric acid, 1 N in methanolic solution: add 3 ml of 96 % sulphuric acid to 100 ml of methanol,
— saturated solution of sodium chloride in water.

2.4.   **Apparatus**

— 50 ml flat-bottomed volumetric flask with long, narrow, ground neck,

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— reflux condenser: air condenser (1 m long) with ground joint appropriate to the neck of the flask,

— boiling chips,

— glass funnel.

2.5.   **Procedure**

Transfer about 0,25 g of the oil sample into a 50 ml ground-necked volumetric flask. With the aid of a funnel, add 10 ml of 0,2 N sodium methylate in methanol and the boiling chips. Fit a reflux condenser, shake, and bring to the boil. The solution should become clear, which usually occurs in about 10 minutes. The reaction is complete after 15 minutes. Remove the flask from the source of heat, wait until the reflux stops, remove the condenser, and add two drops of phenolphthalein solution. Add a few ml of 1 N sulphuric acid in methanol solution until the solution becomes colourless and then add 1 ml in excess. Fit the condenser and boil again for 20 minutes. Withdraw from the source of heat and cool the flask under running water. Remove the condenser, add 20 ml of saturated sodium chloride solution, and shake. Add 5 ml of heptane, plug the flask, and shake vigorously for 15 seconds.

Leave to settle until the two phases have separated. Add saturated sodium chloride solution again until the aqueous layer reaches the lower end of the flask neck. The upper layer containing the methyl esters fills the flask neck. This solution is ready to be injected in the GC.

Caution: Methylation by method B must be done under a hood.

2.6.   **Alternatives to methylation Method B**

2.6.1.   *Method C*

2.6.1.1. Principle

The fatty matter undergoing analysis is treated with methanol-hydrochloric acid, in a sealed vial, at 100 ºC.

2.6.1.2. Apparatus

— Strong glass vial of a capacity of about 5 ml (height 40 to 45 mm, diameter 14 to 16 mm).

— 1 and 2 ml graduated pipettes.

2.6.1.3. Reagents

Solution of hydrochloric acid in 2 % methanol. This is prepared from gaseous hydrochloric acid and anhydrous methanol (Note 1).

Hexane, chromatographic quality.

Note 1: Commercial solutions of hydrogen chloride in methanol can be used. Small amounts of gaseous hydrochloric acid can easily be prepared in the laboratory by simple displacement from the commercial solution (p = 1,18) by dripping concentrated sulphuric acid. Since hydrochloric acid is very rapidly absorbed by methanol, it is advisable to take the usual precautions when dissolving it, e.g. introduce the gas through a small inverted funnel with the rim just touching the surface of the liquid. Large quantities of methanolic hydrochloric acid solution can be prepared in advance, as it keeps perfectly in glass-stoppered bottles stored in the dark. Alternatively, this reagent can be prepared by dissolution of acetyl chloride in anhydrous methanol.

2.6.1.4. Procedure

— Place in the glass vial 0,2 g of the fatty matter, which has previously been dried out on sodium sulphate and filtered, and 2 ml of hydrochloric acid-methanol solution. Heat seal the vial.

— Immerse the vial at 100 ºC for 40 minutes.

— Cool the vial under running water, open, add 2 ml of distilled water and 1 ml of hexane.

— Centrifuge and remove the hexane phase, which is ready for use.

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2.6.2. *Method D*

2.6.2.1. Principle

The fatty matter undergoing analysis is heated under reflux with methanol-hexane-sulphuric acid. The methyl esters obtained are extracted with petroleum ether.

2.6.2.2. Apparatus

— Test tube of a capacity of about 20 ml, fitted with an air reflux condenser approximately 1 m in length, with ground glass joints.
— 5 ml graduated pipette.
— 50 ml separating funnel.
— 10 ml and 25 ml measuring beakers.
— 15 ml test tube with conical base.

2.6.2.3. Reagents

— Methylation reagent: anhydrous methanol-hexane-concentrated sulphuric acid (p = 1,84) in the ratio 75:25:1 (V/V/V).
— 40 to 60 ºC petroleum ether.
— Anhydrous sodium sulphate.

2.6.2.4. Procedure

Place 0,1 g of oil in the 20 ml test tube and add 5 ml of methylation reagent.

Fit the reflux condenser and heat for 30 minutes in a boiling water bath (Note 2).

Transfer quantitatively the mixture into a 50 ml separating funnel, with the aid of 10 ml distilled water and 10 ml petroleum ether. Shake vigorously, and allow the phases to separate, remove the aqueous phase and wash the ether layer twice with 20 ml distilled water. Add to the separating funnel a small quantity of anhydrous sodium sulphate, shake, allow to settle for a few minutes and filter, collecting the filtrate in a 15 ml test tube with a conical base.

Evaporate the solvent over a water bath in a current of nitrogen.

Note 2: To control boiling, insert a glass rod into the test tube and limit the temperature of the water bath to 90 ºC.

3. *Precision parameters*

The statistical evaluation of the precision of methods A and B was published by the International Olive Oil Council in its method COI/T.20/CO. No 24.

RECOMMENDATIONS FOR GAS CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACID ESTERS FROM OLIVE OIL AND OLIVE-POMACE OIL

1. *Procedure*

The gas chromatographic analysis of solutions of fatty esters in heptane is to be carried out according to standard ISO-5508 using a capillary column (50 m length x 0,25 or 0,32 mm i.d.) impregnated with cyanopropylsilicone phase as indicated for the determination of fatty acid trans-isomers (COI/T.20/Doc. no. 17).

Figure 1 gives the typical gas chromatographic profile of an olive-pomace oil containing methyl and ethyl esters of fatty acids, and trans-isomers of methyl esters.

2. *Calculations*

2.1. For the calculation of the fatty acid composition and ΔECN42, all the following fatty acids will be taken into account:

Myristic (C14:0).

Palmitic (C16:0). Sum of the areas of the peaks corresponding to the methyl and ethyl esters.

Palmitoleic (C16:1). Sum of the areas of the peaks corresponding to the ω9 and ω7 isomers of the methyl ester.

Margaric (C17:0).

Margaroleic (C17:1).

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Stearic (C18:0).

Oleic (C18:1). Sum of the areas of the peaks corresponding to the ω9 and ω7 isomers of the methyl ester, ethyl ester, and trans-isomers of the methyl ester.

Linoleic (C18:2). Sum of the areas of the peaks corresponding to the methyl and ethyl esters, and the trans-isomers of the methyl ester.

Arachidic (C20:0).

Linolenic (C18:3). Sum of the areas of the methyl ester and the trans-isomers of the methyl ester.

Eicosenoic (C20:1).

Behenic (C22:0).

Lignoceric (C24:0).

Squalene will not be taken into account for the calculation of the total area.

2.2.   For the calculation of the percentage of trans-C18:1 the peak corresponding to the methyl esters of this fatty acid is to be used. For the sum [trans-C18:2 + trans-C18:3], all the peaks corresponding to the trans-isomers of these two fatty acids are to be added together. For the calculation of the total area, all the peaks mentioned in 2.1. are to be taken into account (see COI/T.20/Doc. No. 17).

The calculation of the percentage of each fatty acid will be carried out according to the formula:% X = (Area X x 100)/(total area)
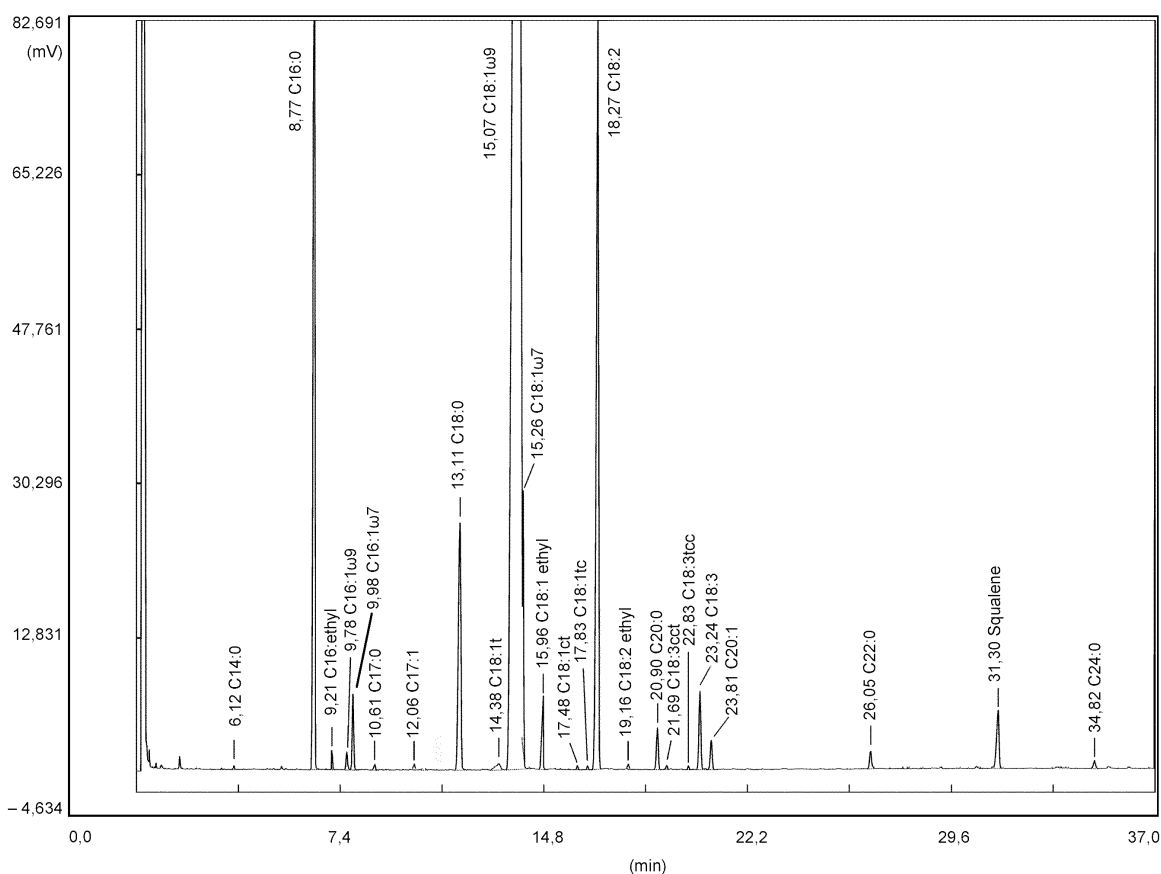

Figure 1: Gas chromatographic profile obtained by the cold methylation method from olive-pomace oil. The chromatographic peaks correspond to the methyl and ethyl esters except where otherwise indicated.

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ANNEX XI

DETERMINATION OF VOLATILE HALOGENATED SOLVENTS CONTENT OF OLIVE OIL

1. METHOD

   Analysis by gas chromatography using the head space technique.

2. EQUIPMENT

2.1. Gas chromatography apparatus fitted with an electron capture detector (ECD).

2.2. Head space apparatus.

2.3. Gas chromatography column, of glass, 2 m long and 2 mm in diameter, stationary phase. OV101 10 % or equivalent, impregnating a calcined diatomaceous earth, acid washed and silanised and of a particle size of 80 to 100 mesh.

2.4. Carrier and auxiliary gas: nitrogen for gas chromatography, suitable for detection by electron capture.

2.5. Glass flasks, 10 to 15 ml, with teflon coating and aluminium stopper with fitment for entry of syringe.

2.6. Hermetically sealing clamps.

2.7. Gas syringe 0,5 to 2 ml.

3. REAGENTS

   Standard: halogenated solvents of a degree of purity suitable for gas chromatography.

4. PROCEDURE

4.1. Exactly weigh around 3 g of oil in a glass flask (not to be reused); hermetically seal it. Place it in a thermostat at 70 ºC for one hour. Using a syringe carefully remove 0,2 to 0,5 ml of the head space. Inject this into the column of the gas chromatography apparatus regulated as follows:

   — injector temperature: 150 ºC,

   — column temperature: 70 to 80 ºC,

   — detector temperature: 200 to 250 ºC.

   Other temperatures may also be used provided the results remain equivalent.

4.2. Reference solutions: prepare standard solutions using refined olive oil with no trace of solvents with concentrations ranging from 0,05 to 1 ppm (mg/kg) and corresponding to the presumed content of the sample. The halogenated solvents may be diluted using pentane.

4.3. Quantitative assessment: correlate the surfaces or the elevations of the peaks of the sample and of the standard solution of the concentration presumed closest. If the deviation is greater than 10 % the analysis must be repeated in comparison with another standard solution until the deviation is within 10 %. The content is determined on the basis of the average of the elementary injections.

4.4. Expression of results: in ppm (mg/kg). The detection limit for the method is 0,01 mg/kg.

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ANNEX XII

ORGANOLEPTIC ASSESSMENT OF VIRGIN OLIVE OILS

1. PURPOSE AND SCOPE

   This Annex sets the criteria required for organoleptic assessment of the virgin oils defined at 1 in the Annex to Regulation No 136/66/EEC and describes the method of grading them with reference to characteristics.

