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COMMISSION REGULATION (EC) No 2870/2000

of 19 December 2000

laying down Community reference methods for the analysis of spirits drinks

(OJ L 333, 29.12.2000, p. 20)

Amended by:

		Official Journal		
		No	page	date
►**M1**	Commission Regulation (EC) No 2091/2002 of 26 November 2002	L 322	11	27.11.2002
►**M2**	Commission Implementing Regulation (EU) 2016/635 of 22 April 2016	L 108	1	23.4.2016
►**M3**	Commission Implementing Regulation (EU) 2023/383 of 16 February 2023	L 53	3	21.2.2023

Corrected by:

►**C1**	Corrigendum, OJ L 200, 10.8.2023, p. 48 (2870/2000)

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COMMISSION REGULATION (EC) No 2870/2000

of 19 December 2000

laying down Community reference methods for the analysis of spirits drinks

Article 1

The Community reference methods for the analysis of spirits drinks to ensure compliance with Regulation (EEC) No 1576/89 and Regulation (EEC) No 1014/90:

— when any official control takes place, or

— in the event of a dispute,

shall be those set out in the Annex hereto.

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Article 1a

1. This Regulation applies to ethyl alcohol of agricultural origin as defined by Article 5 of Regulation (EU) 2019/787 of the European Parliament and of the Council ([1]).

2. The Union reference methods of analysis for ethyl alcohol of agricultural origin shall be those set out in the Annex to this Regulation.

3. For the purposes of this Regulation, ethyl alcohol of agricultural origin shall be considered as a distillate whose alcoholic strength by volume shall be measured directly as set out in Appendix II of Chapter I of the Annex.

However, where the alcohol sample is not clear or suspended particles are visible, the sample shall be distilled.

4. For the determination of volatile substances the calibration with the standard solution C prepared in absolute ethanol shall be required to achieve appropriate matrix matching between samples and standard solutions detailed in Chapter III.2 of the Annex.

([1]) Regulation (EU) 2019/787 of the European Parliament and of the Council of 17 April 2019 on the definition, description, presentation and labelling of spirit drinks, the use of the names of spirit drinks in the presentation and labelling of other foodstuffs, the protection of geographical indications for spirit drinks, the use of ethyl alcohol and distillates of agricultural origin in alcoholic beverages, and repealing Regulation (EC) No 110/2008 (OJ L 130, 17.5.2019, p. 1).

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5. For the determination of furfural, as detailed in Chapter X of the Annex, ethyl alcohol of agricultural origin shall be diluted by two through the addition of water so as to double its initial volume and reach an alcoholic strength by volume compatible with the calibration solutions. Results for the analysis of furfural shall be converted to grams per hectolitre of 100 % vol. alcohol in accordance with the equation 'Concentration of furfural in grams per hectolitre of 100 % vol. alcohol = Concentration of furfural in mg/l x 10/alcoholic strength by volume (% vol)', where alcoholic strength by volume (% vol) is the alcoholic strength of the measured sample as determined in Chapter I of the Annex.

6. For the determination of ^{14}C content in ethanol, the method set out in Chapter XI of the Annex shall be used.

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Article 2

Notwithstanding the first indent of Article 1, other analytical methods shall be permitted, under the responsibility of the director of the laboratory, on condition that the accuracy and precision (repeatability and reproducibility) of the methods are at least equivalent to those of the relevant reference analytical methods given in the Annex.

Article 3

Where Community analytical reference methods are not laid down for the detection and quantification of substances contained in a particular spirit drink, the following methods shall be used:

(a) analytical methods which are validated to internationally recognised procedures and in particular meet the criteria set in the Annex to Directive 85/591/EEC;

(b) analytical methods conforming to the recommended standards of the International Organisation for Standardisation (ISO);

(c) analytical methods recognised by the General Assembly of the International Vine and Wine Office (OIV) and published by that Office;

(d) in the absence of a method as indicated at (a), (b) or (c), by reason of its accuracy, repeatability and reproducibility:

— an analytical method approved by the Member State concerned,

— where necessary, any other suitable analytical method.

Article 4

For the purposes of this Regulation:

(a) 'repeatability limit': shall be the value less than or equal to which the absolute difference between two test results obtained under the repeatability conditions (same operator, same apparatus, same laboratory and a short interval of time) may be expected to be with a probability of 95 % {ISO 3534-1};

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(b) ‘reproducibility limit’: shall be the value less than or equal to which the absolute difference between two test results obtained under the reproducibility conditions (different operators, different apparatus and different laboratories), may be expected to be with a probability of 95 % {ISO 3534-1};

(c) ‘accuracy’: shall be the closeness of agreement between a test result and the accepted reference value {ISO 3534-1}.

Article 5

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

It shall apply from 1 January 2001.

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

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ANNEX

DESCRIPTION OF ANALYTICAL REFERENCE METHODS

I. Appendix I: Preparation of distillate

Appendix II: Measurement of density of distillate

— Method A = pycnometry

— Method B = electronic densimetry

— Method C = densimetry using hydrostatic balance

II. Determination of total dry extract by gravimetry

III. Determination of volatile substances and methanol

III.1. General remarks

III.2. Volatile congeners: aldehydes, higher alcohols, ethyl acetate and methanol (gas chromatography)

III.3. Volatile acidity ►M2 ———————— ◄

IV. Hydrocyanic acid (p.m.)

V. Anethole ►M1 ———————— ◄

VI. Glycyrrhizic acid ►M1 ———————— ◄

VII. Chalcones ►M1 ———————— ◄

VIII. Total sugars ►M2 ———————— ◄

IX. Egg yolk ►M1 ———————— ◄

▼M2

X. Determination of wood compounds: furfural, 5-hydroxymethylfurfural, 5-methylfurfural, vanillin, syringaldehyde, coniferaldehyde, sinapaldehyde, gallic acid, ellagic acid, vanillic acid, syringic acid and scopoletin

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XI. Determination of ^{14}C content in ethanol

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I. DETERMINATION OF ALCOHOLIC STRENGTH BY VOLUME OF SPIRIT DRINKS

Introduction

The reference method includes two Appendices:

Appendix I: Preparation of distillate

Appendix II: Measurement of density of distillate

1. **Scope**

The method is suitable for the determination of the real alcoholic strength by volume of spirit drinks.

2. **Normative References**

ISO 3696:1987: Water for analytical laboratory use — Specifications and test methods.

3. **Terms and Definitions**

3.1. *Reference temperature:*

The reference temperature for the determination of alcoholic strength by volume, density and specific gravity of spirit drinks is 20 °C.

Note 1: The term 'at t °C' is reserved for all determinations (of density or alcoholic strength by volume) expressed at a temperature other than the reference temperature of 20 °C.

3.2. *Density:*

The density is the mass per unit volume in vacuo of spirit drinks at 20 °C. It is expressed in kilograms per cubic metre and its symbol is $\rho_{20\ °C}$ or ρ_{20}.

3.3. *Specific gravity:*

The specific gravity is the ratio, expressed as a decimal number, of the density of spirit drinks at 20 °C to the density of water at the same temperature. It is denoted by the symbol $d_{20\ °C/20\ °C}$ or $d_{20/20}$, or simply d when there is no possibility of confusion. The characteristic that was measured must be specified on the assay certificate using the above-defined symbols only.

Note 2: It is possible to obtain the specific gravity from the density ρ_{20} at 20 °C:

$\rho_{20} = 998{,}203 \times d_{20/20}$ or $d_{20/20} = \rho_{20}/998{,}203$ where 998,203 is the density of water at 20 °C.

3.4. *Real alcoholic strength by volume:*

The real alcoholic strength by volume of spirit drinks is equal to the number of litres of ethyl alcohol contained in 100 l of a water-alcohol mixture having the same density as the alcohol or spirit after distillation. The reference values for alcoholic strength by volume (% vol) at 20 °C versus density at 20 °C for different water-alcohol mixtures that are to be used are those given in the international table adopted by the International Legal Metrology Organisation in its Recommendation No 22.

The general equation relating the alcoholic strength by volume and density of a water-alcohol mixture at a given temperature is given on page 40 in Chapter 3 'Alcoholic strength by volume' of the Annex to Commission Regulation (EEC) No 2676/90 (OJ L 272, 3.10.1990, p. 1) or in the manual of analysis methods of the OIV (1994) (p. 17).

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Note 3: For liqueurs and crèmes for which it is very difficult to measure volume accurately the sample must be weighed and the alcoholic strength is calculated first by mass.

Conversion formula:

$$\text{alcoholic strength by volume (\% vol)} = \frac{\text{ASM (\% mass)} \times P_{20}\text{ (sample)}}{P_{20}\text{ (alcohol)}}$$

where

ASM = alcoholic strength by mass,

ρ_{20} (alcohol) = 789,24 kg/m^3

4. **Principle**

Following distillation, the alcoholic strength by volume of the distillate is determined by pycnometry, electronic densimetry, or densimetry using a hydrostatic balance.

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APPENDIX I: PREPARATION OF DISTILLATE

1. **Scope**

The method is suitable for the preparation of distillates to be used to determine the real alcoholic strength by volume of spirit drinks.

2. **Principle**

The spirits are distilled to separate the ethyl alcohol and other volatile compounds from the extractive matter (substances which do not distil).

3. **Reagents and Materials**

3.1. Anti-bumping granules.

3.2. Concentrated antifoam emulsion (for crème liqueurs).

4. **Apparatus and equipment**

Usual laboratory apparatus and in particular the following.

4.1. Water bath capable of being maintained at 10 °C to 15 °C.

Water bath capable of being maintained at 20 °C (± 0,2 °C).

4.2. Class A volumetric flasks, 100 ml and 200 ml, that have been certified to 0,1 % and 0,15 % respectively.

4.3. Distillation apparatus:

4.3.1. General requirements

The distillation apparatus must meet the following specifications:

— the number of joints must be no more than the strict minimum needed to ensure the system is leak-tight,

— inclusion of a device designed to prevent priming (entrainment of the boiling liquid by the vapour) and to regularise the distillation rate of alcohol-rich vapours,

— rapid and complete condensation of the alcohol vapours,

— collection of the first distillation fractions in an aqueous medium.

The heat source must be used with a suitable heat-diffuser to prevent any pyrogenic reaction involving the extractive matter.

4.3.2. An example of a suitable distillation apparatus is shown in Figure 1 and includes the following parts:

— round-bottomed flask, 1 litre, with a standardised ground-glass joint,

— rectifying column at least 20-cm high (a Vigreux column, for example),

— elbow connector with an approximately 10-cm-long straight-rimmed condenser (a West-type condenser) fitted vertically,

— cooling coil, 40-cm long,

— drawn-out tube, taking the distillate to the bottom of a graduated collecting flask containing a small amount of water.

Note: The apparatus described above is intended for a sample of least 200 ml. However, a smaller sample size (100 ml) can be distilled by using a smaller distillation flask, provided a splashhead or some other device to prevent entrainment is used.

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5. **Storage of test samples**

Samples are stored at room temperature prior to analysis.

6. **Procedure**

Preliminary remark:

Distillation may also be by the procedure published by IUPAC (1968).

6.1. Distillation apparatus verification.

The apparatus used must be capable of the following:

The distillation of 200 ml of a water-alcohol solution with known concentration close to 50 % vol must not cause a loss of alcohol of more than 0,1 % vol.

6.2. Spirit drinks with alcoholic strength below 50 % vol.

Measure out 200 ml of the spirit into a volumetric flask.

Record the temperature of this liquid, or maintain at standard temperature (20 °C).

Pour the sample into the round-bottomed flask of the distillation apparatus and rinse the volumetric flask with three aliquots each of approximately 20 ml of distilled water. Add each rinse water aliquot to the contents of the distillation flask.

Note: This 60-ml dilution is sufficient for spirits containing less than 250 g of dry extract per litre. Otherwise, to prevent pyrolysis, the volume of rinse water must be at least 70 ml if the dry extract concentration is 300 g/l, 85 ml for 400 g/l dry extract, and 100 ml for 500 g/l dry extract (some fruit liqueurs or crèmes). Adjust these volumes proportionally for different sample volumes.

Add a few anti-bumping granules (3.1) (and antifoam for crème liqueurs).

Pour 20 ml of distilled water into the original 200 ml volumetric flask that will be used to hold the distillate. This flask must then be placed in a cold water bath (4.1) (10 to 15 °C for aniseed-flavoured spirit drinks).

Distil, avoiding entrainment and charring, occasionally agitating the contents of the flask, until the level of distillate is a few millimetres below the calibration mark of the volumetric flask.

When the temperature of this distillate has been brought down to within 0,5 °C of the liquid's initial temperature, make up to the mark with distilled water and mix thoroughly.

This distillate is used for the determination of alcoholic strength by volume (Appendix II)

6.3. Spirit drinks with alcoholic strength above 50 % vol.

Measure out 100 ml of the spirit drink into a 100-ml volumetric flask and pour into the round bottomed flask of the distillation apparatus.

Rinse the volumetric flask several times with distilled water and add the washings to the contents of the round-bottomed distillation flask. Use enough water to bring the flask's contents up to approximately 230 ml.

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Pour 20 ml of distilled water into a 200-ml volumetric flask that will be used to hold the distillate. This flask must then be placed in a cold water bath (4.1) (10 to 15 °C for aniseed-flavoured spirits).

Distil, agitating the contents occasionally, until the level of distillate is a few millimetres below the calibration mark of the 200-ml volumetric flask.

When the temperature of this distillate has been brought down to within 0,5 °C of the liquid's initial temperature, make up to the mark with distilled water and mix thoroughly.

This distillate is used for the determination of alcoholic strength by volume (Appendix II)

Note: The alcoholic strength by volume of the spirit drink is twice the alcoholic strength of the distillate.

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APPENDIX II: MEASUREMENT OF DENSITY OF DISTILLATE

METHOD A: DETERMINATION OF REAL ALCOHOLIC STRENGTH BY VOLUME OF SPIRIT DRINKS — MEASUREMENT BY PYCNOMETRY

A.1. **Principle**

The alcoholic strength by volume is obtained from the density of the distillate measured by pycnometry.

A.2. **Reagents and materials**

During the analysis, unless otherwise is stated, use only reagents of recognised analytical grade and water of at least grade 3 as defined in ISO 3696:1987.

A.2.1. Sodium chloride solution (2 % w/v)

To prepare 1 litre, weigh out 20 g of sodium chloride and dissolve to 1 litre using water.

A.3. **Apparatus and Equipment**

Usual laboratory apparatus and in particular the following:

A.3.1. Analytical balance capable of reading 0,1 mg.

A.3.2. Thermometer, with ground glass joint, calibrated in tenths of a degree from 10 to 30 °C. This thermometer must be certified or checked against a certified thermometer.