   This method can be used only for grading virgin oils on the basis of fruitiness and intensity of defects by a group of selected trained tasters operating as a panel in line with section 4.

2. GENERAL

   For the general basic vocabulary, the tasting room, the general methodology and the tasting glass compliance with the stipulations of the International Olive Oil Council is recommended.

3. SPECIFIC VOCABULARY

   3.1. **Positive attributes**

   Fruity: range of smells (dependent on variety) characteristic of oil from healthy fresh fruit, green or white, perceived directly or retronasally.

   Bitter: characteristic taste of oil from green olives or olives turning colour.

   Pungent: tingling sensation characteristic of oil made at the beginning of the season mainly from olives that are still green.

   3.2. **Negative attributes**

   'Atrojado' (fusty): characteristic flavour of oil from piled olives in advanced anaerobic fermentation.

   Mustiness/humidity: characteristic flavour of oil from olives in which large numbers of fungi and yeasts had developed as a result of storage for several days in humid conditions.

   Muddy sediment: characteristic flavour of oil that has remained in contact with sediment in vats and tanks.

   Winey/vinegary: characteristic flavour of certain oils reminiscent of wine or vinegar, due basically to formation of acetic acid, ethyl acetate and ethanol by fermentation of the olives.

   Metallic: flavour reminiscent of metal, characteristic of oil that has been in prolonged contact with metal surfaces during crushing, mixing, pressing or storage.

   Rancid: flavour of oil that has become oxidised.

   Heated or burnt: characteristic flavour caused by excessive and/or prolonged heating during production, particularly by thermo-mixing of the paste in unsuitable conditions.

   Hay/wood: characteristic flavour of certain oils from dry olives.

   Rough: thick and pasty mouthfeel produced by some oils.

   Greasy: flavour reminiscent of diesel, grease or mineral oil.

   Vegetable water: flavour acquired by oil through prolonged contact with the vegetable water.

   Brine: flavour of oil from olives preserved in salt solution.

   Esparto: characteristic flavour of oil from olives pressed in new esparto mats. It can vary according to whether the mats are of green or dried esparto.

   Earthy: flavour of oil from olives collected with earth or mud on them and not washed.

   Grubby: flavour of oil from olives heavily attacked by grubs of the olive fly (Bactrocera oleae).

   Cucumber: characteristic flavour of oil kept too long in hermetically sealed containers, notably in tins, attributed to formation of 2,6-nonadienal.

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4. PANEL

The panel is appointed by the Member State and consists of a panel head and from eight to twelve tasters. However, for the 2001/02 marketing year, the panel may consist of fewer than eight tasters.

The panel head must be a soundly trained expert in the various types of oil. He or she is responsible for the panel and its organisation and operation, including preparation, coding and presentation of the samples to the tasters and collection and processing of the data.

He or she selects the testers, sees to their training and checks that their performance remains of adequate standard.

The testers must be selected and trained on account of their skill in distinguishing between similar samples. The International Olive Oil Council's manual on the selection, training and monitoring of qualified virgin oil tasters must be followed.

Panels must undertake to participate in national, Community and international organoleptic assessments organised for the purposes of periodic monitoring and harmonisation of perception criteria. They must also provide the Member State concerned with full information each year on the composition of the panel and the number of assessments made in their capacity as an approved panel.

5. PROCEDURE FOR ORGANOLEPTIC ASSESSMENT AND GRADING

5.1. **Use of profile sheet by taster**

The profile sheet to be used by the taster is reproduced as Appendix A.

Tasters must each smell and then taste [1] the oil submitted for examination contained in the tasting glass, analysing their olfactory, gustatory, tactile and kinaesthetic perceptions and mark on the sheet the intensity of their perception of each negative and positive attribute.

If negative attributes not listed on the profile sheet are perceived these must be noted under 'Other' using those of the terms defined in 3.2 above that best describe them.

5.2. **Processing of data by panel head**

The panel head collects the profile sheets completed by the tasters and scrutinises the intensities assigned. In the event of an anomaly he or she will ask tasters to re-examine their sheet and if necessary repeat the test.

The panel head may feed each tester's data into a computer programme for calculating the median (Appendix B). Input of each sample shall be made with the help of a grid of 10 vertical columns for the 10 sensory attributes and one line for each panel member.

If a negative attribute is mentioned under 'Other' by 50 % of the panel the head must calculate the median for this attribute and grade accordingly.

In cases of assessment in connection with monitoring of conformity to standards and of counter-assessment the panel head shall arrange for the assessment to be repeated twice at intervals of at least one day. The attribute medians shall be calculated using the data from the profile sheets of the three assessments.

5.3. **Grading of oils**

The oil is graded as follows in line with the median of the defects and the median for 'fruity'. By this is understood the median of the negative attribute perceived with greatest intensity. The value of the robust variation coefficient for this negative attribute must be no greater than 20 %.

(a) *extra virgin olive oil*: the median of the defects is 0 and the median for 'fruity' is above 0;

(b) *virgin olive oil*: the median of the defects is above 0 but not above 2,5 and the median for 'fruity' is above 0;

(c) *ordinary virgin olive oil*: the median of the defects is above 2,5 but not above 6,0; or the median of the defects is not above 2,5 and the median for 'fruity' is 0;

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[1] But may refrain from tasting if they note some extremely intense negative attributes; they will note this exceptional circumstance on the profile sheet.

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(d) *lampante virgin olive oil*: the median of the defects is above 6,0.

From 1 November 2003 categories c) and d) are replaced by:

(c) *lampante olive oil*: the median of the defects is above 2,5; or the median of the defects is not above 2,5 and the median for 'fruity' is 0.

5.4. **Special case**

If the median of a positive attribute other than 'fruity' is above 5,0 the panel head must note this on the analysis certificate.

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APPENDIX A

Profile sheet

(for use by taster)

DEFECTS PERCEIVED   INTENSITY

"Atrojado" (fusty) |————————————————————————————————————➡————

Mustiness/humidity |————————————————————————————————————➡————

Winey/vinegary |————————————————————————————————————➡————

Muddy sediment |————————————————————————————————————➡————

Metallic |————————————————————————————————————➡————

Rancid |————————————————————————————————————➡————

Other (specify) |————————————————————————————————————➡————

POSITIVE ATTRIBUTES PERCEIVED

Fruity |————————————————————————————————————➡————

Bitter |————————————————————————————————————➡————

Pungent |————————————————————————————————————➡————

Name of taster   **Sample code**

                                                Date

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APPENDIX B

**METHOD OF CALCULATING MEDIAN AND CONFIDENCE INTER-
VALS**

Median

$$Me = [P (X < Xm) \leq 1/2 \wedge P (X \leq Xm) \geq 1/2]$$

The median is the real number Xm characterised by the fact that the probability (P) that the values of the distribution (X) are below that number (Xm) is not more than 0,5 and that simultaneously the probability (P) that the values of the distribution (X) are not above Xm is not less than 0,5. Another definition considers the median to be the 50th percentile of a distribution of numbers ranked in order of increase. In other terms the median represents the central value of an ordered series of uneven numbers or the average of the two central values of an ordered series of even numbers.

Robust standard deviation

$$S = \frac{1,25 \, IQR}{1,35 \, \sqrt{N}}$$

To obtain a reliable estimate of the variability that arises around the median recourse is required to the Stuart and Kendall method of estimating the robust standard deviation. The formula for the asymptotic standard deviation S involves N and IQR. N is the number of observations and IQR the interquartile range, i.e. the robust estimate of the variability of the data under consideration (the inter-quartile range covers exactly 50 % of the cases of any probability distribution). The interquartile range is given by calculating the size of the deviation between the 75th and the 25th percentiles.

IQR = 75th percentile — 25th percentile

The percentile is the value Xpc characterised by the fact that the probability (P) that the valves of the distribution are below Xpc is not more than a determined hundredth and that simultaneously the probability (P) that the valves of the distribution are not above Xpc is not less than the said hundredth. The hundredth indicates the distribution fraction used. In the case of the median this is 50/100.

$$Percentile = [P (X < Xpc) \leq \frac{n}{100} \wedge P (X \leq Xpc) \geq \frac{n}{100}]$$

In other words the percentile is the distribution value corresponding to a deter-mined area plotted from the distribution or density curve. For example, the 25th percentile represents the distribution value corresponding to an area equal to 0,25 or 25/100.

Robust variation coefficient %

$$CVR = \frac{S}{Me} \, 100$$

The RVC represents a pure number, i.e. without dimension, that indicates the percentage of variability of the series of numbers analysed against the Av value of the median. For that reason it is very useful for verifying the reliability of the panel members.

Confidence intervals at 95 % on the median

The confidence intervals (C.I.) at 95 % (value of the error of first kind equal to 0,05 or 5 %) represent the range in which the value of the median would be able to vary should it be possible to repeat the experiment an infinite number of

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times. In practice this interval indicates the range of variability of the test under the operating conditions selected should it be possible to repeat the test several times. The interval helps evaluate, as in the case of the RVC, the reliability of the test.

Upper C.I. = Av + (c.S)Lower C.I = Av - (c.S)where c in the case of a confidence interval of 0,95 is equal to 1,96.

Grading is effected by comparing the median values with the reference ranges set in section 5.3. The software package permits a visualised grading on a table of the statistics or on a graph.

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ANNEX XIII

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NEUTRALIZATION AND DECOLORIZATION OF OLIVE OIL IN THE LABORATORY

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1. NEUTRALIZATION AND DECOLORIZATION OF OLIVE OIL IN THE LABORATORY

1.1. **Neutralization of the oil**

1.1.1. Apparatus

— beaker, 300 ml, tall,
— laboratory centrifuge with 100 ml tubes,
— beaker, 250 ml,
— round-bottomed flasks, 100 ml,
— separating funnel, 1 litre.

1.1.2. Reagents

— aqueous solution of 12 % sodium hydroxide,
— ethyl alcohol solution of 1 % phenolphtalein,
— pure hexane, AR,
— pure propan-2-ol of AR.

1.1.3. Procedure

(a) *Oils with a free fatty acid content, expressed as oleic acid, of less than 30 %*

Place 50 g of crude oil in a tall 300 ml beaker and heat to 65º C in a water bath. Add a quantity of 12 % solution of sodium hydroxide corresponding to the free acid of the oil, with an excess of 5 %, stirring gently all the time. Continue to stir for five minutes, keeping the temperature at 65º C.

Transfer the mixture into 100 ml centrifuge tubes and separate the soapy paste by centrifugation. Pour the decanted oil into a 250 ml beaker and wash with 50 to 60 ml of boiling distilled water, removing the water by means of a siphon. Repeat the washings until all traces of residual soap are removed (disappearance of the pink colouring in the phenolphtalein).

Centrifuge the oil to eliminate any small quantities of residual water.

(b) *Oils with a free fatty acid content expressed as oleic acid exceeding 30 %.*

In a 1 litre separating funnel place 50 g of crude oil, 200 ml of hexane, 100 ml of propan-2-ol and a quantity of 12 % solution of sodium hydroxide corresponding to the free acid of the oil, with an excess of 0,3 %.

Stir vigorously for one minute. Add 100 ml of distilled water, stir again and allow to stand.

After separation of the layers, allow the lower layer containing soaps to drain off. Between the two layers (oily on top and aqueous underneath) an intermediary layer often forms made up of mucilages and insoluble substances which must also be eliminated.

1.2. **Decolorization of neutralized oil**

1.2.1. Apparatus

— round-bottomed flask, 250 ml, with three ground glass necks for the insertion of:

(a) a thermometer graduated in degrees and allowing readings to be taken at 90º C;

(b) a mechanical stirrer operating at 250 to 300 revolutions per minute, equipped to operate in a vacuum;

(c) a vacuum pump connection,

— vacuum pump, with a manometer, capable of giving residual pressure of 15 to 30 millibars.

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1.2.2. Procedure

Weigh about 100 g of neutralized oil in the three-necked flask. Insert the thermometer and the stirrer, connect the vacuum pump and heat to 90º C, stirring all the time. Maintain that temperature, continuing to stir, until the oil to be analysed is entirely free from water (about 30 minutes). Then break the vacuum and add 2 to 3 g of activated earth.

Re-establish the vacuum until a residual pressure of 15 to 30 millibars is obtained and, maintaining a temperature of 90º C, stir for 30 minutes at about 250 revolutions per minute.

Filter while still hot in a thermostatic oven (50 to 60º C).

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ANNEX XV

1. OIL CONTENT OF OLIVE RESIDUE

1.1. **Apparatus**

— suitable extraction apparatus fitted with a 200 to 250 ml round-bottomed flask,

— electrically heated bath (e.g., sand bath, water bath) or hotplate,

— analytical balance,

— oven regulated to a maximum of 80º C,

— electrically heated oven fitted with a thermostatic device regulated to 103 ± 2º C and one that can be swept with a stream of air or operated at reduced pressure,

— mechanical mill, easy to clean, and one that allows the olive residues to be ground without a rise in their temperature or any appreciable alteration in their content of moisture, volatile matter or substances extractable with hexane,

— extraction thimble and cotton wool or filter paper from which substances extractable with hexane have already been removed,

— dessicator,

— sieve with 1 mm diameter apertures,

— small particles of previously dried pumice stone.