A.3.3. Pyrex-glass pycnometer of approximately 100 ml capacity fitted with a removable ground-glass thermometer (A.3.2). The pycnometer has a side tube 25 mm in length and 1 mm (maximum) in internal diameter ending in a conical ground joint. Other pycnometers as described in ISO 3507, e.g. 50 ml may be used if appropriate.

A.3.4. A tare bottle of the same external volume (to within 1 ml) as the pycnometer and with a mass equal to the mass of the pycnometer filled with a liquid of density 1,01 (sodium chloride solution A.2.1).

A.3.5. Thermally insulated jacket that fits the body of the pycnometer exactly.

Note 1: The method for determining the densities *in vacuo* of spirits calls for the use of a twin-pan balance, a pycnometer and a tare bottle of the same outside external volume to cancel out the effect of air buoyancy at any given moment. This simple technique may be applied using a single-pan balance provided that the tare bottle is weighed again to monitor changes in air buoyancy over time.

A.4. **Procedure**

Preliminary remarks:

The following procedure is described for the use of 100-ml pycnometer for determination of the alcoholic strength; this gives the best accuracy. However, it is also possible to use a smaller pycnometer, for example 50 ml.

A.4.1. Calibration of pycnometer

The pycnometer is calibrated by determining the following parameters:

— tare of the empty pycnometer,

— volume of the pycnometer at 20 °C,

— mass of the water-filled pycnometer at 20 °C.

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A.4.1.1. Calibration using a single-pan balance:

Determine:

— the mass of the clean, dry pycnometer (P),

— the mass of the water-filled pycnometer at t °C (P1),

— the mass of the tare bottle (T0).

A.4.1.1.1. Weigh the clean, dry pycnometer (P).

A.4.1.1.2. Fill the pycnometer carefully with distilled water at ambient temperature and fit the thermometer.

Carefully wipe the pycnometer dry and place it in the thermally-insulated jacket. Agitate by inverting the container until the thermometer's temperature reading is constant.

Set the pycnometer flush with the upper rim of the side tube. Read the temperature t °C carefully and if necessary correct for any inaccuracies in the temperature scale.

Weigh the water-filled pycnometer (P1).

A.4.1.1.3. Weigh the tare bottle (T0).

A.4.1.1.4. Calculation

— Tare of the empty pycnometer = P – m

where m is the mass of air in the pycnometer.

$m = 0{,}0012 \times (P1 - P)$

Note 2: 0,0012 is the density of dry air at 20 °C at a pressure of 760 mm Hg

— Volume of the pycnometer at 20 °C:

$V_{20\,°C} = [P1 - (P - m)] \times F_t,1$

where F_t is the factor for temperature t °C taken from Table I of Chapter 1 ‘Density and specific gravity’ of the Annex to Regulation (EEC) No 2676/90 (p. 10).

$V_{20\,°C}$ must be known to the nearest 0,001 ml.

— Mass of water in the pycnometer at 20 °C:

$M_{20\,°C} = V_{20\,°C} \times 0{,}998203$

where 0,998203 is the density of water at 20 °C.

Note 3: If necessary, the value 0,99715 of the density in air can be used and the alcoholic strength calculated with reference to the corresponding density in HM Customs and Excise tables in air.

A.4.1.2. Calibration method using a twin-pan balance:

A.4.1.2.1. Place the tare bottle on the left-hand pan and the clean, dry pycnometer with its collecting stopper on the right-hand pan. Balance them by placing weights on the pycnometer side: p grams.

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A.4.1.2.2. Fill the pycnometer carefully with distilled water at ambient temperature and fit the thermometer; carefully wipe the pycnometer dry and place it in the thermally insulated jacket; agitate by inverting the container until the thermometer's temperature reading is constant.

Accurately adjust the level to the upper rim of the side tube. Clean the side tube, fit the collecting stopper; read the temperature t °C carefully and if necessary correct for any inaccuracies in the temperature scale.

Weigh the water-filled pycnometer, with p′ the weight in grams making up the equilibrium.

A.4.1.2.3. Calculation

— Tare of the empty pycnometer = p + m

where m is the mass of air in the pycnometer.

$m = 0{,}0012 \times (p - p')$

— Volume of the pycnometer at 20 °C:

$V_{20\,°C} = (p + m - p') \times F_t,1$

where F_t is the factor for temperature t °C taken from Table I of Chapter 1 ‘Density and specific gravity’ of the Annex to Regulation (EEC) No 2676/90 (p. 10).

$V_{20\,°C}$ must be known to the nearest 0,001 ml.

— Mass of water in the pycnometer at 20 °C:

$M_{20\,°C} = V_{20\,°C} \times 0{,}998203$

where 0,998203 is the density of water at 20 °C.

A.4.2. Determination of alcoholic strength of test sample

A.4.2.1. Using a single-pan balance.

A.4.2.1.1. Weigh the tare bottle, weight T1.

A.4.2.1.2. Weigh the pycnometer with the prepared distillate (see Appendix I), P2 is its weight at t °C.

A.4.2.1.3. Calculation

— $dT = T1 - T0$

— Mass of empty pycnometer at moment of measuring

= P - m + dT

— Mass of the liquid in the pycnometer at t °C

= P2 - (P - m + dT)

— Density at t °C in g/ml

— $P_{t\,°C} = [P_2 - (P - m + dT)]/V_{20\,°C}$

— Express the density at t °C in kilograms per m^3 by multiplying $\rho_{t\,°C}$ by 1 000, the value being known as ρ_t.

— Correct ρ_t to 20 using the table of densities ρ_T for water-alcohol mixtures (Table II of Appendix II to the OIV's manual of analysis methods (1994), pp. 17-29).

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In the table find the horizontal line corresponding to temperature T in whole degrees immediately below t °C, the smallest density above ρ_t. Use the table difference found below that density to calculate the density ρ_t of the spirit at that temperature T in whole degrees.

— Using the whole temperature line, calculate the difference between density ρ′ in the table immediately above ρ_t and the calculated density ρ_t. Divide that difference by the table difference found to the right of density ρ′. The quotient provides the decimal portion of the alcoholic strength while the integer of the alcoholic strength is found at the top of the column in which density ρ′ is found (Dt, the alcoholic strength).

Note 4: Alternatively keep the pycnometer in a water bath maintained at 20 °C (± 0,2 °C) when making up to the mark.

A.4.2.1.4. Result

Using the density ρ_{20} calculate the real alcoholic strength using the alcoholic strength tables identified below:

The table giving the value of the alcoholic strength by volume (% vol) at 20 °C as a function of the density at 20 °C of water-alcohol mixtures is the international table adopted by the International Legal Metrology Organisation in its Recommendation No 22.

A.4.2.2. Method using a single-pan balance

A.4.2.2.1. Weigh the pycnometer with the distillate prepared (see part I), p″ is mass at t °C.

A.4.2.2.2. Calculation

— Mass of the liquid in the pycnometer at t °C

$= p + m - p''$

— Density at t °C in g/ml

$P_{t\,°C} = (p + m - p'')/V_{20\,°C}$

— Express the density at t °C in kilograms per m^3 and carry out the temperature correction in order to calculate the alcoholic strength at 20 °C, as indicated above for use of the single-pan balance.

A.5. **Method performance characteristics (precision)**

A.5.1. Statistical results of the interlaboratory test

The following data were obtained from an international method performance study carried out to internationally agreed procedures [1] [2].

Year of interlaboratory test	1997
Number of laboratories	20
Number of samples	6

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Samples	A	B	C	D	E	F
Number of laboratories retained after eliminating outliers	19	20	17	19	19	17
Number of outliers (laboratories)	1	—	2	1	1	3
Number of accepted results	38	40	34	38	38	34
Mean value ($\bar{\times}$) % vol	23,77	40,04	40,29	39,20	42,24	57,03
	26,51 (*)			42,93 (*)	45,73 (*)	63,03 (*)
Repeatability standard (S_r) % vol	0,106	0,176	0,072	0,103	0,171	0,190
Repeatability relative standard deviation (RSD_r) (%)	0,42	0,44	0,18	0,25	0,39	0,32
Repeatability limit (r) in % vol	0,30	0,49	0,20	0,29	0,48	0,53
Reproductibility standard deviation (S_R) % vol	0,131	0,236	0,154	0,233	0,238	0,322
Reproductibility relative standard deviation (RSD_R) (%)	0,52	0,59	0,38	0,57	0,54	0,53
Reproductibility limit (R) in % vol	0,37	0,66	0,43	0,65	0,67	0,90

Sample types
A Fruit liqueur; split level (*).
B Brandy; blind duplicates.
C Whisky; blind duplicates.
D Grappa; split level (*).
E Aquavit; split level (*).
F Rum; split level (*).

METHOD B: DETERMINATION OF REAL ALCOHOLIC STRENGTH BY VOLUME OF SPIRIT DRINKS — MEASUREMENT BY ELECTRONIC DENSIMETRY (BASED ON THE RESONANT FREQUENCY OSCILLATION OF A SAMPLE IN AN OSCILLATION CELL)

B.1. Principle

The liquid's density is determined by electronic measurement of the oscillations of a vibrating U-tube. To perform this measurement, the sample is added to an oscillating system, whose specific oscillation frequency is thus modified by the added mass.

B.2. Reagents and materials

During the analysis, unless otherwise is stated, use only reagents of recognised analytical grade and water of at least grade 3 as defined in ISO 3696:1987.

B.2.1. Acetone (CAS 666-52-4) or absolute alcohol

B.2.2. Dry air.

B.3. Apparatus and equipment

Usual laboratory apparatus and in particular the following:

B.3.1. Digital display densimeter

Electronic densimeter for performing such measurements must be capable of expressing density in g/ml to 5 decimal places.

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Note 1: The densimeter should be placed on a perfectly stable stand that is insulated from all vibrations.

B.3.2. Temperature regulation

The densimeter's performance is valid only if the measuring cell is connected to a built-in temperature regulator that can achieve the same temperature stability of ± 0,02 °C or better.

Note 2: The precise setting and monitoring of the temperature in the measuring cell are very important, for an error of 0,1 °C can lead to a variation in density of the order of 0,1 kg/m^3.

B.3.3. Sample injection syringes or auto sampler.

B.4. **Procedure**

B.4.1. Calibration of the densimeter

The apparatus must be calibrated according to the instrument manufacturer's instructions when it is first put into service. It must be recalibrated regularly and checked against a certified reference standard or an internal laboratory reference solution based on a certified reference standard.

B.4.2. Determination of sample density

B.4.2.1. If required prior to measurement clean and dry the cell with acetone or absolute alcohol and dry air. Rinse the cell with the sample.

B.4.2.2. Inject the sample into the cell (using a syringe or autosampler) so that the cell is completely filled. During the filling operation make sure that all air bubbles are completely eliminated. The sample must be homogeneous and must not contain any solid particles. Any suspended matter should be removed by filtration prior to analysis.

B.4.2.3. Once the reading has stabilised, record the density ρ_{20} or the alcoholic strength displayed by the densimeter.

B.4.3. Result

When the density ρ_{20} is used, calculate the real alcoholic strength using the alcoholic strength tables identified below:

The table giving the value of the alcoholic strength by volume (% vol) at 20 °C as a function of the density at 20 °C of water-alcohol mixtures is the international table adopted by the International Legal Metrology Organisation in its Recommendation No 22.

B.5. **Method performance characteristics (precision)**

B.5.1. Statistical results of the interlaboratory test

The following data were obtained from an international method performance study carried out to internationally agreed procedures [1] [2].

Year of interlaboratory test	1997
Number of laboratories	16
Number of samples	6

Samples	A	B	C	D	E	F
Number of laboratories retained after eliminating outliers	11	13	15	16	14	13
Number of outliers (laboratories)	2	3	1	—	1	2

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Samples	A	B	C	D	E	F
Number of accepted results	22	26	30	32	28	26
Mean value ($\bar{x}$) % vol	23,81	40,12	40,35	39,27	42,39	56,99
	26,52 (*)			43,10 (*)	45,91 (*)	63,31 (*)
Repeatability standard deviation (S_r) % vol	0,044	0,046	0,027	0,079	0,172	0,144
Repeatability relative standard deviation (RSD_r) (%)	0,17	0,12	0,07	0,19	0,39	0,24
Repeatability limit (r) % vol	0,12	0,13	0,08	0,22	0,48	0,40
Reproducibility standard deviation (S_R) % vol	0,054	0,069	0,083	0,141	0,197	0,205
Reproducibility relative standard deviation (RSD_R) (%)	0,21	0,17	0,21	0,34	0,45	0,34
Reproducibility limit (R) % vol	0,15	0,19	0,23	0,40	0,55	0,58

Sample types

A Fruit liqueur; split level (*).
B Brandy; blind duplicates.
C Whisky; blind duplicates.
D Grappa; split level (*).
E Aquavit; split level (*).
F Rum; split level (*).

METHOD C: DETERMINATION OF REAL ALCOHOLIC STRENGTH BY VOLUME OF SPIRIT DRINKS — MEASUREMENT BY DENSIMETRY USING HYDROSTATIC BALANCE

C.1. **Principle**

The alcoholic strength of spirits can be measured by densimetry using a hydrostatic balance based on Archimedes' principle according to which a body immersed in a liquid receives a vertical upward thrust from the liquid equal to the weight of liquid displaced.

C.2. **Reagents and materials**

During the analysis, unless otherwise is stated, use only reagents of recognised analytical grade and water of at least grade 3 as defined in ISO 3696:1987.

C.2.1. Float cleaning solution (sodium hydroxide, 30 % w/v)

To prepare 100 ml, weigh 30 g of sodium hydroxide and make up to volume using 96 % volume ethanol.

C.3. **Apparatus and Equipment**

Usual laboratory apparatus and in particular the following:

C.3.1. Single-pan hydrostatic balance with a sensitivity of 1 mg.

C.3.2. Float with a volume of at least 20 ml, specially adapted to the balance, suspended with a thread of diameter not exceeding 0,1 mm.

C.3.3. Measuring cylinder bearing a level mark. The float must be capable of being contained completely within the volume of the cylinder located below the mark; the surface of the liquid may only be penetrated by the supporting thread. The measuring cylinder must have an internal diameter at least 6 mm larger than that of the float.

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C.3.4. Thermometer (or temperature-measuring probe) graduated in degrees and tenths of a degree from 10 to 40 °C, calibrated to 0,05 °C.

C.3.5. Weights, calibrated by a recognised certifying body.

Note 1: Use of a twin-pan balance is also possible; the principle is described in Chapter 1 'Density and specific gravity' of the Annex to Regulation (EEC) No 2676/90 (p. 7).