1.2. **Reagent**

Normal hexane, technical grade, which must leave a residue of less than 0,002 g per 100 ml, on complete evaporation.

2. PROCEDURE

2.1. **Preparation of the test sample**

If necessary, use the mechanical mill, which has previously been properly cleaned, to grind the laboratory sample in order to reduce it to particles that can pass completely through the sieve.

Use about one twentieth of the sample to complete the process of cleaning the mill, discard the ground material, grind the remainder and collect, mix carefully and analyze without delay.

2.2. **Test portion**

As soon as the grinding operation has been completed, weigh out about 10 g of the sample to the nearest 0,01 g for testing.

2.3. **Preparation of the extraction thimble**

Place the test portion in the thimble and plug with cotton wool. If a filter paper is used, envelope the test portion in it.

2.4. **Peliminary drying**

If the olive residues are very moist (i.e., moisture and volatile matter content more than 10 %), carry out preliminary drying by placing the loaded thimble (or filter paper) in the oven heated for an appropriate time at not more than 80º C in order to reduce the moisture and volatile matter content to less than 10 %.

2.5. **Preparation of the round-bottomed flask**

Weigh to the nearest 1 mg the flask containing one or two particles of pumice stone, previously dried in the stove at 103 ± 2º C and then cooled in a dessicator for not less than one hour.

2.6. **Initial extraction**

Into the extraction apparatus insert the thimble (or filter paper) containing the test portion. Pour into the flask the requisite quantity of hexane. Fit the flask to the extraction apparatus and place the whole on the electrically heated bath. Adjust the rate of heating in such a way that the reflux rate is not less than three drops per second (moderate, not violent boiling). After four hours extraction, allow to cool. Remove the thimble from the extrac-

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tion apparatus and place it in a stream of air in order to drive off most of the impregnating solvent.

2.7. **Second extraction**

Tip the contents of the thimble into the micro-grinder and grind as finely as possible. Return the ground mixture to the thimble without loss and place it back in the extraction apparatus.

Continue the extraction for a further two hours using the same round-bottomed flask containing the initial extract.

The resultant solution in the extraction flask must be clear. If not, filter it through a filter paper and wash the original flask and the filter paper several times with hexane. Collect the filtrate and the washing solvent in a second round-bottomed flask which has been dried and tared to the nearest 1 mg.

2.8. **Removal of solvent and weighing of extract**

Remove the greater part of the solvent by distillation on an electrically heated bath. Remove the last traces of solvent by heating the flask in the oven at $103 \pm 2°$ C for 20 minutes. Assist the elimination process either by blowing in air, or preferably an inert gas, at intervals or by using reduced pressure.

Leave the flask in a dessicator to cool for at least one hour and weigh to the nearest 1 mg.

Heat again for 10 minutes under the same conditions, cool in a dessicator and reweigh.

The difference between the two weighings shall not exceed 10 mg. If it does, heat again for periods of 10 minutes followed by cooling and weighing until the weight difference is 10 mg or less. Note the last weight of the flask.

Carry out duplicate determinations on the test sample.

3. EXPRESSION OF RESULTS

3.1. **Method of calculation and formula**

(a) The extract expressed as a percentage by mass of the product as received is equal to:

$$S = m_1 \times \frac{100}{m_0}$$

where: S   is the percentage by mass of extract of the product as received,

m_0 = is the mass, in grams, of the test portion,

m_1 = is the mass, in grams, of the extract after drying.

Take as the result the arithmetic mean of the duplicate determinations, providing the repeatability conditions are satisfied.

Express the result to the first decimal place.

(b) The extract is expressed on a dry matter basis by using the formula:

$$S \times \frac{100}{100 - U} = \text{oil percentage of extract on a dry basis}$$

where:

S   = is the percentage of extract by means of the product as received (see (a)),

U   = is its moisture and volatile matter content.

3.2. **Repeatability**

The difference between the duplicate determinations carried out simultaneously or in rapid sucession by the same analyst shall not exceed 0,2 g of hexane extract per 100 g of sample.

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If this condition is not satisfied, repeat the analysis on two other test portions. If, in this case too, the difference exceeds 0,2 g, take as the result the arithmetic mean of the four determinations.

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ANNEX XVI

DETERMINATION OF IODINE VALUE

1. SCOPE

    This International Standard specifies a method for the determination of the iodine value of animal and vegetable fats and oils, referred to hereafter as fats.

2. DEFINITION

    For the purposes of this International Standard, the following definition applies:

2.1. *iodine value.* The mass of iodine absorbed by the sample under the operating conditions specified in this International Standard.

    The iodine value is expressed as grams of iodine per 100 g of sample.

3. PRINCIPLE

    Dissolution of a test portion in solvent and addition of Wijs reagent. After a specified time, addition of potassium iodide solution and water, and titration of the liberated iodine with sodium thiosulfate solution.

4. REAGENTS

    All reagents shall be of recognized analytical grade:

4.1. *water,* complying with the requirements of ISO 3696, Grade 3.

4.2. *potassium iodide,* 100 g/l solution, not containing iodate or free iodine.

4.3. *starch,* solution.

    Mix 5 g of soluble starch in 30 ml of water, add this mixture to 1 000 ml of boiling water, boil for three minutes and allow to cool.

4.4. *sodium thiosulfate,* standard volumetric solution c $(Na_2S_2O_3.5H_2O) = 0,1$ mol/l, standardized not more than seven days before use.

4.5. *solvent,* prepared by mixing equal volumes of cyclohexane and acetic acid.

4.6. *Wijs reagent,* containing iodine monochloride in acetic acid. Commercially available Wijs reagent shall be used.

5. APPARATUS

    Usual laboratory apparatus and, in particular, the following:

5.1. *glass weighing scoops,* suitable for the test portion and for inserting into the flasks (6.2).

5.2. *conical flasks,* of 500 ml capacity, fitted with ground glass stoppers and completely dry.

6. PREPARATION OF THE TEST SAMPLE

    The homogenized sample is dried over sodium sulphate and filtered.

7. PROCEDURE

7.1. Test portion

    The mass of the test portion varies according to its expected iodine value as shown in Table 1.

▼**B**

Table 1

Expected iodine value	Mass of test portion (g)
less than 5	3,00
5 to 20	1,00
21 to 50	0,40
51 to 100	0,20
101 to 150	0,13
151 to 200	0,10

Weigh the test portion to the nearest 0,1 mg in a glass weighing scoop (5.1).

7.2. Determination

Place the test portion in a 500 ml flask (6.2). Add 20 ml of the solvent (4.5) to dissolve the fat. Add exactly 25 ml of the Wijs reagent (4.6), insert the stopper, swirl the contents and place the flask in the dark. Do not use a mouth pipette for the Wijs reagent.

Similarly, prepare a blank with the solvent and the reagent but omitting the test portion.

For samples having an iodine valve below 150, leave the flasks in the dark for one hour; for those with an iodine value above 150 and for polymerized products or products oxidized to a considerable extent, leave for two hours.

At the end of the time, add 20 ml of the potassium iodide solution (4.2) and 150 ml of water (4.1) to each of the flasks.

Titrate with the standard volumetric sodium thiosulfate solution (4.4) until the yellow colour due to iodine has almost disappeared. Add a few drops of the starch solution (4.3) and continue the titration until the blue colour just disappears after very vigorous shaking.

Note: Potentiometric determination of the end point is permissible.

7.3. Number of determinations

Carry out two determinations on the same test sample.

8. EXPRESSION OF RESULTS

The iodine value is given by the expression

$$\frac{12,69 \ c \ (V_1 - V_2)}{m}$$

where:

c = is the numerical value of the exact concentration, in moles per litre, of the standard volumetric sodium thiosulfate solution (4.4) used;

V_1 = is the numerical value of the volume, in millilitres, of the standard volumetric sodium thiosulfate solution (4.4) used for the blank test;

V_2 = is the numerical value of the volume, in millilitres, of the standard volumetric sodium thiosulfate solution (4.4) used for the determination;

m = is the numerical value of the mass, in grams, of the test portion (7.1).

Take as the result the arithmetic mean of the two determinations, provided that the requirement for repeatability (9.2) is satisfied.

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ANNEX XVII

METHOD FOR THE DETERMINATION OF STIGMASTADIENES IN VEGETABLE OILS

1. PURPOSE

   Determination of stigmastadienes in vegetable oils containing low concentrations of these hydrocarbons, particularly in virgin olive oil and crude olive-residue oil.

2. SCOPE

   The standard may be applied to all vegetable oils although measurements are reliable only where the content of these hydrocarbons lies between 0,01 and 4,0 mg/kg. The method is particularly suited to detecting the presence of refined vegetable oils (olive, olive residue, sunflower, palm, etc.) in virgin olive oil since refined oils contained stigmastadienes and virgin oils do not.

3. PRINCIPLE

   Isolation of unsaponifiable matter. Separation of steroidal hydrocarbon fraction by column chromatography on silica gel and analysis by capillary gas chromatography.

4. APPARATUS

4.1. 250 ml flasks suitable for use with a reflux condenser.

4.2. Separating funnels of 500 ml capacity.

4.3. 100 ml round-bottom flasks.

4.4. Rotary evaporator.

4.5. Glass chromatography column (1,5 to 2,0 cm internal diameter by 50 cm length) with Teflon tap and a plug of glass wool fibre or sintered glass disc at the bottom. To prepare silica gel column, pour hexane into the chromatography column to a depth of approximately 5 cm and then fill with a slurry of silica gel in hexane (15 g in 40 ml) with the help of hexane portions. Allow to settle and finish settling by applying slight vibration. Add anhydrous sodium sulphate to a height of approximately 0,5 cm, finally elute the excess hexane.

4.6. Gas chromatograph with flame ionization detector, split or cold on-column injector and oven programmable to within ± 1 ºC.

4.7. Fused silica capillary column for gas chromatography (0,25 or 0,32 mm internal diameter by 25 m length) coated with 5 %-phenylmethylsilicone phase, 0,25 mm film thickness.

   Note 1:

   Other columns of similar or lower polarity can be used.

4.8. Integrator-recorder with possibility of valley-valley integration mode.

4.9. 5 to 10 ml microsyringe for gas chromatography with cemented needle.

4.10. Electrical heating mantle or hot place.

5. REAGENTS

   All reagents should be of analytical grade unless otherwise specified. The water used should be distilled water, or water of at least equivalent purity.

5.1. Hexane or mixture of alkanes of bp interval 65 to 70 ºC, distilled with rectifying column.

   Note 2:

   The solvent must be distilled to remove impurities.

5.2. 96 v/v ethanol.

5.3. Anhydrous sodium sulphate.

5.4. Alcoholic potassium hydroxide solution at 10 %. Add 10 ml of water to 50 g potassium hydroxide, stir, and then dissolve the mixture in ethanol to 500 ml.

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Note 3:

Alcoholic potash turns brown on standing. It should be prepared freshly each day and kept in well stoppered dark glass bottles.

5.5. Silica gel 60 for column chromatography, 70 to 230 mesh, (Merck, reference 7734 or similar).

Note 4:

Usually, silica gel can be used directly from the container without any treatment. However, some batches of silica gel may show low activity resulting in bad chromatographic separations. Under this circumstance, the silica gel should be treated in the following way: Activate the silica gel by heating for a minimum of four hours at 550 ºC. After heating, place the silica gel in a desiccator while the gel is cooling and then transfer the silica gel to a stoppered flask. Add 2 % of water and shake until no lumps can be seen and the powder flows freely.

If batches of silica gel result in chromatograms with interfering peaks, the silica gel should be treated as above. An alternative could be the use of extra pure silica gel 60 (Merck, reference 7754).

5.6. Stock solution (200 ppm) of cholesta-3,5-diene (Sigma, 99 % purity) in hexane (10 mg in 50 ml).

5.7. Standard solution of cholesta-3,5-diene hexane at concentration of 20 ppm, obtained by dilution of above solution.

Note 5:

The solutions 5.6 and 5.7 are stable for a period of at least four months if kept at less than 4 ºC.

5.8. Solution of n-nonacosane in hexane at concentration of approximately 100 ppm.

5.9. Carrier gas for chromatography: helium or hydrogen of 99,9990 % purity.

5.10. Auxiliary gases for flame ionization detector: hydrogen of 99,9990 % purity and purified air.

6. PROCEDURE

6.1. **Preparation of unsaponifiable matter**

6.1.1. Weigh 20 ± 0,1 g of oil into a 250-ml flask (4.1), add 1 ml of the standard solution of cholesta-3,5-diene (20μg) and 75 ml of alcoholic potash at 10 %, fit reflux condenser, and heat to slight boiling for 30 minutes, Remove the flask containing the sample from the heat and allow the solution to cool slightly (do not allow to cool completely as the sample will set). Add 100 ml of water and transfer the solution to a separating funnel (4.2) with the aid of 100 ml of hexane. Shake the mixture vigorously for 30 seconds and allow the separate.

Note 6:

If an emulsion is produced which does not rapidly disappear, add small quantities of ethanol.

6.1.2. Transfer the aqueous phase beneath to a second separating funnel and extract again with 100 ml of hexane. Once more run off the lower phase and wash the hexane extracts (combined in another separating funnel) three times with 100 ml each time of a mixture of ethanol-water (1: 1) until neutral pH is reached.

6.1.3. Pass the hexane solution through anhydrous sodium sulphate (50 g), wash with 20 ml hexane and evaporate in a rotary evaporator at 30 ºC under reduced pressure until dryness.

6.2. **Separation of steroidal hydrocarbon fraction**

6.2.1. Take the residue to the fractioning column with the aid of two 1-ml portions of hexane, run the sample onto the column by allowing the solution level to drop to the top of the sodium sulphate and start the chromatographic elution with hexane at a flow rate of 1 ml/min approximately. Discard the first 25 to 30 ml of eluate and then collect the following 40 ml fraction. After collection, transfer this fraction to a 100-ml round bottomed flask (4.3).

Note 7:

The first fraction contains saturated hydrocarbons (Figure 1 a) and the second fraction the steroidal ones. Further elution provides squalene and

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related compounds. To achieve a good separation between saturated and steroidal hydrocarbons, the optimization of fraction volumes is required. For this, the volume of the first fraction should be adjusted so that when the second fraction is analysed the peaks representing the saturated hydrocarbons are low (see Figure 1 c); if they do not appear but the intensity of the standard peak is low, the volume should be reduced. Anyway, a complete separation between the components of the first and second fractions is unnecessary; as there is no overlapping of peaks during GC analysis if GC conditions are ajusted as indicated in 6.3.1. The optimization of the volume of the second fraction if generally not needed as a good separation exists with the further components. Nevertheless, the presence of a large peak at approximately 1,5 minutes lower retention time than the standard is due to squalene, and it is indicative of a bad separation.

6.2.2. Evaporate the second fraction in a rotary evaporator at 30 ºC under reduced pressure until dryness, and immediately dissolve the residue in 0,2 ml of hexane. Keep the solution in the refrigerator until analysis.

Note 8:

Residues 6.1.3 and 6.2.2 should not be kept dry and at room temperature. As soon as they are obtained, the solvent should be added and the solutions should be kept in the refrigerator.

6.3. **Gas chromatography**

6.3.1. Working conditions for split injection:

— injector temperature: 300 ºC,

— detector temperature: 320 ºC,

— integrator-recorder: the parameters for integration should be fixed so as to give a correct assessment of the areas. Valley-valley integration mode is recommended,

— sensitivity: about 16 times the minimum attenuation,

— amount of solution injected: 1μl,

— oven programming temperatures: initial 235 ºC for six minutes and then rising at 2 ºC/minute up to 285 ºC,

— injector with 1: 15 flow divider,

— carrier: helium or hydrogen at about 120 kPa pressure.

These conditions may be adjusted in accordance with the characteristics of the chromatograph and the column to give chromatograms meeting the following requirements: internal standard peak within approximately five minutes of the time given in 6.3.2; the internal standard peak should be at least 80 % of the full scale.

The gas chromatographic system must be checked injecting a mixture of the stock solution of cholestadiene (5.6) and n-nonacosane solution (5.8). The cholesta-3,5-diene peak must appear before the n-nonacosane (Figure 1c); if it does not occur two actions can be undertaken: reduce the oven temperature and/or use a less polar column.

6.3.2. Peak identification

The internal standard peak appears at approximately 19 minutes and the 3,5-stigmastadiene at a relative retention time of approximately 1,29 (see Figure 1b). The 3,5-stigmastadiene occurs with small quantities of an isomer, and usually, both elute together as a single chromatographic peak. Nevertheless, if the column is too polar or shows a high resolving power, the isomer can appear as a small peak before and close to that of stigmasta-3,5-diene (Figure 2). In order to ensure that the stigmastadienes are eluted as one peak, it is advisable to replace the column by one which is either less polar or has a wider internal diameter.

Note 9:

Stigmastadienes for reference can be obtained from the analysis of a refined vegetable oil by using less amount of sample (1 to 2 g). Stigmastadienes originate a prominent and easily identifiable peak.

6.3.3. Quantitative analysis

The stigmastadienes content is determined according to the formula:

$$\text{mg/kg of stigmastadienes} = \frac{A_s \times M_c}{A_c \times M_o}$$

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where: A_s = area of stigmastadienes peak (if the peak is resolved into two isomers, sum of areas of the two peaks),

A_c = area of internal standard (cholestadiene),

M_c = mass of standard added, in micrograms,

M_o = mass of oil taken, in grams.
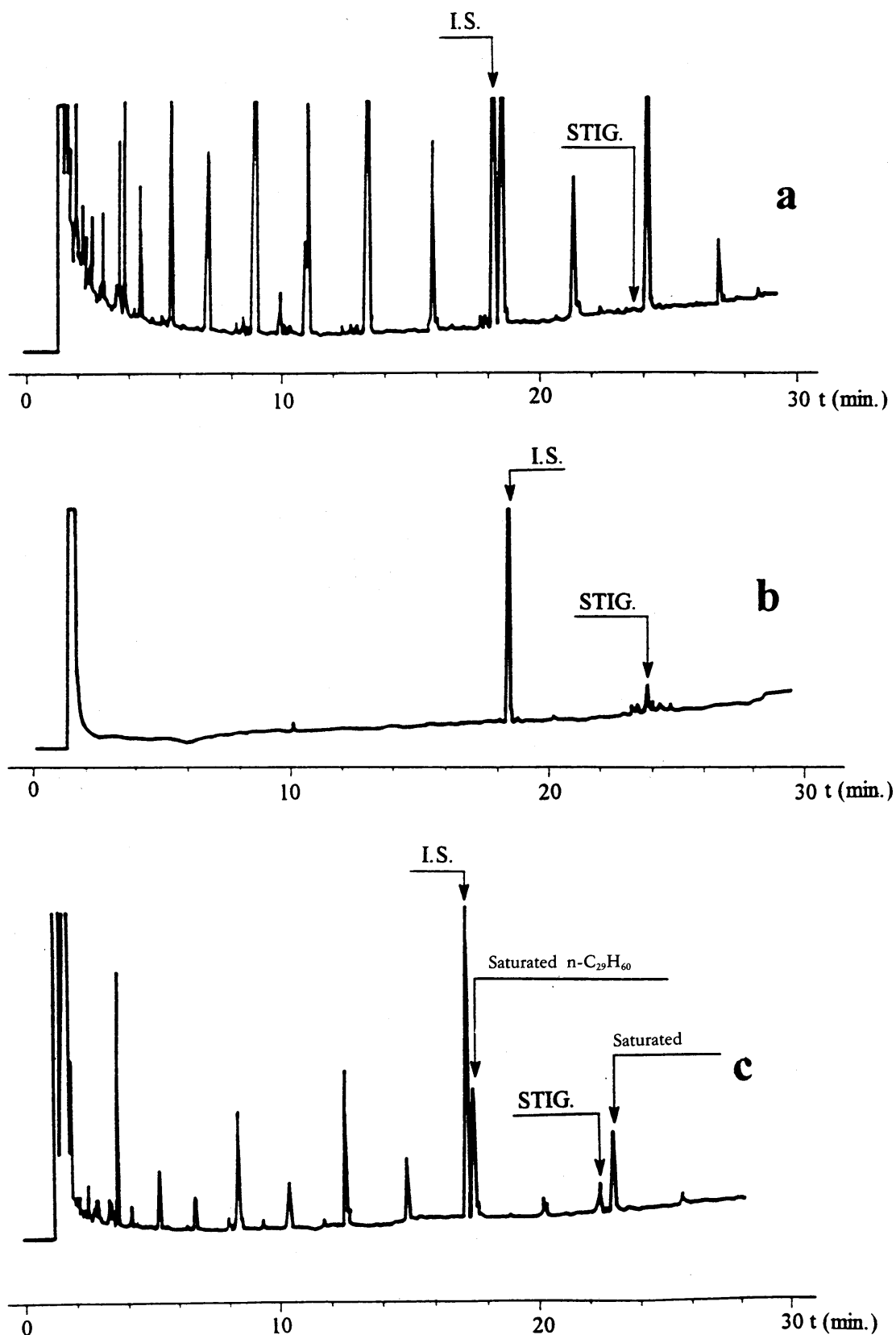
Detection limit: about 0,01 mg/kg.

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where: A_s = area of stigmastadienes peak (if the peak is resolved into two isomers, sum of areas of the two peaks),

A_c = area of internal standard (cholestadiene),

M_c = mass of standard added, in micrograms,

M_o = mass of oil taken, in grams.

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Figure 1

Gas chromatograms obtained from olive oil samples analysed on a fused silica capillary column (0,25 mm internal diameter by 25 m) coated with 5 %-phenylmethylsilicone, 0,25 μm film thickness.

(a) First fraction (30 ml) from a virgin oil, spiked with standard.

(b) Second fraction (40 ml) from an olive oil containing 0,10 mg/kg of stigmastadienes.

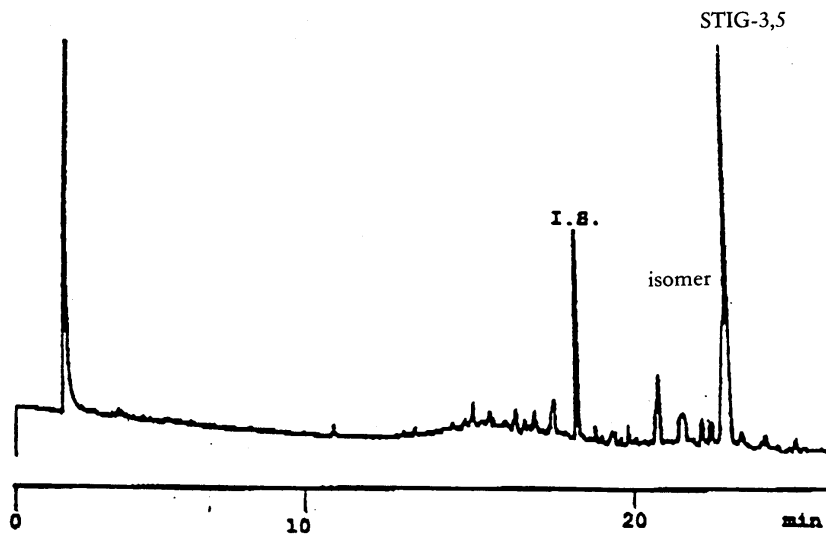(c) Second fraction (40 ml) containing a small proportion of the first fraction.

Figure 2

Gas chromatogram obtained from a refined olive oil sample analysed on DB-5 column showing the isomer of 3,5-stigmastadiene.

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ANNEX XVIII

DETERMINATION OF TRIACYLGLYCEROLS WITH ECN 42 (DIFFERENCE BETWEEN HPLC DATA AND THEORETICAL CONTENT)

1. **Scope**

   Determination of the composition of triacylglycerols (TAGs) in olive oils, in terms of their equivalent carbon number by differences between the analytical results obtained by high performance liquid chromatography (HPLC) and the theoretical content, calculated starting from the fatty acid composition.

2. **Field application**

   The standard is applicable to olive oils. The method is applicable to the detection of the presence of small amounts of seed oils (rich in linoleic acid) in every class of olive oils.

3. **Principle**

   The content of triacylglycerols with ECN42 determined by HPLC analysis and the theoretical content of triacylglycerols with ECN42 (calculated on the basis of GLC determination of fatty acid composition) correspond within a certain limit for pure oils. A difference larger than the values stated in the Regulation for each type of oil points out that the oil contains seed oils.

4. **Method**

   The method for calculation of theoretical content of triacylglycerols with ECN42 and of the difference between the HPLC data and this one essentially is made by the coordination of analytical data obtained by means of other methods: it is possible to distinguish three phases: determination of fatty acid composition by capillary gas chromatography, calculation of theoretical composition of triacylglycerols with ECN42, HPLC determination of ECN42 triacylglycerols

4.1. **Apparatus**

4.1.1. Round bottom flasks, 250 and 500 ml.

4.1.2. Beakers 100 ml.

4.1.3. Glass chromatographic column, 21 mm internal diameter, 450 mm length, with cock and normalized cone (female) at the top.

4.1.4. Separator funnels, 250 ml, with normalized cone (male) at the bottom, suitable to be connected with the top of the column.

4.1.5. Glass rod, 600 mm length.

4.1.6. Glass funnel, 80 mm diameter.

4.1.7. Volumetric flasks, 50 ml.

4.1.8. Volumetic flasks, 20 ml.

4.1.9. Rotative evaporator.

4.1.10. High performance liquid chromatography, allowing thermostatic control of column temperature.

4.1.11. Injection units for 10 μl delivery.

4.1.12. Detector: differential refractometer. The full scale sensitivity should be at least 10^{-4} units of refractive index.