C.4. **Procedure**

The float and measuring cylinder must be cleaned between each measurement with distilled water, dried with soft laboratory paper which does not shed fibres and rinsed with the solution whose density is to be determined. Measurements must be made as soon as the apparatus has reached stability so as to restrict alcohol loss by evaporation.

C.4.1. Calibration of the balance

Although balances usually have an internal calibration system, the hydrostatic balance must be capable of calibration with weights checked by an official certifying body.

C.4.2. Calibration of the float

C.4.2.1. Fill the measuring cylinder to the mark with double-distilled water (or water of equivalent purity, e.g. microfiltered water with a conductivity of 18,2 MΩ/cm) at a temperature between 15 and 25 °C but preferably at 20 °C.

C.4.2.2. Immerse the float and the thermometer, stir, read off the density of the liquid from the apparatus and, if necessary, correct the reading so that it is equal to that of the water at measurement temperature.

C.4.3. Control using a water-alcohol solution

C.4.3.1. Fill the measuring cylinder to the mark with a water-alcohol mixture of known strength at a temperature between 15 and 25 °C but preferably at 20 °C.

C.4.3.2. Immerse the float and the thermometer, stir, read off the density of the liquid (or the alcoholic strength if this is possible) from the apparatus. The alcoholic strength thus established should be equal to the previously determined alcoholic strength.

Note 2: This solution of known alcoholic strength can also be used to calibrate the float instead of double-distilled water.

C.4.4. Measurement of the density of a distillate (or of its alcoholic strength if the apparatus allows)

C.4.4.1. Pour the test sample into the measuring cylinder up to the graduation mark.

C.4.4.2. Immerse the float and the thermometer, stir, read off the density of the liquid (or the alcoholic strength if this is possible) from the apparatus. Note the temperature if the density is measured at t °C (ρ_t).

C.4.4.3. Correct ρ_t to 20 using the table of densities ρT for water-alcohol mixtures (Table II of Annex II to the OIV's Manual of analysis methods (1994), pp. 17-29).

C.4.5. Cleaning of float and measuring cylinder

C.4.5.1. Immerse the float in the float cleaning solution in the measuring cylinder.

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C.4.5.2. Allow to soak for one hour spinning the float periodically.

C.4.5.3. Rinse with copious amounts of tap water followed by distilled water.

C.4.5.4. Dry with soft laboratory paper which does not shed fibres.

Carry out this procedure when the float is first used and then regularly as required.

C.4.6. Result

Using the density ρ_{20} calculate the real alcoholic strength using the alcoholic strength tables identified below.

The table giving the value of the alcoholic strength by volume (% vol) at 20 °C as a function of the density at 20 °C of water-alcohol mixtures is the international table adopted by the International Legal Metrology Organisation in its Recommendation No 22.

C.5. **Method performance characteristics (precision)**

C.5.1. Statistical results of the interlaboratory test

The following data were obtained from an international method performance study carried out to internationally agreed procedures [1] [2].

Year of interlaboratory test	1997
Number of laboratories	12
Number of samples	6

Samples	A	B	C	D	E	F
Number of laboratories retained after eliminating outliers	12	10	11	12	11	9
Number of outliers (laboratories)	—	2	1	—	1	2
Number of accepted results	24	20	22	24	22	18
Mean value ($\bar{x}$) % vol	23,80	40,09	40,29	39,26	42,38	57,16
	26,51 (*)			43,09 (*)	45,89 (*)	63,44 (*)
Repeatability standard deviation (S_r) % vol	0,048	0,065	0,042	0,099	0,094	0,106
Repeatability relative standard deviation (RSD_r) (%)	0,19	0,16	0,10	0,24	0,21	0,18
Repeatability limit (r) % vol	0,13	0,18	0,12	0,28	0,26	0,30
Reproducibility standard deviation (S_R) % vol	0,060	0,076	0,073	0,118	0,103	0,125
Reproducibility relative standard deviation (RSD_R) (%)	0,24	0,19	0,18	0,29	0,23	0,21
Reproducibility limit (R) % vol	0,17	0,21	0,20	0,33	0,29	0,35

Sample types

A Fruit liqueur; split level (*).

B Brandy; blind duplicates.

C Whisky; blind duplicates.

D Grappa; split level (*).

E Aquavit; split level (*).

F Rum; split level (*).

▼B

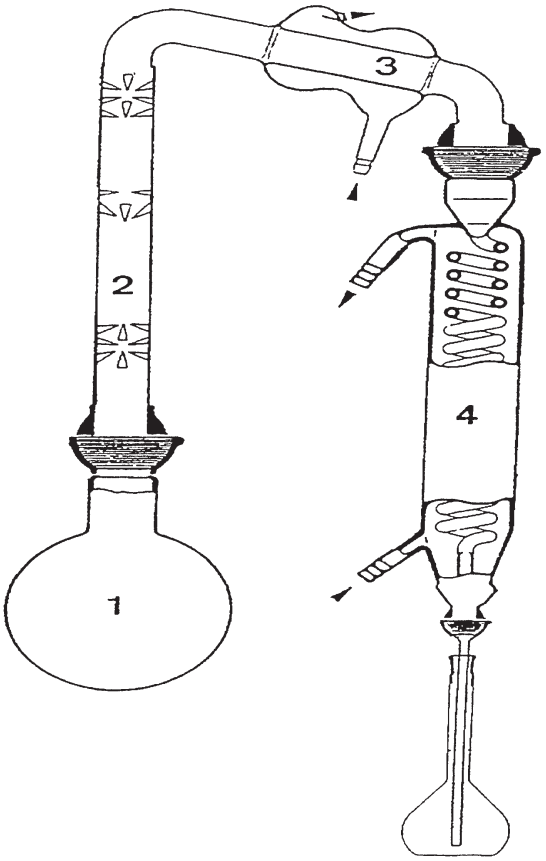


Figure 1. Distillation apparatus for measuring the real alcoholic strength by volume of spirits

1. 1-litre round-bottomed flask with standardised spherical ground-glass joint.

2. 20-cm Vigreux rectifying column.

3. 10-cm straight-rimmed West condenser.

4. 40-cm cooling coil.

▼B

II. DETERMINATION OF TOTAL DRY EXTRACT OF SPIRIT DRINKS BY GRAVIMETRY

1. Scope

Regulation (EEC) No 1576/89 provides for this method only for aquavit for which the dry extract is limited to 15 g/l.

2. Normative References

ISO 3696:1987: Water for analytical laboratory use — Specifications and test methods.

3. Definition

The total dry extract or total dry matter includes all matter that is non-volatile under specified physical conditions.

4. Principle

Weighing of the residue left by evaporation of the spirit on a boiling water bath and drying in a drying oven.

5. Apparatus and Equipment

5.1. Flat-bottomed cylindrical evaporating dish 55 mm in diameter.

5.2. Boiling water bath.

5.3. 25 ml pipette, class A.

5.4. Drying oven.

5.5. Desiccator.

5.6. Analytical balance accurate to 0,1 mg.

6. Sampling and samples

Samples are stored at room temperature prior to analysis.

7. Procedure

7.1. Pipette 25 ml of the spirit containing less than 15 g/l dry matter into a previously weighed flat-bottomed cylindrical evaporating dish 55 mm in diameter. During the first hour of evaporation the evaporating dish is placed on the lid of a boiling water bath so that the liquid will not boil, as this could lead to losses through splattering. Leave one more hour directly in contact with the steam of the boiling water bath.

7.2. Complete the drying by placing the evaporating dish in a drying oven at 105 °C ± 3 °C for two hours. Allow the evaporating dish to cool in a desiccator and weigh the evaporating dish and its contents.

8. Calculation

The mass of the residue multiplied by 40 is equal to the dry extract contained in the spirit and it must be expressed in g/l to one decimal place.

9. Method performance characteristics (precision)

9.1. Statistical results of the interlaboratory test

The following data were obtained from an international method performance study carried out to internationally agreed procedures [1] [2].

Year of interlaboratory test	1997
Number of laboratories	10
Number of samples	4

▼B

Samples	A	B	C	D
Number of laboratories retained after eliminating outliers	9	9	8	9
Number of outliers (laboratories)	1	1	2	—
Number of accepted results	18	18	16	18
Mean value ($\bar{\times}$) g/l	9,0	9,1	10,0	11,8
		7,8	9,4	11,1
Repeatabilities standard deviation (S_r) g/l	0,075	0,441	0,028	0,123
Repeatabilities relative standard deviation (RSD_r) (%)	0,8	5,2	0,3	1,1
Repeatabilities limit (r) g/l	0,2	1,2	0,1	0,3
Reproductibility standard deviation (S_R) g/l	0,148	0,451	0,058	0,210
Reproductibility relative standard deviation (RSD_R) (%)	1,6	5,3	0,6	1,8
Reproductibility limit (R) g/l	0,4	1,3	0,2	0,6

Sample types

A Brandy; blind duplicaties.
B Rum; split levels.
C Grappa; split levels.
D Aquavit; split levels.

▼B

III. DETERMINATION OF VOLATILE SUBSTANCES AND METHANOL OF SPIRIT DRINKS

III.1. GENERAL REMARKS

1. **Definitions**

Regulation (EEC) No 1576/89 sets minimum levels of volatile compounds other than ethanol and methanol for a series of spirit drinks (rum, spirits of viticultural origin, fruit spirits, etc.). For this series of drinks only, these levels are conventionally considered to be equivalent to the sum of the concentrations of:

1. volatile acids expressed as acetic acid;

2. aldehydes expressed as ethanal by the sum of ethanal (acetaldehyde) and the ethanal fraction contained in 1,1-diethoxyethane (acetal);

3. the following higher alcohols: propan-1-ol, butan-1-ol, butan-2-ol, 2-methylpropan-1-ol, assayed by individual alcohol and 2-methyl-butan-1-ol, and 3-methylbutan-1-ol assayed as individual alcohol or the sum of the two;

4. ethyl acetate.

The following are the conventional methods for measuring volatile compounds:

— the volatile acids by means of volatile acidity,

— the aldehydes (ethanal and acetal), ethyl acetate and the alcohols by means of gas chromatography (GPC).

2. **Gas chromatographic analysis of volatile compounds**

Gas chromatographic assays of volatile compounds other than those set out above may prove particularly interesting as a means of determining both the origin of the raw material used in the distillation and the actual conditions of distillation.

Some spirits contain other volatile components, such as aromatic compounds, which are characteristic of the raw materials used to obtain the alcohol, of the aroma of the spirit drink and of the special features of the preparation of the spirit. These compounds are important for evaluating the requirements set out in Regulation (EEC) No 1576/89.

III.2. GAS CHROMATOGRAPHIC DETERMINATION OF VOLATILE CONGENERS: ALDEHYDES, HIGHER ALCOHOLS, ETHYL ACETATE AND METHANOL

1. **Scope**

This method is suitable for use for the determination of 1,1-diethoxyethane (acetal), 2-methylbutan-1-ol (active amyl alcohol), 3-methyl-butan-1-ol (isoamyl alcohol), methanol (methyl alcohol), ethyl ethanoate (ethyl acetate), butan-1-ol (n-butanol), butan-2-ol (sec-butanol), 2-methylpropan-1-ol (isobutyl alcohol), propan-1-ol (n-propanol) and ethanal (acetaldehyde) in spirit drinks using gas chromatography. The method uses an internal standard, for example pentan-3-ol. The concentrations of the analytes are expressed as grams per 100 litres of absolute alcohol; the alcoholic strength of the product must be determined prior to analysis. The spirit drinks that can be analysed using this method include whisky, brandy, rum, wine spirit, fruit spirit and grape marc spirit.

2. **Normative References**

ISO 3696:1987: Water for analytical laboratory use — Specifications and test methods.

▼B

3. **Definition**

Congeners are volatile substances formed along with ethanol during fermentation, distillation and maturation of spirit drinks.

4. **Principle**

Congeners in spirit drinks are determined by direct injection of the spirit drink, or appropriately diluted spirit drink, into a gas chromatography (GC) system. A suitable internal standard is added to the spirit drink prior to injection. The congeners are separated by temperature programming on a suitable column and are detected using a flame ionisation detector (FID). The concentration of each congener is determined with respect to the internal standard from response factors, which are obtained during calibration under the same chromatographic conditions as those of the spirit drink analysis.

5. **Reagents and materials**

Unless otherwise stated, use only reagents of a purity greater than 97 %, purchased from an ISO-accredited supplier with a certificate of purity, free from other congeners at test dilution (this may be confirmed by injection of individual congener standards at the test dilution using GC conditions as in 6.4) and only water of at least grade 3 as defined in ISO 3696. Acetal and acetaldehyde must be stored in the dark at < 5 °C, all other reagents may be stored at room temperature.

5.1. Ethanol absolute (CAS 64-17-5).

5.2. Methanol (CAS 67-56-1).

5.3. Propan-1-ol (CAS 71-23-8).

5.4. ►C1 2-methylpropan-1-ol (CAS 78-83-1). ◄

5.5. Acceptable internal standards: pentan-3-ol (CAS 584-02-1), pentan-1-ol (CAS 71-41-0), 4-methylpentan-1-ol (CAS 626-89-1) or methyl nonanoate (CAS 1731-84-6).

5.6. 2-methylbutan-1-ol (CAS 137-32-6).

5.7. 3-methylbutan-1-ol (CAS 123-51-3).

5.8. Ethyl acetate (CAS 141-78-6).

5.9. Butan-1-ol (CAS 71-36-3).

5.10. Butan-2-ol (CAS 78-92-2).

5.11. Acetaldehyde (CAS 75-07-0).

5.12. Acetal (CAS 105-57-7).

5.13. 40 % v/v ethanol solution

To prepare 400 ml/l ethanol solution pour 400 ml ethanol (5.1) into a 1-litre volumetric flask, make up to volume with distilled water and mix.

▼M3

5.13a. For ethyl alcohol of agricultural origin only, ethanol absolute (CAS 64-17-5).

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5.14. Preparation and storage of standard solutions (procedure used for the validated method).

All standard solutions must be stored at < 5 °C and be prepared freshly on a monthly basis. Masses of components and solutions should be recorded to the nearest 0,1 mg.

5.14.1. Standard solution — A

Pipette the following reagents into a 100-ml volumetric flask, containing approximately 60-ml ethanol solution (5.13) to minimise component evaporation, make up to volume with ethanol solution (5.13) and mix thoroughly. Record the weight of the flask, each component added and the total final weight of contents.

Component	Volume (ml)
Methanol (5.2)	3,0
Propan-1-ol (5.3)	3,0
2-methylpropan-1-ol (5.4)	3,0
2-methylbutan-1-ol (5.6)	3,0
3-methylbutan-1-ol (5.7)	3,0
Ethyl acetate (5.8)	3,0
Butan-1-ol (5.9)	3,0
Butan-2-ol (5.10)	3,0
Acetaldehyde (5.11)	3,0
Acetal (5.12)	3,0

Note 1: It is preferable to add acetal and acetaldehyde last in order to minimise losses through evaporation.

▼M3

5.14.1a. For ethyl alcohol of agricultural origin only, standard solution A shall be prepared pipetting the reagents with reduced volumes of higher alcohols with the purpose of having standard solutions with concentrations close to the legal limits for ethyl alcohol of agricultural origin.

▼B

5.14.2. Standard solution — B

Pipette 3 ml of pentan-3-ol, or other suitable internal standard, (5.5) into a 100-ml volumetric flask, containing approximately 80 ml ethanol solution (5.13), make up to volume with ethanol solution (5.13) and mix thoroughly.

Record the weight of the flask, the weight of pentan-3-ol or other internal standard added and the total final weight of contents.

▼M3

5.14.2a. For ethyl alcohol of agricultural origin only, standard solution B shall be prepared pipetting a suitable internal standard with the reduced volumes with the purpose of having standard solutions with concentrations close to the legal limits for ethyl alcohol of agricultural origin.

▼B

5.14.3. Standard solution — C

Pipette 1 ml solution A (5.14.1) and 1 ml solution B (5.14.2) into a 100-ml volumetric flask containing approximately 80 ml ethanol solution (5.13), make up to volume with ethanol solution (5.13) and mix thoroughly.

Record the weight of the flask, each component added and the total final weight of contents.

5.14.4. Standard solution — D

In order to maintain analytical continuity, prepare a quality control standard using the previously prepared standard A (5.14.1). Pipette 1 ml solution A (5.14.1) into a 100-ml volumetric flask containing approximately 80 ml ethanol solution (5.13), make up to volume with ethanol solution (5.13) and mix thoroughly.

Record the weight of the flask, each component added and the total final weight of contents.

5.14.5. Standard solution — E

Pipette 10 ml solution B (5.14.2) into a 100-ml volumetric flask containing approximately 80 ml ethanol solution (5.13), make up to volume with ethanol solution (5.13) and mix thoroughly.

Record the weight of the flask, each component added and the total final weight of contents.

5.14.6. Standard solutions used to check the linearity of response of FID

Into separate 100-ml volumetric flasks, containing approximately 80 ml ethanol (5.13), pipette 0, 0,1, 0,5, 1,0, 2,0 ml solution A (5.14.1) and 1 ml solution B (5.14.2), make up to volume with ethanol solution (5.13) and mix thoroughly.

Record the weight of the flask, each component added and the total final weight of contents.

5.14.7. QC standard solution

Pipette 9 ml standard solution D (5.14.4) and 1 ml of standard solution E (5.14.5) into a weighing vessel and mix thoroughly.

Record the weight of the flask, each component added and the total final weight of contents.

▼B

6. **Apparatus and equipment**

6.1. Apparatus capable of measuring the density and alcoholic strength.

6.2. Analytical balance, capable of measuring to four decimal places.

6.3. A temperature programmed gas chromatograph fitted with a flame ionisation detector and integrator or other data handling system capable of measuring peak areas or peak heights.

6.4. Gas chromatographic column(s), capable of separating the analytes such that the minimum resolution between the individual components (other than 2-methylbutan-1-ol and 3-methylbutan-1-ol) is at least 1.3.

Note 2: The following columns and GC conditions are suitable examples:

1. A retention gap 1 m × 0,32 mm i.d. connected to a CP-WAX 57 CB column 50 m × 0,32 mm i.d. 0,2 µm film thickness (stabilised polyethylene glycol) followed by a Carbowax 400 column 50 m × 0,32 mm i.d. 0,2 µm film thickness. (Columns are connected using press-fit connectors.)

Carrier gas and pressure:	Helium (135 kPa)
Column temperature:	35 °C for 17 min., 35 to 70 °C at 12 °C/min., hold at 70 °C for 25 min.
Injector temperature:	150 °C
Detector temperature:	250 °C
Injection volume:	1 µl, split 20 to 100:1

2. A retention gap 1 m × 0,32 mm i.d. connected to a CP-WAX 57 CB column 50 m × 0,32 mm i.d. 0,2 µm film thickness (stabilised polyethylene glycol). (Retention gap is connected using a press-fit connector.)

Carrier gas and pressure:	Helium (65 kPa)
Column temperature:	35 °C for 10 min., 35 to 110 °C at 5 °C/min., 110 to 190 °C at 30 °C/min., hold at 190 °C for 2 min.
Injector temperature:	260 °C
Detector temperature:	300 °C
Injection volume:	1 µl, split 55:1

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3. A packed column (5 % CW 20M, Carbopak B), 2 m × 2 mm i.d.

Column temperature:	65 °C for 4 min., 65 to 140 °C at 10 °C/min., hold at 140 °C for 5 min., 140 to 150 °C at 5 °C/min., hold at 150 °C for 3 min.
Injector temperature:	65 °C
Detector temperature:	200 °C
Injection volume:	1 µl

7. **Sampling and samples.**

7.1. Laboratory sample

On receipt, the alcoholic strength of each sample is measured (6.1).

8. **Procedure (used for the validated method)**

8.1. Test portion

8.1.1. Weigh an appropriate sealed weighing vessel and record the weight.

8.1.2. Pipette 9 ml laboratory sample into the vessel and record the weight (M_{SAMPLE}).

8.1.3. Add 1 ml of standard solution E (5.14.5) and record the weight (M_{IS}).

8.1.4. Shake the test material vigorously (at least 20 inversions). Samples must be stored at less than 5 °C prior to analysis in order to minimise any volatile losses.

8.2. Blank test

8.2.1. Using a four decimal place balance (6.2), weigh an appropriate sealed weighing vessel and record the weight.

8.2.2. Pipette 9 ml 400 ml/l ethanol solution (5.13) into the vessel and record the weight.

8.2.3. Add 1 ml of standard solution E (5.14.5) and record the weight.

8.2.4. Shake the test material vigorously (at least 20 inversions). Samples must be stored at less than 5 °C prior to analysis in order to minimise any volatile losses.

8.3. Preliminary test

Inject standard solution C (5.14.3) to ensure that all of the analytes are separated with a minimum resolution of 1.3 (except 2-methylbutan-1-ol and 3-methylbutan-1-ol).

8.4. Calibration

The calibration should be checked using the following procedure. Ensure that the response is linear by successively analysing in triplicate each
of the linearity standard solutions (5.14.6) containing internal standard (IS). From the integrator peak areas or peak heights for each injection

▼B

calculate the ratio R for each congener and plot a graph of R versus the concentration ratio of congener to internal standard (IS), C. A linear plot should be obtained, with a correlation coefficient of at least 0,99.

$$R = \frac{\text{Peak area or height of congener}}{\text{Peak area or height of IS}}$$

$$C = \frac{\text{Concentration of congener (µg / g)}}{\text{Concentration of IS (µg / g)}}$$

8.5. Determination

Inject standard solution C (5.14.3) and 2 QC standard solutions (5.14.7). Follow with unknown samples (prepared according to 8.1 and 8.2) inserting one QC standard every 10 samples to ensure analytical stability. Inject one standard solution C (5.14.3) after every 5 samples.

9. **Calculation**

An automated system of data handling can be used, provided the data can be checked using the principles described in the method below.

Measure either peak areas or peak heights for congener and internal standard peaks.

9.1. Response factor calculation.

From the chromatogram of the injection of standard solution C (5.14.3), calculate response factors for each congener using equation (1).

$$(1)\ \text{Response factor} = \frac{\text{Peak area or height IS}}{\text{Peak area or height congener}} \times \frac{\text{Conc. congener (µg / g)}}{\text{Conc. IS (µg / g)}}$$

where:

IS = Internal Standard

Conc. congener = concentration of congener in solution C (5.14.3)

Conc. IS = concentration of internal standard in solution C (5.14.3).

9.1.2. Sample analysis

Using equation (2) below, calculate the concentration of each congener in the samples.

$$(2)\ \text{Congener concentrations, (µg/g)} = \frac{\text{Peak area or height congener}}{\text{Peak area or height IS}} \times \frac{M_{IS}\ (g)}{M_{SAMPLE}\ (g)} \times \text{Conc. IS (µg / g)} \times RF$$

where:

M_{SAMPLE} = weight of sample (8.1.2);

M_{IS} = weight of internal standard (8.1.3);

Conc. IS = concentration of internal standard in solution E (5.14.5);

RF = response factor calculated using equation 1.

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9.1.3. Quality control standard solution analysis

Using equation (3) below, calculate the percentage recovery of the target value for each congener in the quality control standards (5.14.7):

$$(3)\ \%\ \text{recovery of QC sample} = \frac{\text{concentration of analyte in QC standard}}{\text{concentration of analyte in solution D}} \times 100$$

The concentration of the analyte in the QC standard is calculated using equations (1) and (2) above.

9.2. Final presentation of results

Results are converted from μ/g to g per 100 litres absolute alcohol for samples using equation (4):

$$(4)\ \text{Concentration in g per 100 literes absolute alcohol} = \text{Conc}\ (\mu g / g) \times \rho \times 10/(\text{strengh}\ (\%\ \text{vol}) \times 1\ 000)$$

where

ρ = density in kg/m^3.

Results are quoted to 3 significant figures and a maximum of one decimal place e.g. 11,4 g per 100 l absolute alcohol.

10. **Quality assurance and control (used for the validated method)**

Using equation (2) above, calculate the concentration of each congener in the quality control standard solutions prepared by following the procedure as in 8.1.1 to 8.1.4. Using equation (3), calculate the percentage recovery of the target value. If the analysed results are within ± 10 % of their theoretical values for each congener, analysis may proceed. If not, an investigation should be made to find the cause of the inaccuracy and remedial action taken as appropriate.

11. **Method performance characteristics (precision)**

Statistical results of the interlaboratory test: the following tables give the values for the following compounds: ethanal, ethyl acetate, acetal, total ethanal, methanol, butan-2-ol, propan-1-ol, butan-1-ol, 2-methyl-propan-1-ol, 2 methyl-butan-1-ol, 3 methyl-butan-1-ol.

The following data were obtained from an international method performance study carried out to internationally agreed procedures.

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	ethanal

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	28	26	27	27	28
Number of outliers (laboratories)	2	4	3	3	2

▼B

Samples	A	B	C	D	E
Number of accepted results	56	52	54	54	56
Mean value ($\bar{x}$) µg/g	63,4	71,67	130,4	38,4	28,6
				13,8 (*)	52,2 (*)
Repeatability standard deviation (S_r) µg/g	3,3	1,9	6,8	4,1	3,6
Repeatability relative standard deviation (RSD_r) (%)	5,2	2,6	5,2	15,8	8,9
Repeatability limit (r) µg/g	9,3	5,3	19,1	11,6	10,1
Reproducibility standard deviation (S_R) µg/g	12	14	22	6,8	8,9
Reproducibility relative standard deviation (RSD_R) (%)	18,9	19,4	17,1	26,2	22,2
Reproducibility limit (R) µg/g	33,5	38,9	62,4	19,1	25,1

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	ethyl acetate

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	24	24	25	24	24
Number of outliers (laboratories)	2	2	1	2	2
Number of accepted results	48	48	50	48	48
Mean value ($\bar{x}$) µg/g	96,8	1 046	120,3	112,5	99,1
				91,8 (*)	117,0 (*)
Repeatability standard deviation (S_r) µg/g	2,2	15	2,6	2,1	2,6
Repeatability relative standard deviation (RSD_r) (%)	2,3	1,4	2,1	2,0	2,4
Repeatability limit (r) µg/g	6,2	40,7	7,2	5,8	7,3
Reproducibility standard deviation (S_R) µg/g	6,4	79	8,2	6,2	7,1
Reproducibility relative standard deviation (RSD_R) (%)	6,6	7,6	6,8	6,2	6,6
Reproducibility limit (R) µg/g	17,9	221,9	22,9	17,5	20,0

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

▼B

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	acetal

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	20	21	22	17	21
Number of outliers (laboratories)	4	3	2	4	3
Number of accepted results	40	42	44	34	42
Mean value ($\bar{x}$) µg/g	35,04	36,46	68,5	20,36	15,1
				6,60 (*)	28,3 (*)
Repeatability standard deviation (S_r) µg/g	0,58	0,84	1,6	0,82	1,9
Repeatability relative standard deviation (RSD_r) (%)	1,7	2,3	2,3	6,1	8,7
Repeatability limit (r) µg/g	1,6	2,4	4,4	2,3	5,3
Reproducibility standard deviation (S_R) µg/g	4,2	4,4	8,9	1,4	3,1
Reproducibility relative standard deviation (RSD_R) (%)	12,1	12,0	13,0	10,7	14,2
Reproducibility limit (R) µg/g	11,8	12,2	25,0	4,0	8,7

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	total ethanal

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	23	19	22	21	22
Number of outliers (laboratories)	1	5	2	3	2
Number of accepted results	46	38	44	42	44
Mean value ($\bar{x}$) µg/g	76,5	85,3	156,5	45,4	32,7
				15,8 (*)	61,8 (*)
Repeatability standard deviation (S_r) µg/g	3,5	1,3	6,5	4,4	3,6
Repeatability relative standard deviation (RSD_r) (%)	4,6	1,5	4,2	14,2	7,6
Repeatability limit (r) µg/g	9,8	3,5	18,3	12,2	10,0

▼B

Samples	A	B	C	D	E
Reproducibility standard deviation (S_R) µg/g	13	15	24,1	7,3	9,0
Reproducibility relative standard deviation (RSD_R) (%)	16,4	17,5	15,4	23,7	19,1
Reproducibility limit (R) µg/g	35,2	41,8	67,4	20,3	25,2

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	Methanol

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	26	27	27	28	25
Number of outliers (laboratories)	4	3	3	1	4
Number of accepted results	52	54	54	56	50
Mean value ($\bar{\times}$) µg/g	319,8	2 245	1 326	83,0	18,6
				61,5 (*)	28,9 (*)
Repeatability standard deviation (S_r) µg/g	4,4	27	22	1,5	1,3
Repeatability relative standard deviation (RSD_r) (%)	1,4	1,2	1,7	2,1	5,6
Repeatability limit (r) µg/g	12,3	74,4	62,5	4,3	3,8
Reproducibility standard deviation (S_R) µg/g	13	99	60	4,5	2,8
Reproducibility relative standard deviation (RSD_R) (%)	3,9	4,4	4,6	6,2	11,8
Reproducibility limit (R) µg/g	35,2	278,3	169,1	12,5	7,9