4.1.13. Column: stainless steel tube 250 mm length and 4,5 mm internal diameter packed with 5 μm diameter particles of slica with 22 to 23 % carbon in the form of octadecylsilane (note 2).

4.1.14. Recorder and/or integrator.

4.2. **Reagents**

   The reagents should be of analytical purity. Elution solvents should be de-gassed, and may be recycled several times without effect on the separations.

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4.2.1. Petroleum ether 40 to 60 ºC chromatographic grade.

4.2.2. Ethil ether, peroxides free, freshly distilled.

4.2.3. Glass chromatographic elution solvent: mixture petroleum ether/ethil ether 87/13 (v/v).

4.2.4. Silicagel, 70-230 mesh, type Merck 7734, with water content standardized at 5 % (w/w).

4.2.5. Glass wool.

4.2.6. Acetone.

4.2.7. Acetonitrile.

4.2.8. HPLC elution solvent: acetonitrile + acetone (proportions to be adjusted to obtain the desired separation; begin with 50:50 mixture).

4.2.9. Solubilization solvent: acetone.

4.2.10. Reference triglycerides commercial triglycerides tripalmitin, triolein, etc.) may be used and the retention times thence plotted in accordance with the equivalent carbon number, or alternatively reference chromatograms obtained from soya oil, mixture 30:70 soya oil/olive oil and pure olive oil (see notes 3 and 4 and figure 1, 2, 3, 4).

4.3. **Sample preparation**

As a number of interfering substances can rise false positive results, the sample must always be purified according to IUPAC method 2.507, used for determination of polar substances in oxidised oils.

4.3.1. Chromatographic column preparation

Fill the column (4.1.3) with about 30 ml of elution solvent (4.2.3), then introduce inside the column some glass wool (4.2.5) pushing it to the bottom of the column by means of the glass rod (4.1.5).

In a 100 ml beaker, suspend 25 g of silicagel (4.2.4) in 80 ml of elution mixture (4.2.3), then transfer it inside the column, by means of a glass funnel (4.1.6).

To ensure the complete transfer of silicagel inside the column, wash the beaker with the elution mixture and transfer the washing portions inside the column, too.

Open the cock and let solvent elute from the column until its level is about 1 cm over the silicagel.

4.3.2. Column chromatography

Weigh with the accuracy of 0,001 g, 2,5 ± 0,1 g of oil, previously filtered, homogenized and anhydrified, if necessary, in a 50 ml volumetric flask (4.1.7). Solve it in about 20 ml of elution solvent (4.2.3), if necessary, slightly heat it to make the dissolution easily. Cool at room temperature and adjust the volume with elution solvent.

By means of a volumetric pipette, introduce 20 ml of solution inside the column prepared according to 4.3.1, open the cock and let solvent elute to the silicagel layer level.

Then elute with 150 ml of elution solvent (4.2.3), adjusting the solvent rate at about 2 ml/min (150 ml will take about 60 to 70 minutes to pass through the column).

The eluated is recovered in a 250 ml round bottom flask (4.1.1) previously tared in an oven and exactly weighted. Eliminate solvent at reduce pressure (Rotavapor) and weigh the residue that will be used to prepare the solution for HPLC analysis and for methyl ester preparation.

The sample recovery from the column must be 90 % at least for extra virgin, virgin, ordinary refined and olive oil categories, and a minimum of 80 % for lampante and residue olive oils.

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4.4. **HPLC analysis**

4.4.1. Preparation of the samples for chromatographic analysis

A 5 % solution of the sample to be analysed is prepared by weighing $0,5 \pm 0,001$ g of the sample into a 10 ml graduated flask and making up to 10 ml with the solubilization solvent (4.2.9).

4.4.2. Procedure

Set up the chromatographic system. Pump elution solvent (4.2.8) at a rate of 1,5 ml/min to purge the entire system. Wait until a stable base line is obtained. Inject 10 μl of the sample prepared as in 4.3.

4.4.3. Calculation and expression of results

Use the area normalization method, i.e. assume that the sum of the areas of the peaks corresponding to TAGs from ECN42 up to ECN52 is equal to 100 %. Calculate the relative percentage of each triglyceride using the formula:

% triglyceride = area of peak × 100/ sum of peak areas.

The results are to be given to within at least two decimal places.

Note 1: The elution order can be determined by calculating the equivalent carbon numbers, often defined by the relation ECN = CN-2n, where CN is the carbon number and n is the number of double bounds, it can be calculated more precisely by taking into account the origin of the double bond. If n_o, n_l and n_{ln} are the numbers of double bonds attributed to oleic, linoleic and linolenic acids respectively, the equivalent carbon number can be calculated by means of the relation of the formula:

$$ECN = CN - d_o n_o - d_l n_l - d_{ln} n_{ln},$$

where the coefficient d_o, d_l and d_{ln} can be calculated by means of the reference triglycerides. Under the conditions specified in this method, the relation obtained will be close in:

$$ECN = CN - (2,60 \ n_o) - (2,35 \ n_l) - (2,17 \ n_{ln})$$

Note 2: Examples: Lichrosorb (Merck) RP 18 Art 50333

      Lichrosphere or equivalent (Merck) 100 CH18 Art 50377.

Note 3: With several reference triglycerides, it is also possible to calculate the resolution with respect to triolein:

$$\alpha = RT' \ / \ RT \ triolein$$

by use of the reduced retention time $RT' = RT - RT$ solvent.

The graph of log α against f (number of double bonds) enables the retention values to be determined for all the triglycerides of fatty acids contained in the reference triglycerides — see figure 2.

Note 4: The efficiency of the column should permit clear separation of the peak of trilinoein from the peaks of the triglycerides with an adjacent RT. The elution is carried out up to ECN52 peak.

Note 5: A correct measure of the areas of all peaks of interest for the present determination is ensured if the second peak corresponding to ECN50 is 50 % of full scale of the recorder.

4.5. **Calculation of triacylglycerols composition**

4.5.1. Determination of fatty acid composition

Fatty acid composition is carried out by means of the EEC gas chromatographic method reported in Annex X A of Regulation (EEC) No 2568/91, by means of a capillary column. The methyl esters preparation is carried out according to Annex X B (sodium methylate alcohol solution).

4.5.2. Fatty acids for calculation

Glycerides are grouped by their equivalent carbon number (ECN), taking into account the following equivalencies between ECN and fatty acids. Only fatty acids with 16 and 18 carbon atoms were taken in consideration, because only these are important for olive oil.

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Fatty acid (FA)	Abbreviation	Molecular weight (MW)	ECN
Palmatic acid	P	256,4	16
Palmatoleic acid	Po	254,4	14
Stearic acid	S	284,5	18
Oleic acid	O	282,5	16
Linoleic acid	L	280,4	14
Linolenic acid	Ln	278,4	12

4.5.3. Conversion of area % into moles for all fatty acids

$$\text{moles P} = \frac{\text{area \% P}}{\text{MW P}} \quad \text{moles S} = \frac{\text{area \% S}}{\text{MW S}} \quad \text{moles Po} = \frac{\text{area \% Po}}{\text{MW Po}}$$

$$\text{moles O} = \frac{\text{area \% O}}{\text{MW O}} \quad \text{moles L} = \frac{\text{area \% L}}{\text{MW L}} \quad \text{moles Ln} = \frac{\text{area \% Ln}}{\text{MW Ln}}$$

(1)

4.5.4. Normalization of fatty acids to 100 %

$$\text{moles \% P } (1,2,3) = \frac{\text{moles P} * 100}{\text{moles } (P + S + Po + O + L + Ln)}$$

$$\text{moles \% S } (1,2,3) = \frac{\text{moles S} * 100}{\text{moles } (P + S + Po + O + L + Ln)}$$

$$\text{moles \% Po } (1,2,3) = \frac{\text{moles Po} * 100}{\text{moles } (P + S + Po + O + L + Ln)}$$

$$\text{moles \% O } (1,2,3) = \frac{\text{moles O} * 100}{\text{moles } (P + S + Po + O + L + Ln)}$$

$$\text{moles \% L } (1,2,3) = \frac{\text{moles L} * 100}{\text{moles } (P + S + Po + O + L + Ln)}$$

$$\text{moles \% Ln } (1,2,3) = \frac{\text{moles Ln} * 100}{\text{moles } (P + S + Po + O + L + Ln)}$$

(2)

The result gives the percentage of each fatty acid in moles % in the overall (1,2,3-) position of the TAGs.

Then the sum of the saturated fatty acids P and S (SFA) and the unsaturated fatty acids Po, O, L and Ln (UFA) are calculated:

moles % SFA = moles % P + moles % S

moles UFA = 100 − moles % SFA

(3)

4.5.5. Calculation of the fatty acid composition in 2- and 1,3- positions of TAGs

The fatty acids are distributed to three pools as follows: two identical for 1- and 3- positions and one for 2- position, with different coefficients for the saturated (P and S) and unsaturated acids (Po, O, L and Ln).

4.5.5.1. Saturated fatty acids in 2- position [P(2) and S(2)]

moles % P(2) = moles % P (1,2,3) * 0,06

moles % S(2) = moles % S (1,2,3) * 0,06

(4)

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4.5.5.2. Unsaturated fatty acids in 2- position [Po(2), O(2), L(2) and Ln(2)]:

$$
\left.
\begin{aligned}
\text{moles \% Po(2)} &= \frac{\text{moles \% Po(1,2,3)}}{\text{moles \% UFA}} \ast [\,100 - \text{moles \% P(2)} - \text{moles \% S(2)}\,] \\[2ex]
\text{moles \% O(2)} &= \frac{\text{moles \% O(1,2,3)}}{\text{moles \% UFA}} \ast [\,100 - \text{moles \% P(2)} - \text{mol \% S(2)}\,] \\[2ex]
\text{moles \% L(2)} &= \frac{\text{moles \% L(1,2,3)}}{\text{moles \% UFA}} \ast [\,100 - \text{moles \% P(2)} - \text{moles \% S(2)}\,] \\[2ex]
\text{moles \% Ln(2)} &= \frac{\text{moles \% Ln(1,2,3)}}{\text{moles \% UFA}} \ast [\,100 - \text{moles \% P(2)} - \text{moles \% S(2)}\,]
\end{aligned}
\right\} \quad (5)
$$

4.5.5.3. Fatty acids in 1,3-positions [P(1,3), S(1,3), Po(1,3) O(1,3), L(1,3) and Ln(1,3)]:

$$
\left.
\begin{aligned}
\text{moles \% P1,3} &= \frac{\text{moles \% P1,2,3} - \text{moles \% P(2)}}{2} + \text{moles \% P(1,2,3)} \\[2ex]
\text{moles \% S(1,3)} &= \frac{\text{moles \% S(1,2,3)} - \text{moles \% S(2)}}{2} + \text{moles \% S(1,2,3)} \\[2ex]
\text{moles \% Po(1,3)} &= \frac{\text{moles \% Po(1,2,3)} - \text{moles \% Po(2)}}{2} + \text{moles \% Po(1,2,3)} \\[2ex]
\text{moles \% O(1,3)} &= \frac{\text{moles \% O(1,2,3)} - \text{moles \% O(2)}}{2} + \text{moles \% O(1,2,3)} \\[2ex]
\text{moles \% L(1,3)} &= \frac{\text{moles \% L(1,2,3)} - \text{moles \% L(2)}}{2} + \text{moles \% L(1,2,3)} \\[2ex]
\text{moles \% Ln(1,3)} &= \frac{\text{moles \% Ln(1,2,3)} - \text{moles \% Ln(2)}}{2} + \text{moles \% Ln(1,2,3)}
\end{aligned}
\right\} \quad (6)
$$

4.5.6. Calculation of triacylglycerols

4.5.6.1. TAGs with one fatty acid (AAA, here LLL, PoPoPo)

$$
\text{moles \% AAA} = \frac{\text{moles \% A(1,3)} \ast \text{moles \% A(2)} \ast \text{moles \% A(1,3)}}{10\,000} \quad (7)
$$

4.5.6.2. TAGs with two fatty acids (AAB, here PoPoL, PoLL)

$$
\left.
\begin{aligned}
\text{moles \% AAB} &= \frac{\text{moles \% A(1,3)} \ast \text{moles \% A(2)} \ast \text{moles \% B(1,3)} \ast 2}{10\,000} \\[2ex]
\text{moles \% ABA} &= \frac{\text{moles \% A(1,3)} \ast \text{moles \% B(2)} \ast \text{moles \% A(1,3)}}{10\,000}
\end{aligned}
\right\} \quad (8)
$$

4.5.6.3. TAGs with three different fatty acids (ABC, here OLLn, PLLn, PoOLn, PPoLn)

$$
\left.
\begin{aligned}
\text{moles \% ABC} &= \frac{\text{moles \% A(1,3)} \ast \text{moles \% B(2)} \ast \text{moles \% C(1,3)} \ast 2}{10\,000} \\[2ex]
\text{moles \% BCA} &= \frac{\text{moles \% B(1,3)} \ast \text{moles \% C(2)} \ast \text{moles \% A(1,3)} \ast 2}{10\,000} \\[2ex]
\text{moles \% CAB} &= \frac{\text{moles \% C(1,3)} \ast \text{moles \% A(2)} \ast \text{moles \% B(1,3)} \ast 2}{10\,000}
\end{aligned}
\right\} \quad (9)
$$

▼__M13__

4.5.6.4. Triacylglycerides with ECN42

The following triglycerides with ECN42 are calculated according to equation 7, 8 and 9 in order of expected elution in HPLC (normally only three peaks).