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	4
Analyte	butan-2-ol

▼B

Samples	A	B	C	E
Number of laboratories retained after eliminating outliers	21	27	29	22
Number of outliers (laboratories)	4	3	1	3
Number of accepted results	42	54	58	44
Mean value ($\bar{x}$) µg/g	5,88	250,2	27,57	5,83
				14,12 (*)
Repeatability standard deviation (S_r) µg/g	0,40	2,2	0,87	0,64
Repeatability relative standard deviation (RSD_r) (%)	6,8	0,9	3,2	6,4
Repeatability limit (r) µg/g	1,1	6,1	2,5	1,8
Reproducibility standard deviation (S_R) µg/g	0,89	13	3,2	0,87
Reproducibility relative standard deviation (RSD_R) (%)	15,2	5,1	11,5	8,7
Reproducibility limit (R) µg/g	2,5	35,5	8,9	2,4

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	propan-1-ol

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	29	27	27	29	29
Number of outliers (laboratories)	2	4	3	2	2
Number of accepted results	58	54	54	58	58
Mean value ($\bar{x}$) µg/g	86,4	3 541	159,1	272,1	177,1
				229,3 (*)	222,1 (*)
Repeatability standard deviation (S_r) µg/g	3,0	24	3,6	2,3	3,3
Repeatability relative standard deviation (RSD_r) (%)	3,4	0,7	2,3	0,9	1,6
Repeatability limit (r) µg/g	8,3	68,5	10,0	6,4	9,1
Reproducibility standard deviation (S_R) µg/g	5,3	150	6,5	9,0	8,1

▼B

Samples	A	B	C	D	E
Reproducibility relative standard deviation (RSD_R) (%)	6,1	4,1	4,1	3,6	4,1
Reproducibility limit (R) µg/g	14,8	407,2	18,2	25,2	22,7

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	propan-1-ol

Samples	A	B	C
Number of laboratories retained after eliminating outliers	20	22	22
Number of outliers (laboratories)	4	4	6
Number of accepted results	40	44	44
Mean value ($\bar{x}$) µg/g	3,79	5,57	7,54
Repeatability standard deviation (S_r) µg/g	0,43	0,20	0,43
Repeatability relative standard deviation (RSD_r) (%)	11,2	3,6	5,6
Repeatability limit (r) µg/g	1,1	0,6	1,2
Reproducibility standard deviation (S_R) µg/g	0,59	0,55	0,82
Reproducibility relative standard deviation (RSD_R) (%)	15,7	9,8	10,8
Reproducibility limit (R) µg/g	1,7	1,5	2,3

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates. (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	2-methylpropan-1-ol

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	28	31	30	26	25
Number of outliers (laboratories)	3	0	1	5	6
Number of accepted results	56	62	60	52	50

▼B

Samples	A	B	C	D	E
Mean value ($\bar{x}$) µg/g	174,2	111,7	185,0	291,0	115,99
				246,8 (*)	133,87 (*)
Repeatability standard deviation (S_r) µg/g	2,3	1,6	2,5	1,8	0,74
Repeatability relative standard deviation (RSD_r) (%)	1,3	1,4	1,3	0,7	0,6
Repeatability limit (r) µg/g	6,4	4,5	6,9	5,0	2,1
Reproducibility standard deviation (S_R) µg/g	8,9	8,9	9,7	6,0	6,2
Reproducibility relative standard deviation (RSD_R) (%)	5,1	8,0	5,2	2,2	5,0
Reproducibility limit (R) µg/g	24,9	24,9	27,2	16,9	17,4

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	2-methyl-butan-1-ol

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	25	26	25	27	25
Number of outliers (laboratories)	3	2	3	1	2
Number of accepted results	50	52	50	54	50
Mean value ($\bar{x}$) µg/g	113,0	48,3	91,6	72,1	39,5
				45,2 (*)	61,5 (*)
Repeatability standard deviation (S_r) µg/g	2,1	1,5	1,7	2,3	2,3
Repeatability relative standard deviation (RSD_r) (%)	1,9	3,1	1,8	3,9	4,5
Repeatability limit (r) µg/g	6,0	4,2	4,7	6,4	6,3
Reproducibility standard deviation (S_R) µg/g	7,4	3,8	6,6	4,7	4,5
Reproducibility relative standard deviation (RSD_R) (%)	6,6	7,9	7,2	8,1	8,8
Reproducibility limit (R) µg/g	20,8	10,7	18,4	13,3	12,5

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

▼B

Year of interlaboratory test	1997
Number of laboratories	32
Number of samples	5
Analyte	3-methyl-butan-1-ol

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	23	23	24	27	21
Number of outliers (laboratories)	5	5	4	1	6
Number of accepted results	46	46	48	54	42
Mean value ($\bar{x}$) µg/g	459,4	242,7	288,4	142,2	212,3
				120,4 (*)	245,6 (*)
Repeatability standard deviation (S_r) µg/g	5,0	2,4	3,4	2,4	3,2
Repeatability relative standard deviation (RSD_r) (%)	1,1	1,0	1,2	1,8	1,4
Repeatability limit (r) µg/g	13,9	6,6	9,6	6,6	9,1
Reproducibility standard deviation (S_R) µg/g	29,8	13	21	8,5	6,7
Reproducibility relative standard deviation (RSD_R) (%)	6,5	5,2	7,3	6,5	2,9
Reproducibility limit (R) µg/g	83,4	35,4	58,8	23,8	18,7

Sample types

A Brandy; blind duplicates.
B Kirsch; blind duplicates.
C Grappa; blind duplicates.
D Whisky; split levels (*).
E Rum; split levels (*).

▼M2

III.3. DETERMINATION OF VOLATILE ACIDITY OF SPIRIT DRINKS

1. **Scope**

The method has been validated in an interlaboratory study for rum, brandy, marc and fruit spirits, at levels ranging from 30 mg/l to 641 mg/l.

2. **Normative references**

ISO 3696: 1987 Water for analytical use — Specifications and test methods.

3. **Definitions**

3.1. Volatile acidity is calculated by deducting the fixed acidity from the total acidity.

3.2. Total acidity is the sum of titratable acidities.

3.3. Fixed acidity is the acidity of the residue left after evaporating the spirit to dryness.

4. **Principle**

The total acidity and fixed acidity are determined by titration or by potentiometry.

5. **Reagents and materials**

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and water of at least grade 3 as defined in ISO 3696:1987.

▼M2

5.1. 0,01 M sodium hydroxide solution (NaOH)

5.2. Mixed indicator solution:

Weigh 0,1 g of indigo carmine and 0,1 g of phenol red.

Dissolve in 40 ml water and make up to 100 ml with ethanol.

6. **Apparatus and equipment**

Indirect laboratory apparatus, grade A glassware and the following:

6.1. Water pump

6.2. Rotary evaporator or ultrasonic bath

6.3. Equipment for potentiometric titration (optional).

7. **Sampling and samples**

Samples are stored at room temperature prior to analysis.

8. **Procedure**

8.1. Total acidity

8.1.1. Preparation of sample

The spirit is irradiated with ultrasonic (ultrasonication) or stirred two minutes under a vacuum to rid it of carbon dioxide if required.

8.1.2. Titration

Pipette 25 ml of the spirit into a 500 ml Erlenmeyer flask.

Add about 200 ml of cooled boiled distilled water (prepared fresh daily) and 2-6 drops of the mixed indicator solution (5.2).

Titrate with the 0,01 M sodium hydroxide solution (5.1) until the yellow-green colour changes to violet in the case of colourless spirits, the yellow-brown colour to red-brown in the case of brown-coloured spirits respectively.

The titration may also be carried out by potentiometry, to pH 7,5.

Let n_1 ml be the volume of the 0,01 M sodium hydroxide solution added.

8.1.3. Calculation

The total acidity (TA) expressed in milliequivalents per l of spirit is equal to $0,4 \times n_1$.

The total acidity (TA′) expressed in mg of acetic acid per l of spirit is equal to $24 \times n_1$.

8.2. Fixed acidity

8.2.1. Preparation of sample

Evaporate 25 ml of the spirit to dryness:

Pipette 25 ml of the spirit into a flat-bottomed cylindrical evaporating dish 55 mm in diameter. During the first hour of evaporation the evaporating dish is placed on the lid of a boiling water bath so that the liquid will not boil, as this could lead to losses through splattering.

Complete the drying by placing the evaporating dish in a drying oven at 105 °C for two hours. Allow the evaporating dish to cool in a desiccator.

8.2.2. Titration

Dissolve the residue left after evaporating with cooled boiled distilled water (prepared fresh daily) and make up to a volume to circa 100 ml and add 2-6 drops of the mixed indicator solution (5.2).

▼M2

Titrate with the 0,01 M sodium hydroxide solution (5.1).

The titration may also be carried out by potentiometry, to pH 7,5.

Let n_2 ml be the volume of the 0,01 M sodium hydroxide solution added.

8.2.3. Calculation

The fixed acidity (FA) expressed in milliequivalents per l of spirit is equal to $0,4 \times n_2$.

The fixed acidity (FA) expressed in mg of acetic acid per l of spirit is equal to $24 \times n_2$.

9. **Calculation of volatile acidity**

9.1. Expression in milliequivalents per l:

Let:

TA = total acidity in milliequivalents per l

FA = fixed acidity in milliequivalents per l

Volatile acidity, VA, in milliequivalents per l is equal to:

TA – FA.

9.2. Expression in mg of acetic acid per l:

Let:

TA′ = total acidity in mg of acetic acid per l

FA′ = fixed acidity in mg of acetic acid per l

Volatile acidity, VA, in mg of acetic acid per l is equal to:

TA′ – FA′.

9.3. Expression in g of acetic acid per hl of pure 100 % vol. alcohol is equal to: $\frac{TA' - FA'}{A} \times 10$

where A is the alcoholic strength by volume of the spirit drink.

10. **Method performance characteristics (Precision)**

10.1. Statistical results of the interlaboratory test

The following data were obtained from an international method performance study carried out to internationally agreed procedures (1) (2).

Year of interlaboratory test	**2000**
Number of laboratories	18
Number of samples	6

Samples	A	B	C	D	E	F
Number of laboratories retained after eliminating outliers	16	18	18	14	18	18
Number of outliers (laboratories)	2			4		
Number of accepted results	32	36	36	28	36	36
Mean value ($\bar{x}$) (mg/L)	272* 241*	30	591* 641*	46	107	492
Repeatability standard deviation, s_r (mg/l)	8,0	3,6	15,0	3,7	6,7	8,5

▼**M2**

Samples	A	B	C	D	E	F
Repeatability relative standard deviation, RSDr (%)	3,1	11,8	2,4	8,0	6,2	1,7
Repeatability limit, r (mg/l)	23	10	42	10	19	24
Reproducibility standard deviation, s_R (mg/l)	8,5	8,4	25,0	4,55	13,4	24,4
Reproducibility relative standard deviation, RSD_R (%)	3,3	27,8	4,1	9,9	12,5	5,0
Reproducibility limit, R (mg/l)	24	23	70	13	38	68

Sample types:

A Plum spirit; split level *

B Rum I; blind duplicates

C Rum II; split level *

D Slivovitz; blind duplicates

E Brandy; blind duplicates

F Marc spirit; blind duplicates.

(1) ‘Protocol for the design, conduct and interpretation of method-performance studies’, Horwitz, W. (1995) *Pure and Applied Chemistry*, 67, 332-343.

(2) Horwitz, W. (1982) *Analytical Chemistry*, 54, 67A-76A.

▼M1

V. ANETHOLE. GAS CHROMATOGRAPHIC DETERMINATION OF TRANS-ANETHOLE IN SPIRIT DRINKS

1. Scope

This method is suitable for the determination of trans-anethole in aniseed-flavoured spirit drinks using capillary gas chromatography.

2. Normative references

ISO 3696: 1987 Water for analytical laboratory use — Specifications and test methods.

3. Principle

The trans-anethole concentration of the spirit is determined by gas chromatography (GC). The same quantity of an internal standard, e.g. 4-allylanisole (estragole) when estragole is not naturally present in the sample, is added to the test sample and to a trans-anethole reference solution of known concentration, both of which are then diluted with a 45 % ethanol solution and injected directly into the GC system. An extraction is necessary before sample preparation and analysis for liqueurs that contain large amounts of sugars.

4. Reagents and materials

During the analysis, use only reagents of a purity of at least 98 %. Water of at least grade 3 as defined by ISO 3696 should be used.

Reference chemicals should be stored cold (at 4 °C), away from light, in aluminium containers or in tinted (amber) glass reagent bottles. The stoppers should preferably be fitted with an aluminium seal. Trans-anethole will need to be 'thawed' from its crystalline state before use, but in this case its temperature should never exceed 35 °C.

4.1. Ethanol 96 % vol. (CAS 64-17-5)

4.2. 1-methoxy-4-(1-propenyl) benzene; (trans-anethole) (CAS 4180-23-8)

4.3. 4-allylanisole, (estragole) (CAS 140-67-0), suggested internal standard (IS)

4.4. Ethanol 45 % vol.

Add 560 g of distilled water to 378 g of ethanol 96 % vol.

4.5. Preparation of standard solutions

All standard solutions should be stored at room temperature (15 to 35 °C) away from light in aluminium containers or in tinted (amber) glass reagent bottles. The stopper should preferably be fitted with an aluminium seal.

Trans-anethole and 4-allylanisole are practically insoluble in water, and it is therefore necessary to dissolve the trans-anethole and 4-allylanisole in some 96 % ethanol (4.1) before the addition of 45 % ethanol (4.4).

The stock solutions must be freshly prepared each week.

4.5.1. Standard solution A

Stock solution of trans-anethole (concentration: 2 g/l)

Weigh 40 mg of trans-anethole (4.2) in a 20 ml volumetric flask (or 400 mg in 200 ml, etc.). Add some 96 % ethanol (4.1) and make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

▼M1

4.5.2. Internal standard solution B

Stock solution of internal standard, e.g. estragole (concentration: 2 g/l)

Weigh 40 mg of estragole (4.3) in a 20 ml volumetric flask (400 mg in 200 ml etc.). Add some 96 % vol. ethanol (4.1) make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

4.5.3. Solutions used to check the linearity response of the flame ionisation detector (FID)

The linearity response of the FID must be checked for the analysis taking into account a range of concentrations of trans-anethole in spirits from 0 g/l up to 2.5 g/l. In the procedure of analysis, the unknown samples of spirits to be analysed are diluted 10 times (8.3). For the conditions of the analysis described in the method, stock solutions corresponding to concentrations of 0, 0,05, 0,1, 0,15, 0,2, and 0,25 g/l of trans-anethole in the sample to be analysed are prepared as follows: take 0,5, 1, 1,5, 2, and 2,5 ml of stock solution A (4.5.1) and pipette in separate 20 ml volumetric flasks; pipette into each flask 2 ml of internal standard solution B (4.5.2) and make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

The blank solutions (8.4) is used as the 0 g/l solution.

4.5.4. Standard solution C

Take 2 ml of standard solution A (4.5.1) and pipette into a 20 ml volumetric flask, then add 2 ml of internal standard solution B (4.5.2) and make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

5. **Apparatus and equipment**

5.1. A capillary gas chromatograph fitted with a flame ionisation detector (FID) and integrator or other data handling system capable of measuring peak heights or areas, and with an automatic sampler or the necessary equipment for manual sample injection.

5.2. Split/splitless injector

5.3. Capillary column, for example:

Length: 50 m

Internal diameter: 0,32 mm

Film thickness: 0,2 μm

Stationary phase: FFAP — modified TPA polyethylene glycol cross-linked porous polymer.

5.4. Common laboratory equipment: A grade volumetric glassware, analytical balance (precision: ± 0,1 mg).

6. **Chromatography conditions**

The column type and dimensions, and the GC conditions, should be such that anethole and the internal standard are separated from each other and from any interfering substances. Typical conditions for the column given as an example in 5.3 are:

▼M1

6.1. Carrier gas: analytical helium

6.2. Flow rate: 2 ml/min

6.3. Injector temperature: 250 °C

6.4. Detector temperature: 250 °C

6.5. Oven temperature conditions: isothermal, 180 °C, run time 10 minutes

6.6. Injection volume: 1 µl, split 1:40.

7. **Samples**

Samples should be stored at room temperature, away from light and cold.

8. **Procedure**

8.1. Sample screening for estragole

To ensure that there is no estragole naturally present in the sample, a blank analysis should be carried out without the addition of any internal standard. If estragole is naturally present then another internal standard must be chosen (for instance menthol).

Pipette 2 ml sample into a 20 ml volumetric flask and make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

8.2. Preparation of unknown samples

Pipette 2 ml sample into a 20 ml volumetric flask then add 2 ml of internal standard solution B (4.5.2) and make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

8.3. Blank

Pipette 2 ml of internal standard solution B (4.5.2) into a 20 ml volumetric flask and make up to volume with 45 % vol. ethanol (4.4), mix thoroughly.

8.4. Linearity test

Prior to the commencement of the analysis the linearity of the response of the FID should be checked by successively analysing in triplicate each of the linearity standard solutions (4.5.3).

From the integrator peak areas or peak heights for each injection plot a graph of their mother solution concentration in g/l versus the ratio R for each.

R = trans-anethole peak height or area divided by the estragole peak height or area.

A linear plot should be obtained.

8.5. Determination

Inject the blank solution (8.3), followed by standard solution C (4.5.4), followed by one of the linearity standards (4.5.3) which will act as a quality control sample (this may be chosen with reference to the probable concentration of trans-anethole in the unknown), followed by five unknowns (8.2); insert a linearity (quality control) sample after every five unknown samples, to ensure analytical stability.

▼M1

9. **Calculation of response factor**

Measure either peak areas (using an integrator or other data system) or peak heights (manual integration) for trans-anethole and internal standard peaks.

9.1. Response factor (RF_i) calculation

The response factor is calculated as follows

$RF_i = (C_i/\text{area or height}_i)*(\text{area or height}_{is}/C_{is})$

where:

C_i is the concentration of trans-anethole in the standard solution A (4.5.1)

C_{is} is the concentration of internal standard in the standard solution B (4.5.2)

$area_i$ is the area (or height) of the trans-anethole peak

$area_{is}$ the area (or height) of the internal standard peak

RF_i is calculated from the five samples of solution C (4.5.4).

9.2. Analysis of the linearity response test solutions

Inject the linearity response test solutions (4.5.3).

9.3. Analysis of the sample

Inject the unknown sample solution (8.2).

10. **Calculation of results**

The formula for the calculation of the concentration of trans-anethole is the following:

$c_i = C_{is} * (\text{area or height}_i/\text{area or height}_{is})*RF_i$

where:

c_i is the unknown trans-anethole concentration

C_{is} is the concentration of internal standard in the unknown (4.5.2)

Area or $height_i$ is the area or height of the trans-anethole peak

Area or $height_{is}$ the area or height of the internal standard peak

RF_i is the response coefficient (calculated as in 9.1)

The trans-anethole concentration is expressed as grams per litre, to one decimal place.

11. **Quality assurance and control**

The chromatograms should be such that anethole and the internal standard are separated from each other and from any interfering substances. The RF_i value is calculated from the results for the five injections of solution C (4.5.4). If the coefficient of variation (CV % = (standard deviation/mean)*100)) is within plus or minus 1 %, the RF_i average value is acceptable.

▼M1

The calculation above should be used to calculate the concentration of trans-anethole in the sample selected for the quality control from the linearity control solutions (4.5.3).

If the mean calculated results from analysis of the linearity solution selected for internal quality control sample (IQC) are within plus or minus 2,5 % of their theoretical value, then the results for the unknown samples can be accepted.

12. **Treatment of spirits sample containing large amount of sugar and of liqueur sample prior to GC analysis**

Extraction of alcohol from spirit drink containing a large amount of sugar, in order to be able to determine the trans-anethole concentration using capillary gas chromatography.

12.1. Principle

An aliquot of the liqueur sample is taken and to this is added the internal standard, at a concentration similar to that of the analyte (trans-anethole) in the liqueur. To this are added sodium phosphate dodecahydrate and anhydrous ammonium sulphate. The resulting mixture is well shaken and chilled, two layers develop, and the upper alcohol layer is removed. An aliquot of this alcohol layer is taken and diluted with 45 % ethanol solution (4.4) (Note: no internal standard is added at this stage, because it has already been added). The resulting solution is analysed in gas chromatography.

12.2. Reagents and materials

During the extraction use only reagents of a purity greater than 99 %.

12.2.1. Ammonium sulphate, anhydrous, (CAS 7783-20-2).

12.2.2. Sodium phosphate, dibasic, dodecahydrate, (CAS 10039-32-4).

12.3. Apparatus and equipment

Conical flasks, separating flasks, refrigerator.

12.4. Procedure

12.4.1. Sample screening for estragole

To ensure that there is no estragole naturally present in the sample, a blank extraction (12.6.2) and analysis should be carried out without the addition of any internal standard. If estragole is naturally present then another internal standard must be chosen.

12.4.2. Extraction

Pipette 5 ml of 96 % ethanol (4.1) into a conical flask, weigh into this flask 50 mg of internal standard (4.3), and add 50 ml of the sample. Add 12 g of ammonium sulphate, anhydrous (12.2.1), and 8.6 g of dibasic sodium phosphate, dodecahydrate (12.2.2). Stopper the conical flask.

Shake the flask for at least 30 minutes. A mechanical shaking device may be used, but not a Teflon coated magnetic stirring bar, as the Teflon will absorb some of the analyte. Note that the added salts will not dissolve completely.

Place the stoppered flask in a refrigerator (T < 5 C) for at least two hours.

▼M1

After this time, there should be two distinct liquid layers and a solid residue. The alcohol layer should be clear; if not, replace in the refrigerator until a clear separation is achieved.

When the alcohol layer is clear, carefully take an aliquot (e.g. 10 ml), without disturbing the aqueous layer, place in an amber vial and close securely.

12.4.3. Preparation of the extracted sample to be analysed

Allow extract (12.4.2) to reach room temperature.

Take 2 ml of the alcohol layer of the attemperated extracted sample and pipette into a 20 ml volumetric flask, make up to volume with 45 % ethanol (4.4), mix thoroughly.

12.5. Determination

Follow the procedure as outlined in 8.5.

12.6. Calculation of results

Use the following formula to calculate the results:

$C_i = (m_{is}/V)^* (area_i/area_{is}) * RF_i$

where:

m_{is} is the weight of internal standard (4.3) taken (12.4.2) (in milligrams)

V is the volume of unknown sample (50 ml)

RF_i is the response factor (9.1)

$area_i$ is the area of the trans-anethole peak

$area_{is}$ is the area of the internal standard peak

The results are expressed in grams per litre, to one decimal place.

12.7. Quality control and assurance

Follow the procedure as outlined in 11 above.

13. **Method performance characteristics (precision)**

Statistical results of the interlaboratory test:

the following tables give the values for anethole.

The following data were obtained from an international method performance study carried out to internationally agreed procedures.

Year of interlaboratory test	1998
Number of laboratories	16
Number of samples	10
Analyte	anethole

▼M1

Pastis:

Samples	A	B	C	D	E	F
Number of laboratories retained after eliminating outliers	15	15	15	13	16	16
Number of outliers (laboratories)	1	1	1	3	—	—
Number of accepted results	30	30	30	26	16	16
Mean value g/l	1,477	1,955	1,940	1,833	1,741	1,754
Repeatability standard deviation (S_r) g/l	0,022	0,033	0,034	0,017	—	—
Repeatability relative standard deviation (RSD_r) (%)	1,5	1,7	1,8	0,9	—	—
Repeatability limit (r) g/l	0,062	0,093	0,096	0,047	—	—
Reproducibility standard deviation (S_R) g/l	0,034	0,045	0,063	0,037	0,058	0,042
Reproducibility relative standard deviation (RSD_R) (%)	2,3	2,3	3,2	2,0	3,3	2,4
Reproducibility limit (R) g/l	0,094	0,125	0,176	0,103	0,163	0,119

Sample types:

A pastis, blind duplicates

B pastis, blind duplicates

C pastis, blind duplicates

D pastis, blind duplicates

E pastis, single duplicates

F pastis, single duplicates

Other aniseed-flavoured spirit drinks:

Samples	G	H	I	J
Number of laboratories retained after eliminating outliers	16	14	14	14
Number of outliers (Laboratories)	—	2	1	1
Number of accepted results	32	28	28	28
Mean value g/l	0,778 0,530 (*)	1,742	0,351	0,599
Repeatability standard deviation (S_r) g/l	0,020	0,012	0,013	0,014
Repeatability relative standard deviation (RSD_r) (%)	3,1	0,7	3,8	2,3
Repeatability limit (r) g/l	0,056	0,033	0,038	0,038
Reproducibility standard deviation (S_R) g/l	0,031	0,029	0,021	0,030
Repeatability relative standard deviation (RSD_R) (%)	4,8	1,6	5,9	5,0
Reproducibility limit (R) g/l	0,088	0,080	0,058	0,084

Sample types:

G ouzo, split levels (*)

H anis, blind duplicates

I aniseed-flavoured liqueur, duplicates

J aniseed-flavoured liqueur, duplicates.

▼M1

VI. GLYCYRRHIZIC ACID. DETERMINATION OF GLYCYRRHIZIC ACID USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1. **Scope**

This method is suitable for the determination of glycyrrhizic acid in aniseed-flavoured spirit drinks using high performance liquid chromatography (HPLC). Regulation (EEC) No 1576/89 specifies that any aniseed-flavoured spirit called 'pastis' must contain between 0,05 and 0,5 g of glycyrrhizic acid per litre.

2. **Normative references**

ISO 3696: 1987 Water for analytical laboratory use — Specifications and test methods.

3. **Principle**

The glycyrrhizic acid concentration is determined using high-performance liquid chromatography (HPLC) with UV detection. A standard solution and the test sample are filtered and they are separately injected directly into the HPLC system.

4. **Reagents and materials**

During the analysis, use only reagents of HPLC grade, absolute ethanol and water of grade 3 as defined by ISO 3696.

4.1. Ethanol 96 % vol. (CAS 64-17-5).

4.2. Ammonium glycyrrhizinate, $C_{42}H_{62}O_{16}.NH_3$ (Glycyrrhizic acid ammonium salt)

(Mol. Wt.: 839,98)(CAS 53956-04-0): purity at least 90 %

(Mol. Wt.: glycyrrhizic acid 822,94).

4.3. Glacial acetic acid, CH_3COOH, (CAS 64-19-7).

4.4. Methanol, CH_3OH (CAS 67-56-1).

4.5. Ethanol 50 % vol.

For 1 000 ml at 20 °C:

— 96 % vol. ethanol (4.1): 521 ml

— Water (2.0): 511 ml.

4.6. Preparation of the HPLC elution solutions

4.6.1. Elution solvent A (example)

80 parts (by volume) of water (2.0)

20 parts (by volume) of acetic acid (4.3).

Degas the elution solvent for five minutes.

Note: If the water being used has not been microfiltered, it is advisable to filter the prepared elution solvent on a filter for organic solvents with a pore size less than or equal to 0,45 μm.

4.6.2. Elution solvent B

Methanol (4.4).

4.7. Preparation of standard solutions

All standard solutions must be freshly prepared after two months.

4.7.1. Reference solution C

Weigh to the nearest 0,1 mg, 25 mg of ammonium glycyrrhizinate (4.2) in a 100 ml volumetric flask. Add some 50 % vol. ethanol (4.5) and dissolve the ammonium glycyrrhizinate. When it has dissolved make up to the mark with 50 % vol. ethanol (4.5).

▼M1

Filter through a filter for organic solvents.

4.7.2. Standard solutions used to check the linearity of the response of the instrumentation

A 1,0 g/l stock solution is prepared by weighing, to the nearest 0,1 mg, 100 mg of ammonium glycyrrhizinate in a 100 ml volumetric flask. Add some 50 % vol. ethanol (4.5) and dissolve the ammonium glycyrrhizinate. When it has dissolved make up to the mark with 50 % vol. ethanol (4.5).

At least four other solutions corresponding to 0,05, 0,1, 0,25 and 0,5 g/l of ammonium glycyrrhizinate are prepared by pipetting respectively 5 ml, 10 ml, 25 ml and 50 ml of the 1,0 g/l stock solution in separate 100 ml volumetric flasks. Then make up to the mark with 50 % vol. ethanol (4.5) and mix up thoroughly.

Filter all solutions though a filter for organic solvents.

5. **Apparatus and equipment**

5.1. Separation system

5.1.1. High-performance liquid chromatograph.

5.1.2. Pumping system enabling one to achieve and maintain a constant or programmed rate of flow with great precision.

5.1.3. UV spectrophotometric detection system: to be set at 254 nm.

5.1.4. Solvent degassing system.

5.2. Computational integrator or recorder, the performance of which is compatible with the rest of the system.

5.3. Column (example):

Material: stainless steel or glass

Internal diameter: 4 to 5 mm

Length: 100 to 250 mm

Stationary phase: cross-linked silica with a (preferably spherical) octadecyl functional group (C18), maximum particle size: 5 μm.

5.4. Laboratory equipment

5.4.1. Analytical balance with a precision of 0,1 mg

5.4.2. A-grade volumetric glassware

5.4.3. Micromembrane filtration arrangement for small volumes.

6. **Chromatography conditions**

6.1. Elution characteristics: (example)

— flow rate: 1 ml/minute,

— solvent A = 30 %,

— solvent B = 70 %.