LLL

PoLL and the positional isomer LPoL

OLLn and the positional isomers OLnL and LnOL

PoPoL and the positional isomer PoLPo

PoOLn and the positional isomers OPoLn and OLnPo

PLLn and the positional isomers LLnP and LnPL

PoPoPo

SLnLn and the positional isomer LnSLn

PPoLn and the positional isomers PLnPo and PoPLn

The triacylglycerides with ECN42 are given by the sum of the nine triacylglycerols including their positional isomers. The results to be given with at least two decimal places.

5. **Evaluation of the results**

The calculated theoretical content and the content determined by the HPLC analysis are compared. If the difference between HLPC data minus theoretical data is greater than the values states for the appropriate oil category in the Regulation, the sample contains seed oil.

Note: Results are given to within one decimal figure.

6. **Example** *(The numbers refer to the sections in the text of the method)*

4.5.1. Calculation of moles % fatty acids from GLC data (area %)

The following data are obtained for the fatty acid composition by GLC:

FA MW	P 256,4	S 284,5	Po 254,4	O 282,5	L 280,4	Ln 278,4
area %	10,0	3,0	1,0	75,0	10,0	1,0

4.5.3. Conversion of area % into moles for all fatty acids

$$\text{moles P} = \frac{10}{256,4} = 0,03900 \text{ moles P}$$ See formula (1)

$$\text{moles S} = \frac{3}{284,5} = 0,01054 \text{ moles S}$$ See formula (1)

$$\text{moles Po} = \frac{1}{254,4} = 0,00393 \text{ moles Po}$$ See formula (1)

$$\text{moles O} = \frac{75}{282,5} = 0,26549 \text{ moles O}$$ See formula (1)

$$\text{moles L} = \frac{10}{280,4} = 0,03566 \text{ moles L}$$ See formula (1)

$$\text{moles Ln} = \frac{1}{278,4} = 0,003594 \text{ moles Ln}$$ See formula (1)

Total   = 0,35822 moles TAGs

4.5.4. Normalization of fatty acids to 100 %

$$\text{moles \% P}(1,2,3) = \frac{0,03900 \text{ moles P} * 100}{0,35822 \text{ moles}} = 10,888 \%$$ See formula (2)

▼M13

$$\text{moles \% S(1, 2, 3)} = \frac{0,01054 \text{ moles S } * 100}{0,35822 \text{ moles}} = 2,944 \text{ \%}$$ See formula (2)

$$\text{moles \% Po(1, 2, 3)} = \frac{0,00393 \text{ moles Po } * 100}{0,35822 \text{ moles}} = 1,097 \text{ \%}$$ See formula (2)

$$\text{moles \% O(1, 2, 3)} = \frac{0,26549 \text{ moles O } * 100}{0,35822 \text{ moles}} = 74,113 \text{ \%}$$ See formula (2)

$$\text{moles \% L(1, 2, 3)} = \frac{0,03566 \text{ moles L } * 100}{0,35822 \text{ moles}} = 9,956 \text{ \%}$$ See formula (2)

$$\text{moles \% Ln(1, 2, 3)} = \frac{0,00359 \text{ moles Ln } * 100}{0,35822 \text{ moles}} = 1,003 \text{ \%}$$ See formula (2)

Total moles %   = 100,0 %

Sum of the saturated and unsaturated fatty acids in the 1,2,3- position of TAGs

moles % SFA = 10,888 % + 2,944 % = 13,831 %   See formula (3)

moles % UFA = 100,000 % — 13,831 % = 86,169 %   See formula (3)

4.5.5. Calculation of the fatty acid composition in 2- and 1,3-positions of the TAGs

4.5.5.1. Saturated fatty acids in 2- position [P(2) and S(2)]

moles % P(2) = 10,888 % * 0,06 = 0,653 moles %   See formula (4)

moles % S(2) = 2,944 % * 0,06 = 0,177 moles %   See formula (4)

4.5.5.2. Unsaturated fatty acids in 1,3-position [Po(1,3), O(1,3), L(1,3) and Ln(1,3)]

$$\text{moles \% Po(2)} = \frac{1,097 \text{ \%}}{86,169 \text{ \%}} * (100 - -0,659 - 0,177) = 1,263 \text{ moles \%}$$ See formula (5)

$$\text{moles \% O(2)} = \frac{74,113 \text{ \%}}{86,169 \text{ \%}} * (100 - -0,659 - 0,177) = 85,295 \text{ moles \%}$$ See formula (5)

$$\text{moles \% L(2)} = \frac{9,956 \text{ \%}}{86,169 \text{ \%}} * (100 - -0,659 - 0,177) = 11,458 \text{ moles \%}$$ See formula (5)

$$\text{moles \% Ln(2)} = \frac{1,003 \text{ \%}}{86,169 \text{ \%}} * (100 - -0,659 - 0,177) = 1,154 \text{ moles \%}$$ See formula (5)

4.5.5.3. Fatty acids in 1,3-positions [P(1,3), S(1,3), Po(1,3), O(1,3), L(1,3) and Ln(1,3)]

$$\text{moles \% P(1, 3)} = \frac{10,888 - 0,659}{2} \; 10,888 = 16,005 \text{ moles \%}$$ See formula (6)

$$\text{moles \% S(1, 3)} = \frac{2,944 - 0,177}{2} \; 2,944 = 4,327 \text{ moles \%}$$ See formula (6)

$$\text{moles \% Po(1, 3)} = \frac{1,097 - 1,263}{2} \; 1,097 = 1,015 \text{ moles \%}$$ See formula (6)

$$\text{moles \% O(1, 3)} = \frac{74,113 - 85,295}{2} \; 74,113 = 68,522 \text{ moles \%}$$ See formula (6)

$$\text{moles \% L(1, 3)} = \frac{9,956 - 11,458}{2} \; 9,956 = 9,205 \text{ moles \%}$$ See formula (6)

$$\text{moles \% Ln(1, 3)} = \frac{1,003 - 1,154}{2} \; 1,003 = 0,927 \text{ moles \%}$$ See formula (6)

▼__M13__

4.5.6.   Calculation of triacylglycerols

From the calculated fatty acid composition in sn-2- and sn-1,3- positions (see above):

FA in	1,3-pos.	2-pos.
P	16,005 %	0,653 %
S	4,327 %	0,177 %
Po	1,015 %	1,263 %
O	68,522 %	85,295 %
L	9,205 %	11,458 %
Ln	0,927 %	1,154 %
Sum	100,0 %	100,0 %

the following triacylglycerols are calculated:

LLL

PoPoPo

PoLL with 1 positional isomer

SLnLn with 1 positional isomer

PoPoL with 1 positional isomer

PPoLn with 2 positional isomers

OLLn with 2 positional isomers

PLLn with 2 positional isomers

PoOLn with 2 positional isomers.

4.5.6.1.   TAGs with one fatty acid (LLL, PoPoPo)   See formula (7)

$$\text{mol \% LLL} = \frac{9,205 \text{ \%} \ * \ 11,458 \text{ \%} \ * \ 9,205 \text{ \%}}{10\,000} \qquad = 0,09708 \text{ mol LLL}$$

$$\text{mol \% PoPoPo} = \frac{1,015 \text{ \%} \ * \ 1,263 \text{ \%} \ * \ 1,015 \text{ \%}}{10\,000} \qquad = 0,00013 \text{ mol PoPoPo}$$

4.5.6.2.   TAGs with two fatty acids (PoLL, SLnLn, PoPoL)   See formula (8)

$$\text{mol \% PoLL} + \text{LLPo} = \frac{1,015 \text{ \%} \ * \ 11,458 \text{ \%} \ * \ 9,205 \text{ \%} \ * \ 2}{10\,000} \qquad = 0,02141$$

$$\text{mol \% LPoL} = \frac{9,205 \text{ \%} \ * \ 1,263 \text{ \%} \ * \ 9,205 \text{ \%}}{10\,000} \qquad = 0,01070$$

0,03211 mol PoLL

$$\text{mol \% SLnLn} + \text{LnLnS} = \frac{4,327 \text{ \%} \ * \ 1,154 \text{ \%} \ * \ 0,927 \text{ \%} \ * \ 2}{10\,000} \qquad = 0,00093$$

$$\text{mol \% LnSLn} = \frac{0,927 \text{ \%} \ * \ 0,177 \text{ \%} \ * \ 0,927 \text{ \%}}{10\,000} \qquad = 0,00002$$

0,00095 mol SLnLn

$$\text{mol \% PoPoL} + \text{LPoPo} = \frac{1,015 \text{ \%} \ * \ 1,263 \text{ \%} \ * \ 9,205 \text{ \%} \ * \ 2}{10\,000} \qquad = 0,00236$$

$$\text{mol \% PoLPo} = \frac{1,015 \text{ \%} \ * \ 11,458 \text{ \%} \ * \ 1,015 \text{ \%}}{10\,000} \qquad = 0,00118$$

0,00354 mol PoPoL

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4.5.6.3. TAGs with three different fatty acids (PoPLn, OLLn, PLLn, PoOLn)

See formula (9)

$$\text{mol \% PPoLn} = \frac{16,005 \text{ \%} * 1,263 \text{ \%} * 0,927 \text{ \%} * 2}{10\,000} = 0,00375$$

$$\text{mol \% LnPPo} = \frac{0,927 \text{ \%} * 0,653 \text{ \%} * 1,015 \text{ \%} * 2}{10\,000} = 0,00012$$

$$\text{mol \% PoLnP} = \frac{1,015 \text{ \%} * 1,154 \text{ \%} * 16,005 \text{ \%} * 2}{10\,000} = 0,00375$$

0,00762 mol PPoLn

$$\text{mol \% OLLn} = \frac{68,522 \text{ \%} * 11,458 \text{ \%} * 0,927 \text{ \%} * 2}{10\,000} = 0,14577$$

$$\text{mol \% LnOL} = \frac{0,927 \text{ \%} * 85,295 \text{ \%} * 9,205 \text{ \%} * 2}{10\,000} = 0,14577$$

$$\text{mol \% LLnO} = \frac{9,205 \text{ \%} * 1,154 \text{ \%} * 68,522 \text{ \%} * 2}{10\,000} = 0,14577$$

0,43671 mol OLLn

$$\text{mol \% PLLn} = \frac{16,005 \text{ \%} * 11,458 \text{ \%} * 0,927 \text{ \%} * 2}{10\,000} = 0,03400$$

$$\text{mol \% LnPL} = \frac{0,927 \text{ \%} * 0,653 \text{ \%} * 9,205 \text{ \%} * 2}{10\,000} = 0,00111$$

$$\text{mol \% LLnP} = \frac{9,205 \text{ \%} * 1,154 \text{ \%} * 16,005 \text{ \%} * 2}{10\,000} = 0,03400$$

0,06911 mol PLLn

$$\text{mol \% PoOLn} = \frac{1,015 \text{ \%} * 85,295 \text{ \%} * 0,927 \text{ \%} * 2}{10\,000} = 0,01605$$

$$\text{mol \% LnPoO} = \frac{0,927 \text{ \%} * 1,263 \text{ \%} * 68,522 \text{ \%} * 2}{10\,000} = 0,01605$$

$$\text{mol \% OLnPo} = \frac{68,522 \text{ \%} * 1,154 \text{ \%} * 1,015 \text{ \%} * 2}{10\,000} = 0,01605$$

0,04815 mol PoOLn

ECN42 = 0,69540 mol TAGs

▼M14

Figure 1: *Graph of log α against f (number of double bonds)*
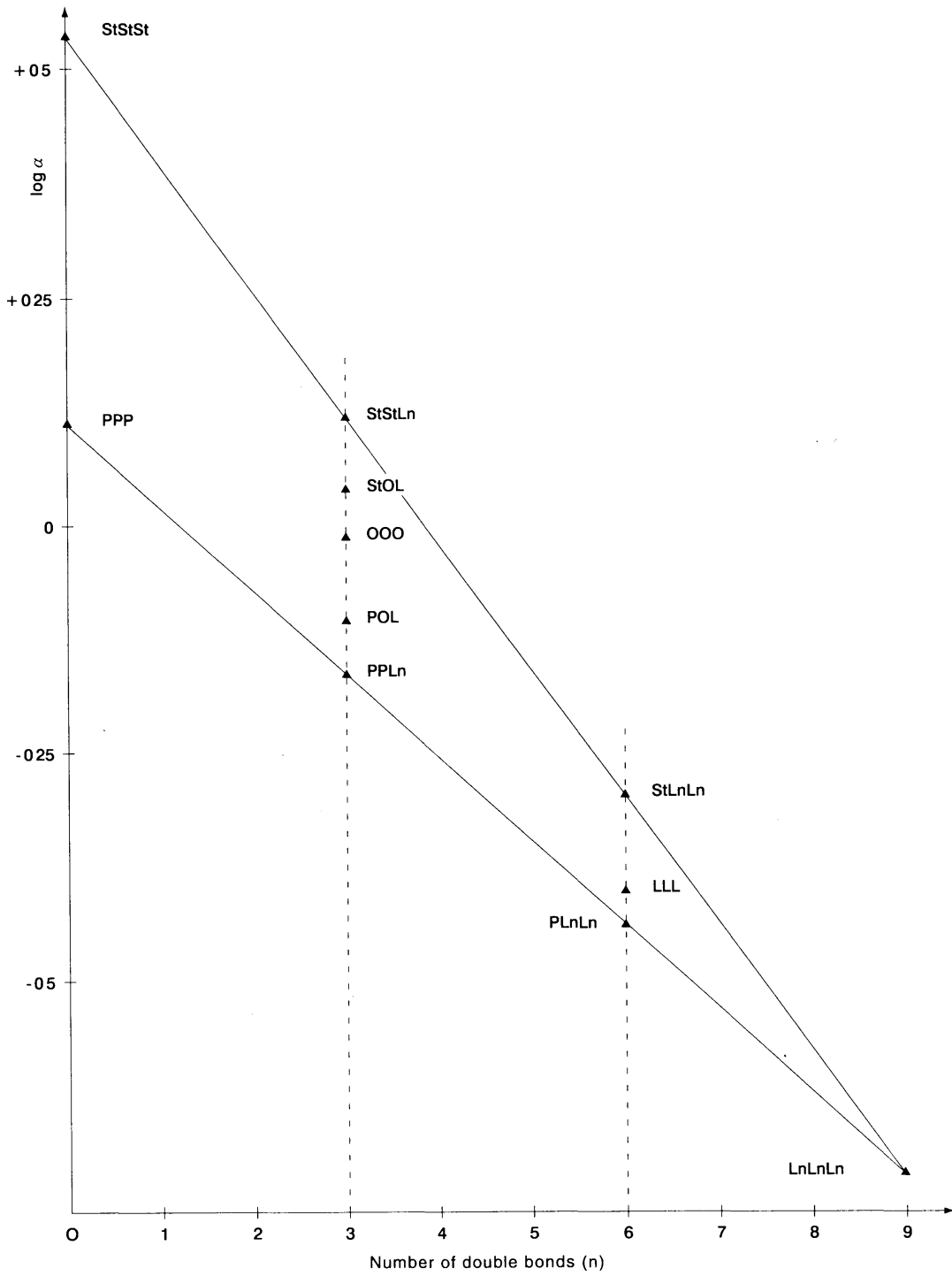
Note: La = lauric acid; My = myristic acid; P = palmitic acid; St = stearic acid; O = oleic acid; L = linoleic acid; Ln = linolenic acid.
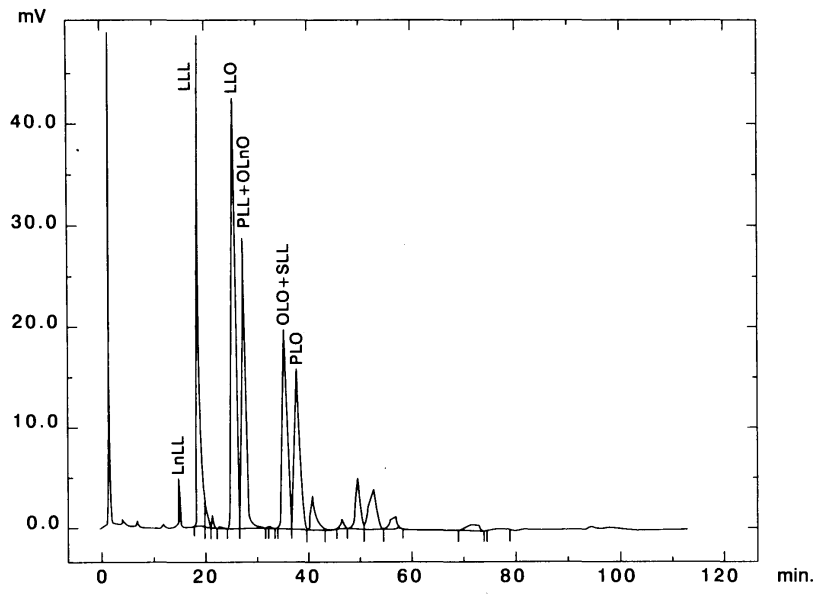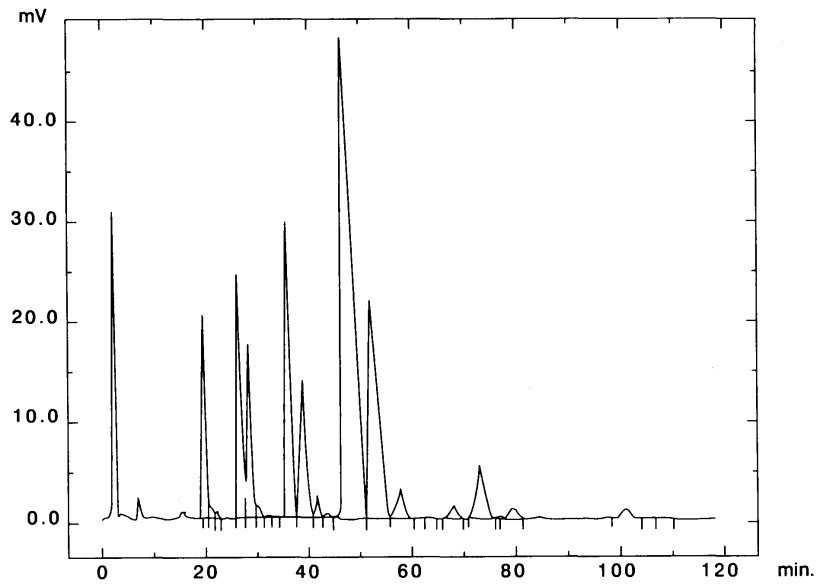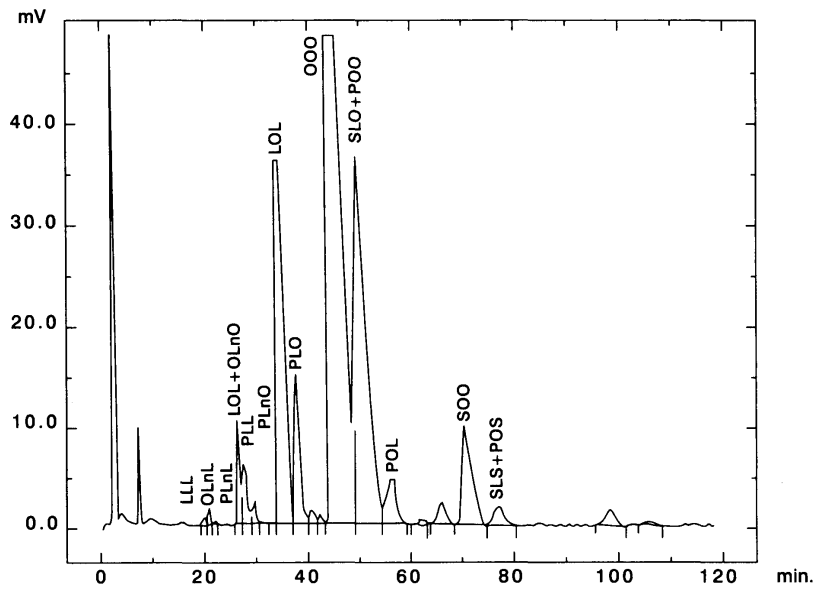
▼M14

Figure 2: *Soyabean oil*

Figure 3: *Soyabean oil / olive oil 30/70*

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Figure 4: *Olive oil*

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ANNEX XIX

DETERMINATION OF ALIPHATIC ALCOHOLS CONTENT BY CAPILLARY GAS CHROMATOGRAPHY

1. OBJECT

   The procedure describes a method for the determination of aliphatic alcohols content in oils and fats.

2. PRINCIPLE OF THE METHOD

   The fatty substance, with 1-eicosanol added as internal standard, is saponified with ethanolic potassium hydroxide and then the unsaponifiable matter extracted with ethyl ether. The alcoholic fraction is separated from the unsaponifiable matter by chromatography on a basic silica gel plate; the alcohols recovered from the silica gel are transformed into trimethylsilyl ethers and analysed by capillary gas chromatography.

3. EQUIPMENT

3.1. 250 ml round-bottomed flask fitted with a reflux condenser having ground-glass joints.

3.2. 500 ml separating funnel.

3.3. 250 ml round-bottomed flasks.

3.4. Chromatographic tank for thin-layer chromatographic analysis, for glass plates of dimensions 20 x 20 cm.

3.5. Ultraviolet lamp having a wavelength of 366 or 254 nm.

3.6. 100 µl and 500 µl microsyringes.

3.7. A cylindrical filter funnel with a G3 porous septum (porosity 15 to 40 µm) of diameter approximately 2 cm and a depth of some 5 cm, with an attachment suitable for filtration under vacuum and a 12/21 male ground glass joint.

3.8. 50 ml vacuum conical flask with a 12/21 ground-glass female joint which can be fitted to the filter funnel (3.7).

3.9. A 10 ml test tube with a tapering bottom and a sealing stopper.

3.10. Gas chromatograph for use with a capillary column, and provided with a splitting system composed of:

3.10.1. Thermostatic chamber for columns (column oven) to hold the temperature desired with a precision of ± 1 ºC.

3.10.2. A temperature-adjustable injection unit with a persilanised glass vaporising element.

3.10.3. A flame ionisation detector and converter-amplifier.

3.10.4. Recorder-integrator for operation with the converter-amplifier (3.10.3), with response time not exceeding one second and with variable paper-speed.

3.11. Glass or fused silica capillary column, of length 20 to 30 m, internal diameter 0,25 to 0,32 mm, with SE-52 or SE-54 liquid phase or equivalent, with a film thickness between 0,10 and 0,30 µm.

3.12. Microsyringe for gas chromatography, of 10 µl capacity with hardened needle.

3.13. Analytical balance sensitive to 1 mg (with 0,1 mg display).

4. REAGENTS

4.1. Potassium hydroxide, approximately 2 N ethanolic solution: 130 g potassium hydroxide (minimum concentration 85 %) is dissolved, with cooling, in 200 ml distilled water and then made up to one litre with ethanol. The solution should be stored in a well-stoppered opaque glass bottle.

4.2. Ethyl ether, pure for analysis.

4.3. Anhydrous sodium sulphate, analytical purity.

▼**M19**

4.4. Glass plates coated with silica gel, without fluorescence indicator, thickness 0,25 mm (commercially available ready for use).

4.5. Potassium hydroxide, approximately 0,2 N ethanolic solution; 13 g of potassium hydroxide are dissolved in 20 ml of distilled water and made up to one litre with ethanol.

4.6. Benzene, for chromatography (see 5.2.2).

4.7. Acetone, for chromatography (See 5.2.2).

4.8. Hexane, for chromatography (see 5.2.2).

4.9. Ethyl ether, for chromatography (see 5.2.2).

4.10. Chloroform, for chromatography.

4.11. Reference solution for thin-layer chromatography: cholesterol or phytosterols, 5 % solution in chloroform.

4.12. 0,2 % solution of 2', 7'-dichlorofluorescein in ethanol. Make slightly basic by adding a few drops of 2 N alcoholic potassium hydroxide solution.

4.13. Anhydrous pyridine, for chromatography.

4.14. Hexamethyl disilazane.

4.15. Trimethylchlorosilane.

4.16. Standard solutions of trimethylsilyl ethers of aliphatic alcohols from C_{20} to C_{28}. They may be prepared from mixtures of pure alcohols at the time they are required for use.

4.17. A 0,1 % (m/v) solution of 1-eicosanol in chloroform (internal standard).

4.18. Carrier gas: hydrogen or helium, gas-chromatographic purity.

4.19. Auxiliary gas: nitrogen, gas-chromatographic purity.

5. PROCEDURE

5.1. **Preparation of the unsaponifiables**

5.1.1. Using a 500 μl microsyringe place, into a 250 ml round-bottom flask, a volume of 0,1 % 1-eicosanol solution in chloroform (4.17) containing a quantity of 1-eicosanol approximately equal to 10 % of the aliphatic alcohols content in that portion of sample to be taken for analysis. For example, to 5 g of sample add 250 μl of the 0,1 % 1-eicosanol solution if olive oil and 1 500 μl if olive pomace oil.