6.2. Detection:

— UV = 254 nm

7. **Procedure**

7.1. Preparation of the spirit sample

Filter, if necessary, through a filter for organic solvents (pore diameter: 0,45 μm).

▼M1

7.2. Determination

Once the chromatography conditions have stabilised,

— inject 20 µl of the reference solution C (4.7.1),

— inject 20 µl of the sample solution,

— compare the two chromatograms. Identify the glycyrrhizic acid peaks from their retention times. Measure their areas (or heights) and calculate the concentration in g/l to two decimal figures using the following equation:

$$c = c \times \frac{h \times P \times 823}{H \times 100 \times 840}$$

where:

c is the concentration in grams per litre of glycyrrhizic acid in the spirit being analysed

C is the concentration in grams per litre of ammonium glycyrrhizinate in the reference solution

h is the area (or height) of the glycyrrhizic acid peak of the spirit being analysed

H is the area (or height) of the glycyrrhizic acid peak of the reference solution

P is the purity of the reference ammonium glycyrrhizinate (in %)

823 is the mass of one mole of glycyrrhizic acid

840 is the mass of one mole of ammonium glycyrrhizinate.

8. **Method performance characteristics (precision)**

Statistical results of the interlaboratory test:

the following table give the values for glycyrrhizic acid.

The following data were obtained from an international method performance study carried out to internationally agreed procedures.

Year of interlaboratory test	1998
Number of laboratories	16
Number of samples	5
Analyte	glycyrrhizic acid

Samples	A	B	C	D	F
Number of laboratories retained after eliminating outliers	13	14	15	16	16
Number of outliers (laboratories)	3	2	1	—	—
Number of accepted results	26	28	30	32	32
Mean value g/l	0,046	0,092 (*) 0,099	0,089	0,249	0,493
Repeatability standard deviation (S_r) g/l	0,001	0,001	0,001	0,002	0,003
Repeatability relative standard deviation (RSD_r) (%)	1,5	1,3	0,7	1,0	0,6
Repeatability limit (r) g/l	0,002	0,004	0,002	0,007	0,009
Reproducibility standard deviation (S_R) g/l	0,004	0,007	0,004	0,006	0,013

▼**M1**

Samples	A	B	C	D	F
Reproducibility relative standard deviation (RSD_R) (%)	8,6	7,2	4,0	2,5	2,7
Reproducibility limit (R) g/l	0,011	0,019	0,010	0,018	0,037

Sample types:

A pastis, blind duplicates

B pastis, split levels (*)

C pastis, blind duplicates

D pastis, blind duplicates

E pastis, blind duplicates

▼M1

VII. CHALCONES. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR VERIFYING THE PRESENCE OF CHALCONES IN PASTIS

1. Scope

This method is suitable for determining whether chalcones are present in aniseed-flavoured drinks or not. Chalcones are natural colorants of the flavonoid family that are present in liquorice root (*Glycyrrhiza glabra*).

For an aniseed-flavoured spirit to be called 'pastis', it must contain chalcones (Regulation (EEC) No 1576/89).

2. Normative references

ISO 3696: 1987, Water for analytical laboratory use — Specifications and test methods.

3. Principle

A reference liquorice extract solution is prepared. The presence or absence of chalcones is determined using high-performance liquid chromatography (HPLC) with UV detection.

4. Reagents and materials

During the analysis, use only reagents of HPLC grade. The ethanol should be 96 % vol. Only water of grade 3 as defined by ISO 3696 should be used.

4.1. Ethanol 96 % vol. (CAS 64-17-5)

4.2. Acetonitrile, CH_3CN, (CAS 75-05-8)

4.3. Reference substance: *Glycyrrhiza glabra*: liquorice, 'sweet root'

Coarsely ground liquorice roots (*Glycyrrhiza glabra*). Average dimensions of the rodlike particles: length: 10 to 15 mm, thickness: 1 to 3 mm.

4.4. Sodium acetate, CH_3COONa, (CAS 127-09-3)

4.5. Glacial acetic acid, CH_3COOH, (CAS 64-19-7)

4.6. Preparation of solutions

4.6.1. Ethanol 50 % volume

For 1 000 ml at 20 °C:

— 96 % vol. ethanol (4.1): 521 ml,

— Water (2.0): 511 ml.

4.6.2. Solvent A: acetonitrile

Acetonitrile (4.2) of HPLC analytical purity.

Degas

4.6.3. Solvent B: 0,1 M sodium acetate buffer solution, pH 4,66.

Weigh 8,203 g of sodium acetate (4.4), add 6,005 g of glacial acetic acid (4.5) and make up to 1 000 ml with water (2) in a volumetric flask.

5. Preparation of the reference extract from *Glycyrrhiza glabra* (4.3)

5.1. Weigh 10 g of ground liquorice root (*Glycyrrhiza glabra*) (4.3) and place in a round-bottomed distillation flask

— add 100 ml of 50 % vol. ethanol (4.6.1),

— boil under reflux for one hour,

— filter,

— set the filtrate aside for later use.

▼M1

5.2. Recover the liquorice extract from the filter

— place in a round-bottomed distillation flask,

— add 100 ml of 50 % vol. ethanol (4.6.1),

— boil under reflux for one hour,

— filter. Set aside the filtrate for later use.

5.3. The liquorice root extraction must be performed three times in succession.

5.4. Combine the three filtrates.

5.5. Evaporate the solvent phase (of 5.4) on a rotary evaporator.

5.6. Take up the residual extract (of 5.5) with 100 ml 50 % vol. ethanol (4.6.1).

6. **Apparatus and equipment**

6.1. Separation system.

6.1.1. High-performance liquid chromatograph.

6.1.2. Pumping system capable of achieving and maintaining a constant or programmed rate of flow at high pressure.

6.1.3. UV/visible spectrophotometric detection system that can be set at 254 and 370 nm.

6.1.4. Solvent degassing system:

6.1.5. Column oven that can be set at a temperature of 40 ± 0,1 °C.

6.2. Computational integrator or recorder, the performance of which is compatible with the rest of the separation system.

6.3. Column

Material: stainless steel or glass

Internal diameter: 4 to 5 mm

Stationary phase: cross-linked silica with an octadecyl derived functional group (C18), particle size: 5 μm at most (cross-linked phase).

6.4. Common laboratory equipment, including:

6.4.1. analytical balance. (precision: ± 0,1 mg);

6.4.2. distillation apparatus with a reflux condenser, comprising, for example:

— a 250 ml round-bottomed flask with a standardised ground-glass joint,

— a 30 cm long reflux condenser, and

— a heat source (any pyrogenic reaction involving the extractive matter must be avoided by using an appropriate arrangement).

6.4.3. Rotary evaporation apparatus.

6.4.4. Filtration set-up (i.e. Buchner funnel).

6.5. Chromatography conditions (example).

6.5.1. Elution characteristics of solvents A (4.6.2) and B (4.6.3):

— shift from 20/80 (v/v) to 50/50 (v/v) gradient in 15 minutes,

— shift from 50/50 (v/v) to 75/25 (v/v) gradient in five minutes,

— equal strength at 75/25 (v/v) for five minutes,

▼M1

— stabilisation of the column between injections,

— equal strength at 20/80 (v/v) for five minutes.

6.5.2. Flow rate: 1 ml/minute.

6.5.3. UV detector settings:

the detector must be set at 370 nm to detect the presence of chalcones and then at 254 nm to detect glycyrrhizic acid.

Note: the change of wavelength (from 370 nm to 254 nm) must be carried out 30 seconds before the beginning of the peak of elution of glycyrrhizic acid.

7. **Procedure**

7.1. Preparation of the spirit sample

Filter through a filter for organic solvents (pore diameter: 0,45 µm).

7.2. Preparation of the residual liquorice extract (5.6)

Make a one in ten dilution with 50 % vol. ethanol (4.6.1) before analysis.

7.3. Determination

7.3.1. Inject 20 µl of the prepared liquorice extract (7.2). Perform the analysis using the chromatography conditions described above (6.5).

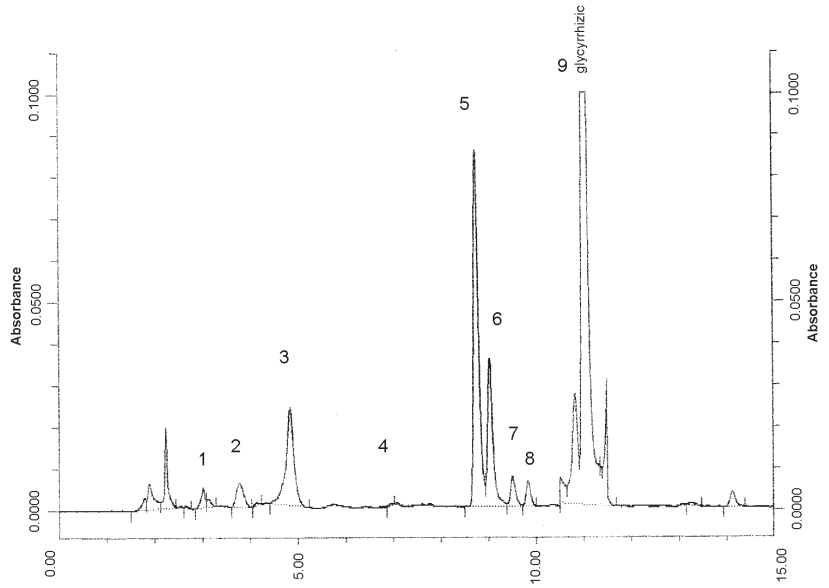
7.3.2. Inject 20 µl of the sample (7.1) (aniseed-flavoured spirit sample). Perform the analysis using the chromatography conditions described above (6.5).

7.3.3. Compare the two chromatograms. There must be a great similarity between the two chromatograms in the chalcone exit zone (during the detection at 370 nm under the analysis conditions described above) (see Figure 1).

8. **Characteristic chromatogram for a pastis**

Figure 1

Chromatogram obtained by the method described above, showing the presence of chalcones in a ‘pastis’. Peaks 1 to 8 are chalcones and peak 9 is glycyrrhizic acid.



▼M1

9. **Method performance characteristics (precision)**

Results of the interlaboratory test:

the following table gives the performance for recognition of presence or absence of chalcones in pastis and aniseed-flavoured spirits.

The following data were obtained from an international method performance study carried out to internationally agreed procedures.

Year of interlaboratory test	1998
Number of laboratories	14
Number of samples	11
Analyte	chalcones

Samples	A	B	C	D	E	F
Number of laboratories retained after eliminating outliers	14	14	14	14	14	13
Number of outliers (laboratories)	—	—	—	—	—	1 (*)
Number of accepted results	28	14	14	28	28	26
Number of results for presence of chalcones	28	14	14	0	28	0
Number of results for absence of chalcones	0	0	0	28	0	26
Percentage of correct results (%)	100	100	100	100	100	100

(*) Inconsistent results between the two duplicates, attributed to a sampling error

Samples	G	H	I	J	K
Number of laboratories retained after eliminating outliers	14	14	14	14	14
Number of outliers (laboratories)	—	—	—	—	—
Number of accepted results	28	14	14	28	28
Number of results for presence of chalcones	0	0	0	0	0
Number of results for absence of chalcone	28	14	14	28	28
Percentage of correct results (%)	100	100	100	100	100

Sample types:

A pastis, blind duplicates

B pastis, single sample

C pastis, single sample

D ‘pastis’ (not containing chalcones), blind duplicates

E ‘pastis’ (not containing chalcones), blind duplicates

F aniseed-flavoured liqueur (not containing chalcones), blind duplicates

▼**M1**

G aniseed-flavoured liqueur (not containing chalcones), blind duplicates

H ouzo (not containing chalcones), single sample

I ouzo (not containing chalcones), single sample

J anis (not containing chalcones), blind duplicates

K ‘pastis’ (not containing chalcones), blind duplicates.

▼M2

VIII. TOTAL SUGARS

1. **Scope**

The HPLC–RI method is applicable for the determination of total sugars (expressed as invert sugar) in spirit drinks, with the exclusion of liqueurs containing egg and milk products.

The method has been validated in an interlaboratory study for pastis, distilled anis, cherry liqueur, crème de (followed by the name of a fruit or the raw material used) and crème de cassis, at levels ranging from 10,86 g/l to 509,7 g/l. However, linearity of the instrument response was proven for the concentration range 2,5 g/l to 20,0 g/l.

This method is not intended for determining low levels of sugars.

2. **Normative references**

ISO 3696:1987 Waters for analytical use — Specifications and test methods.

3. **Principle**

High-performance liquid chromatography assays of sugar solutions, in order to determine their glucose, fructose, sucrose, maltose and lactose concentrations.

This method uses an alkylamine stationary phase and differential refractometry detection and is given as an example. The use of anion exchange resins as stationary phase would also be possible.

4. **Reagents and materials**

4.1. Glucose (CAS 50-99-7), at least 99 % pure.

4.2. Fructose (CAS 57-48-7), at least 99 % pure.

4.3. Sucrose (CAS 57-50-1), at least 99 % pure.

4.4. Lactose (CAS 5965-66-2), at least 99 % pure.

4.5. Maltose monohydrate (CAS 6363-53-7), at least 99 % pure.

4.6. Pure acetonitrile (CAS 75-05-8) for HPLC analysis.

4.7. Distilled or demineralised water, preferably microfiltered.

4.8. Solvents (example)

The elution solvent is composed of:

75 parts by volume of acetonitrile (4.6),

25 parts by volume of distilled water (4.7).

Pass helium through at a slow rate for 5-10 minutes prior to use to degas.

If the water being used has not been microfiltered, the solvent should be filtered with a filter for organic solvents with a pore size less than or equal to 0,45 μm.

4.9. Ethanol absolute (CAS 64-17-5).

4.10. Ethanol solution (5 %, v/v).

4.11. Preparation of stock standard solution (20 g/l)

Weigh 2 g each of the sugars to be analysed (4.1 to 4.5), transfer them without loss to a 100 ml volumetric flask. (NB 2,11 g of maltose monohydrate is equivalent to 2 g of maltose).

▼M2

Adjust to 100 ml with a 5 % vol. alcohol solution (4.10), shake and store at around + 4 °C. Prepare a new stock solution once a week.

4.12. Preparation of working standard solutions (2,5, 5,0, 7,5, 10,0 and 20,0 g/L)

Dilute the stock solution, 20 g/l (4.11) appropriately with a 5 % vol. alcohol solution (4.10) to give five working standards of 2,5, 5,0, 7,5, 10,0 and 20,0 g/l. Filter with a filter of a pore size less than or equal to 0,45 µm (5.3).