Evaporate to dryness in current of nitrogen and then weigh accurately 5 g of the dry filtered sample into the same flask.

5.1.2. Add 50 ml of 2 N potassium hydroxide ethanolic solution, fit the reflux condenser and heat the apparatus to slight boiling on a steam bath, stirring continuously throughout the heating process until saponification has taken place (the solution becomes clear). Continue heating for a further 20 minutes and then add 50 ml of distilled water through the condenser. The condenser is then disconnected and the flask cooled to approximately 30 ºC.

5.1.3. The contents of the flask are quantitatively transferred to a separating funnel of 500 ml capacity by adding distilled water several times, using a total of around 50 ml distilled water. Add approximately 80 ml of ethyl ether, shake vigorously for approximately 30 seconds and allow to settle (Note 1).

Separate off the lower aqueous phase collecting it in a second separating funnel. Two further extractions are effected on the aqueous phase, in the same manner, using each time 60 to 70 ml ethyl ether.

Note 1: Emulsions may be eliminated by adding, using as a spray, small quantities of ethyl alcohol or methyl alcohol.

5.1.4. The ethyl ether extracts are combined in a separating funnel and washed with distilled water (50 ml at a time) until the washing water gives a neutral reaction.

Discard the washing water, dry with anhydrous sodium sulphate and filter, into a flask of 250 ml capacity which has been weighed beforehand, the funnel and filter being washed with small quantities of ethyl ether which are added to the total.

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5.1.5. Distil the ether down to a few ml, then bring to dryness under a slight vacuum or in a current of nitrogen, completing drying in an oven at 100 ºC for approximately a quarter of an hour, and then weigh after cooling in a desiccator.

5.2. **Separation of alcoholic fractions**

5.2.1. Preparation of basic TLC plates: the silica gel plates (4.4) are immersed completely, in 0,2 N potassium hydroxide solution (4.5) for 10 seconds, and then left to dry for two hours under an extractor hood and finally placed in an oven at 100 ºC for one hour.

Remove from the oven and keep in a calcium chloride desiccator until required for use (plates treated in this way must be used within 15 days).

Note 2: When basic silica gel plates are used to separate the alcoholic fraction there is no need to treat the unsaponifiables with alumina. It follows that all acid compounds (fatty acids and others) are retained at the origin thereby obtaining both aliphatic alcohol and terpenic alcohol bands which are both separated distinctly from the sterol band.

5.2.2. Place a 65/35 by volume hexane/ethyl ether mixture in the plate-developing chamber to a depth of approximately 1 cm (*).

Close the chamber using an appropriate cover and leave for half an hour to allow equilibration between vapour and liquid. Strips of filter paper dipping into the eluent may be affixed to the inside surfaces of the tank to reduce the development time by approximately one third and obtain more uniform, regular elution of the components.

Note 3: The developing solution must be replaced for each analysis in order to obtain reproducible developing conditions.

5.2.3. An approximately 5 % solution of unsaponifiable matter (5.1.5) in chloroform is prepared and 0,3 ml of the solution is streaked as a uniform strip of minimum thickness, using the 100 μl microsyringe, on a TLC plate at approximately 2 cm from the bottom of the TLC plate. Aligned with the origin, 2 to 3 μl of the aliphatic alcohols reference solution (4.11) are spotted for the identification of the aliphatic alcohols band after development has been completed.

5.2.4. Place the plate inside the development tank as stated in 5.2.2. The ambient temperature should be maintained between 15 and 20 ºC. Immediately close the chamber with the cover and allow to elute until the solvent front reaches approximately 1 cm from the upper edge of the plate.

The plate is then removed from the development chamber and the solvent evaporated under a hot air current or the plate is left for a while under the extractor hood.

5.2.5. The plate is sprayed lightly and evenly with the solution of 2', 7'-dichlorofluorescein when the plate is observed under ultra violet light. The aliphatic alcohols band can be identified through being aligned with the stain obtained from the reference solution: mark the limits of the band with a black pencil; outlining the band of aliphatic alcohols and the band immediately above that, which is the terpenic alcohols band, together.

Note 4: The aliphatic alcohols band and the terpenic alcohols band are to be grouped together in view of the possible migration of some aliphatic alcohols into the triterpenic alcohols band.

5.2.6. Using a metal spatula scrape off the silica gel in the marked area. Place the finely comminuted material removed into the filter funnel (3.7). Add 10 ml of hot chloroform, mix carefully with the metal spatula and filter under vacuum, collecting the filtrate in the conical flask (3.8) attached to the filter funnel.

Wash the pomace in the flask three times with ethyl ether (approximately 10 ml each time) collecting the filtrate in the same flask attached to the funnel. Evaporate the filtrate to a volume of 4 to 5 ml, transfer the residual solution to the previously weighed 10 ml test tube (3.9), evaporate to dryness by mild heating in a gentle flow of nitrogen, make up again using a few drops of acetone, evaporate again to

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(*) In these cases in particular, a 95/5 by volume benzene/acetone eluent mixture must be used to obtain distinct band separation.

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dryness, place in an oven at 105 ºC for approximately 10 minutes and then allow to cool in a desiccator and weigh.

The pomace inside the test tube is composed of the alcoholic fraction.

5.3. **Preparation of the trimethylsilyl ethers**

5.3.1. The reagent for silylation, consisting of a mixture of 9:3:1 by volume (Note 5) of pyridine-hexamethyldisilazane-trimethylchlorosilane in the proportion of 50 μl for each milligram of aliphatic alcohols, is added to the test tube containing the alcoholic fraction, avoiding all absorption of moisture (Note 6).

Note 5: Solutions which are ready for use are available commercially. Other silanising reagents such as, for example, bis-trimethyl-silyl, trifluor acetamide + 1 % trimethyl chlorosilane, which has to be diluted with an equal volume of anhydrous pyridine, are also available.

Note 6: The slight opalescence which may form is normal and does not cause any interference. The formation of a white floc or the appearance of a pink colour are indicative of the presence of moisture or deterioration of the reagent. If these occur the test must be repeated.

5.3.2. Stopper the test tube, shake carefully (without overturning) until the aliphatic alcohols are completely dissolved. Stand for at least 15 minutes at ambient temperature and then centrifuge for a few minutes. The clear solution is ready for gas chromatographic analysis.

5.4. **Gas chromatography analysis**

5.4.1. Preliminary operations, column packing

5.4.1.1. Fit the column in the gas chromatograph, attaching the inlet end to the injector connected to the splitting system and the outlet end to the detector. Carry out a general check of the gas chromatography assembly (tightness of gas fittings, efficiency of the detector, efficiency of the splitting system and of the recording system, etc.).

5.4.1.2. If the column is being used for the first time it is recommended that it should be subjected to conditioning. A little carrier gas is passed through the capillary column and then the gas chromatography assembly is switched on and gradually heated until a temperature not less than 20 ºC above the operating temperature (see Note 7) is attained. That temperature is held for not less than two hours and then the assembly is brought to the operating conditions (regulation of gas flow, split flame ignition, connection to the electronic recorder, adjustment of the temperature of the capillary column oven, the detector and the injector, etc.) and the signal is adjusted to a sensitivity not less than twice the highest level contemplated for the execution of the analysis. The course of the base line must be linear, without peaks of any kind, and must not drift. A negative straight-line drift indicates leakage from the column connections; a positive drift indicates inadequate conditioning of the column.

Note 7: The conditioning temperature shall be at least 20 ºC less than the maximum temperature contemplated for the liquid phase employed.

5.4.2. Choice of operating conditions

5.4.2.1. The guideline operating conditions are as follows:

— column temperature: the initial isotherm is set at 180 ºC for eight minutes and then programmed at 5 ºC/minute to 260 ºC and a further 15 minutes at 260 ºC,

— temperature of evaporator: 280 ºC,

— temperature of detector: 290 ºC,

— linear velocity of carrier gas: helium 20 to 35 cm/s, hydrogen 30 to 50 cm/s,

— splitting ratio: 1:50 to 1:100,

— sensitivity of instrument: 4 to 16 times the minimum attenuation,

— sensitivity of recording: 1 to 2 mV fs,

— paper speed: 30 to 60 cm/h,

— quantity of substance injected: 0,5 to 1 μl of TMSE solution.

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The above conditions may be modified according to the characteristics of the column and of the gas chromatograph to obtain chromatograms satisfying the following conditions:

— alcohol C_{26} retention time shall be 18 ± 5 minutes,
— the alcohol C_{22} peak shall be 80 ± 20 % of the full scale value for olive oil and 40 ± 20 % of the full scale value for seed oil.

5.4.2.2. The above requirements are checked by repeated injection of the standard TMSE mixture of alcohols and the operating conditions are adjusted to yield the best possible results.

5.4.2.3. The parameters for the integration of peaks shall be set so that a correct appraisal of the areas of the peaks considered is obtained.

5.4.3. Analytical procedure

5.4.3.1. Using the microsyringe of 10 μl capacity draw in 1 μl of hexane followed by 0,5 μl of air and subsequently 0,5 to 1 μl of the sample solution; raise the plunger of the syringe further so the needle is emptied. Push the needle through the membrane of the injection unit and after one to two seconds inject rapidly, then slowly remove the needle after some five seconds.

5.4.3.2. Recording is effected until the TMSE of the aliphatic alcohols present have been eluted completely. The base line shall always correspond to the requirements of 5.4.1.2.

5.4.4. Peak identification

The identification of individual peaks is effected according to the retention times and by comparison with the standard TMSE mixture, analysed under the same conditions.

A chromatogram of the alcoholic fraction of a virgin olive oil is shown in Figure 1.

5.4.5. Quantitative evaluation

5.4.5.1. The peak areas of 1-eicosanol and of the aliphatic alcohols C_{22}, C_{24}, C_{26} and C_{28} are calculated by electronic integration.

5.4.5.2. The contents of each aliphatic alcohol, expressed in mg/1 000 g fatty substance, are calculated as follows:

$$\text{alcohol } x = \frac{A_x \cdot m_s \cdot 1\ 000}{A_s \cdot m}$$

where:

Ax = area of the alcohol peak x

A_s = area of 1-eicosanol

m_s = mass of 1-eicosanol in milligrams

m = mass of sample drawn for determination, in grams.

6. EXPRESSION OF THE RESULTS

The contents of the individual aliphatic alcohols in mg/1 000 g of fatty substance and the sum of the 'total aliphatic alcohols' are reported.

▼M19

APPENDIX

Determination of the linear velocity of the gas

1 to 3 µl of methane or propane are injected into the gas chromatograph set at normal operating conditions and the time taken for the methane or propane to flow through the column from the instant of injection to the instant the peak elutes (tM) is measured using a stop clock.

The linear velocity in cm/s is given by L/tM, where L is the length of the column in centimetres and tM is the measured time in seconds.
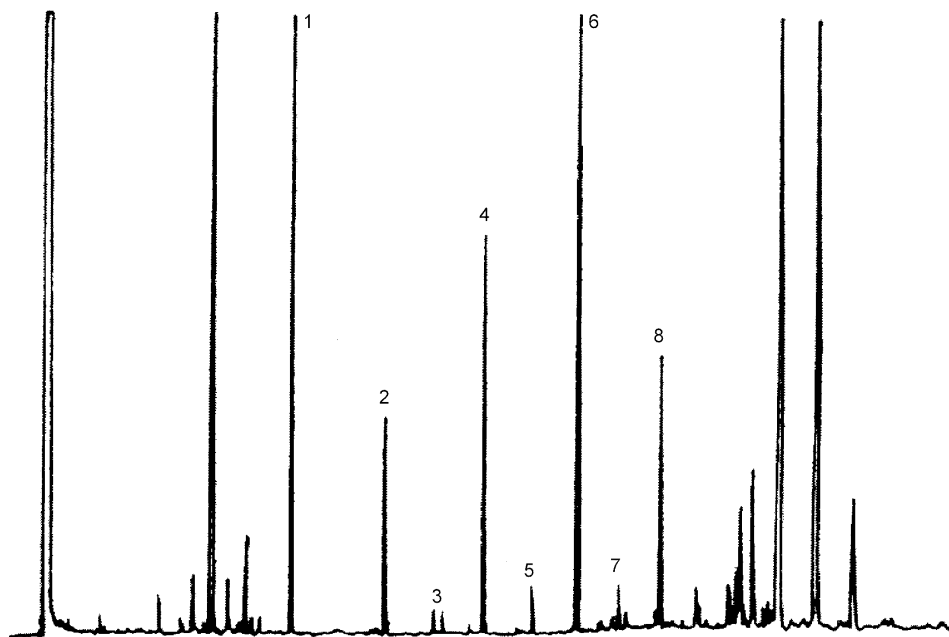
Figure 1 — Chromatogram of the alcoholic fraction of virgin oil

1 = Eicosanol

2 = Decosanol

3 = Tricosanol

4 = Tetracosanol

5 = Pentacosanol

6 = Hexacosanol

7 = Heptacosanol

8 = Octacosanol