5. **Apparatus and Equipment**

5.1. HPLC system capable of achieving baseline resolution of all of the sugars.

5.1.1. High-performance liquid chromatograph with a six-way injection valve fitted with a 10 µl loop or any other device, whether automatic or manual, for the reliable injection of microvolumes.

5.1.2. Pumping system enabling one to achieve and maintain a constant or programmed rate of flow with great precision.

5.1.3. Differential refractometer.

5.1.4. Computational integrator or recorder, the performance of which is compatible with the rest of the set-up.

5.1.5. Pre-column:

It is recommended that a suitable pre-column is attached to the analytical column.

5.1.6. Column (example):

Material:	stainless steel or glass.
Internal diameter:	2-5 mm.
Length:	100-250 mm (depending on the packing particle size), for example, 250 mm if the particles are 5 µm in diameter.
Stationary phase:	alkylamine functional groups bonded to silica, maximum particle size 5 µm.

5.1.7. Chromatography conditions (example):

Elution solvent (4.8), flow rate: 1 ml/minute.

Detection: Differential refractometry.

To make certain that the detector is perfectly stable, it should be switched on a few hours before use. The reference cell must be filled with the elution solvent.

5.2. Analytical balance accurate to 0,1 mg.

5.3. Filtration set-up for small volumes using a 0,45 µm micromembrane.

6. **Sample storage**

On receipt, samples are to be stored at room temperature prior to analysis.

7. **Procedure**

7.1. PART A: Sample preparation

7.1.1. Shake the sample.

▼M2

7.1.2. Filter the sample through a filter with a pore size less than or equal to 0,45 µm (5.3).

7.2. PART B: HPLC

7.2.1. Determination

Inject 10 µl of the standard solutions (4.12) and samples (7.1.2). Perform the analysis under suitable chromatography conditions, for example those described above.

7.2.2. Should any peak of a sample have a greater area (or height) than the corresponding peak in the most concentrated standard, then the sample should be diluted with distilled water and reanalysed.

8. **Calculation**

Compare the two chromatograms obtained for the standard solution and spirit. Identify the peaks by their retention times. Measure their areas (or heights) to calculate the concentrations by the external standard method. Take into account any dilutions made to the sample.

The final result is the sum of sucrose, maltose, lactose, glucose and fructose, expressed as invert sugar in g/l.

Invert sugar is calculated as the sum of all monosaccharides and reducing disaccharides present, plus the stoichiometric amount of glucose and fructose calculated from the sucrose present.

Invert sugar (g/l) = glucose (g/l) + fructose (g/l) + maltose (g/l) + lactose (g/l) + (sucrose (g/l) × 1,05).

1,05 = (molecular weight of fructose + molecular weight of glucose)/molecular weight of sucrose.

9. **Method performance characteristics (precision)**

9.1. Statistical results of the interlaboratory test

The following data were obtained from an international method performance study carried out to internationally agreed procedures ([1]) ([2]).

Year of interlaboratory test	2000
Number of laboratories	24
Number of samples	8

(1) ‘Protocol for the design, conduct and interpretation of method-performance studies’, Horwitz, W. (1995) *Pure and Applied Chemistry*, 67, 332-343.

(2) Horwitz, W. (1982) *Analytical Chemistry*, 54, 67A-76A.

Table 1

Fructose, glucose, maltose

Analyte	Fructose		Glucose			Maltose	
Samples (× 2)	Crème de Cassis	Standard (50 g/l)	Aniseed-flavoured spirit drink	Crème de Cassis	Standard (50 g/l)	Aniseed-flavoure-d spirit drink	Standard (10 g/l)
Mean value (g/l)	92,78	50,61	15,62	93,16	50,06	15,81	9,32
No of labs without outliers	21	22	21	23	19	21	22

▼M2

Analyte	Fructose		Glucose			Maltose	
Samples (× 2)	Crème de Cassis	Standard (50 g/l)	Aniseed-flavoured spirit drink	Crème de Cassis	Standard (50 g/l)	Aniseed-flavoure-d spirit drink	Standard (10 g/l)
Repeatability standard deviation, s_r, (g/l)	2,34	2,12	0,43	3,47	1,01	0,48	0,54
Repeatability relative standard deviation, RSD_r (%)	2,53	4,2	2,76	3,72	2,03	3,02	5,77
Repeatability limit, r (g/l) ($r = 2,8 \times s_r$)	6,56	5,95	1,21	9,71	2,84	1,34	1,51
Reproducibility standard deviation, s_R (g/l)	7,72	3,13	0,84	9,99	2,7	0,88	1,4
Reproducibility relative standard deviation, RSD_R (%)	8,32	6,18	5,37	10,72	5,4	5,54	15,06
Reproducibility limit, R (g/l) ($R = 2,8 \times sR$)	21,62	8,76	2,35	27,97	7,57	2,45	3,93

Table 2

Sucrose

Analyte	Sucrose					
Samples	Pastis	Ouzo	Cherry liqueur	Crème de Menthe	Crème de Cassis	Standard (100 g/l)
Mean value (g/l)	10,83	29,2 19,7 (*)	103,33	349,96	319,84	99,83
No of labs without outliers	19	19	20	18	18	18
Repeatability standard deviation, s_r (g/l)	0,09	0,75	2,17	5,99	4,31	1,25
Repeatability relative standard deviation, RSD_r (%)	0,81	3,07	2,1	1,71	1,35	1,25
Repeatability limit, r (g/l) ($r = 2,8 \times s_r$)	0,25	2,1	6,07	16,76	12,06	3,49
Reproducibility standard deviation, s_R (g/l)	0,79	0,92	4,18	9,94	16,11	4,63
Reproducibility relative standard deviation, RSD_R (%)	7,31	3,76	4,05	2,84	5,04	4,64
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	2,22	2,57	11,7	27,84	45,12	12,97

(*) split level.

▼M2

Table 3

Total Sugars

(*Note:* this data was calculated for total sugars, not invert sugar as defined in Section 8 above.)

Samples	Pastis	Ouzo	Aniseed-flavoured spirit drink	Cherry liqueur	Crème de Menthe	Crème de Cassis	Standard (220 g/l)
Mean value (g/l)	10,86	29,2 19,7 (*)	31,59	103,33	349,73	509,69	218,78
No of Labs without outliers	20	19	20	20	18	18	19
Repeatability standard deviation, s_r (g/l)	0,13	0,75	0,77	2,17	5,89	5,59	2,71
Repeatability relative standard deviation, RSD_r (%)	1,16	3,07	2,45	2,1	1,69	1,1	1,24
Repeatability limit, r (g/l) ($r = 2,8 \times s_r$)	0,35	2,1	2,17	6,07	16,5	15,65	7,59
Reproducibility standard deviation s_R (g/l)	0,79	0,92	1,51	4,18	9,98	14,81	8,53
Reproducibility relative standard deviation, RSD_R (%)	7,25	3,76	4,79	4,04	2,85	2,91	3,9
Reproducibility limit R (g/l) ($R = 2,8 \times s_R$)	2,21	2,57	4,24	11,7	27,94	41,48	23,89

(*) split level.

▼M1

IX. EGG YOLK. DETERMINATION OF EGG YOLK CONCENTRATION IN SPIRIT DRINKS — PHOTOMETRIC METHOD

1. **Scope**

This method is suitable for the determination of egg yolk concentration in the range of 40 to 250 g/l in egg liqueur and liqueur with egg.

2. **Normative references**

ISO 3696:1897 Water for analytical laboratory use — Specifications and test methods.

3. **Principle**

The ethanol-soluble phosphorus compounds found in egg yolk are extracted and assayed photometrically as a phosphorus molybdate complex.

4. **Reagents and materials**

4.1. Double-distilled water

4.2. Diatomaceous earth

4.3. Ethanol 96 % vol. (CAS 64-17-5)

4.4. 15 % magnesium acetate (CAS 16674-78-5) solution

4.5. 10 % sulphuric acid (CAS 7664-93-9)

4.6. 1 N sulphuric acid.

4.7. 0,16 g/l potassium dihydrogen phosphate (CAS 778-77-0), KH_2PO_4 solution

4.8. Reagent for phosphate determination:

dissolve 20 g of ammonium molybdate (CAS 12054-85-2), $(NH_4)_6Mo_7O_{24}.4H_2O$ in 400 ml water at 50 °C;

dissolve, in another vessel, 1 g of ammonium vanadate (CAS 7803-55-6), NH_4VO_3, in 300 ml hot water, allow to cool, then add 140 ml of concentrated nitric acid (CAS 7697-37-2). Combine the cooled solutions in a 1 000 ml volumetric flask and make up to the 1 000 ml mark.

5. **Apparatus and equipment**

5.1. 100 ml conical flask

5.2. Ultrasonic bath (or magnetic stirrer)

5.3. 100 ml volumetric flask

5.4. 20 °C water bath

5.5. Filter (Whatman No 4 or equivalent)

5.6. Porcelain (or platinum) crucible

5.7. Boiling water bath

5.8. Hot plate

5.9. Muffle furnace

5.10. 50 ml volumetric flask

5.11. 20 ml volumetric flask

5.12. Spectrophotometer set at 420 nm

5.13. 1 cm cuvette.

▼M1

6. **Samples**

Samples are stored at room temperature prior to analysis.

7. Procedure

7.1. Sample preparation

7.1.1. Weigh 10 g of the sample into a 100 ml conical flask (5.1).

7.1.2. Add gradually 70 ml of ethanol (4.3) in small portions, swirling with each addition, and place in an ultrasonic bath (5.2) for 15 minutes (or stir the mixture with a magnetic stirrer (5.2) for 10 minutes at room temperature).

7.1.3. Transfer the contents of the flask to a 100 ml volumetric flask (5.3) with washings of ethanol (4.3). Adjust to the calibration mark with ethanol (4.3) and place the flasks in a 20 °C water bath (5.4). Adjust to the calibration mark at 20 °C.

7.1.4. Add a small amount of diatomaceous earth (4.2) and filter (5.5), discarding the first 20 ml.

7.1.5. Transfer 25 ml of the filtrate to a porcelain (or platinum) crucible (5.6). The filtrate must then be concentrated by gentle evaporation in a boiling water bath (5.7), with the addition of 5 ml of 15 % magnesium acetate solution (4.4).

7.1.6. Place the crucibles on a hot plate (5.8) and heat until just dry.

7.1.7. Ash the residue by heating to incandescence at 600 °C in a muffle furnace (5.9) until the ash is white, minimum of one and a half hours but can be left overnight.

7.1.8. Take up the ash with 10 ml of 10 % sulphuric acid (4.5) and transfer it with washings of distilled water (4.1) to a 50 ml volumetric flask (5.10), and fill to the mark at room temperature with distilled water (4.1). A 5 ml aliquot of this ash solution is to be used to prepare the sample solution of the photometric phosphate assay.

7.2. Photometric phosphate assay

7.2.1. Comparative solution

7.2.1.1. Place 10 ml of 10 % sulphuric acid (4.5) in a 50 ml volumetric flask (5.10) and fill to the mark with distilled water (4.1).

7.2.1.2. Add to a 5 ml aliquot of this solution (7.2.1.1), contained in a 20 ml volumetric flask (5.11), 1 ml of 1 N sulphuric acid (4.6) and 2 ml of the phosphate reagent (4.8) and make up to 20 ml with distilled water (4.1).

7.2.1.3. Stopper with a loosely inserted stopper, shake, and heat in a boiling water bath (5.7) for 10 minutes, then cool in a 20 °C water bath (5.4) for 20 minutes.

7.2.1.4. Fill a 1 cm cuvette (5.13) with this comparative solution.

7.2.2. Sample solution

7.2.2.1. Add to a 5 ml aliquot of the ash solution (7.1.8), contained in a 20 ml volumetric flask (5.11), 1 ml of 1 N sulphuric acid (4.6) and 2 ml of the phosphate reagent (4.8) and make up to 20 ml with distilled water (4.1).

▼M1

7.2.2.2. Stopper with a loosely inserted stopper, shake, and heat in a boiling water bath (5.7) for 10 minutes, then cool in a 20 °C water bath (5.4) for 20 minutes.

7.2.2.3. The yellow solution that develops is immediately analysed spectrophotometrically (5.12) in a 1 cm cuvette (5.13) at 420 nm against the comparative solution (7.2.1.4).

7.2.3. Calibration curve

7.2.3.1. To construct the calibration curve, add 2 ml aliquots of the phosphate reagent (4.8) to 20 ml volumetric flasks (5.11) each containing 1 ml of 1 N sulphuric acid (4.6) and 0, 2, 4, 6, 8, and 10 ml of the potassium dihydrogen phosphate solution (4.7) respectively, and make up to the 20 ml mark with distilled water (4.1).

7.2.3.2. Stopper with a loosely inserted stopper, shake, and heat in a boiling water bath (5.7) for 10 minutes, then cool in a 20 °C water bath (5.4) for 20 minutes and analyse spectrophotometrically (5.12) in a 1 cm cuvette (5.13) at 420 nm against the comparative solution (7.2.1.4).

7.2.3.3. Construction of the calibration curve:

dihydrogen phosphate solution (ml)	0	2	4	6	8	10
P_2O_5 (mg)	0	0,167	0,334	0,501	0,668	0,835

8. **Expression of results**

The egg yolk content in g/l is calculated from the following formula:

$$\text{g/l egg yolk} = \text{mg } P_2O_5 \times \frac{110 \times \text{density}}{E/40}$$

where:

110	conversion factor for total P_2O_5 in g in 100 g of egg yolk
mg P_2O_5	value established from the calibration curve
density	mass per unit volume (g/ml) of the egg-based liqueur at 20 °C
E	weight of the egg-based liqueur in g
40	dilution factor for a 5 ml aliquot of ash solution.

9. **Method performance characteristics (precision)**

Statistical results of the interlaboratory test:

the following table gives the values for egg yolk.

The following data were obtained from an international method performance study carried out to internationally agreed procedures.

Year of interlaboratory test:	1998
Number of laboratories:	24
Number of samples:	5
Analyte:	Egg yolk

▼**M1**

Samples	A	B	C	D	E
Number of laboratories retained after eliminating outliers	19	20	22	20	22
Number of outliers (laboratories)	3	4	2	4	2
Number of accepted results	38	40	44	40	44
Mean value	147,3	241,1	227,4	51,9 (*) 72,8 (*)	191,1
Repeatability standard deviation (S_r) g/l	2,44	4,24	3,93	1,83	3,25
Repeatability relative standard deviation (RSD_r) (%)	1,7	1,8	1,8	2,9	1,7
Repeatability limit (r) g/l	6,8	11,9	11,0	5,1	9,1
Reproducibility standard deviation (S_R) g/l	5,01	6,06	6,66	3,42	6,87
Reproducibility relative standard deviation (RSD_R) (%)	3,4	2,5	2,9	5,5	3,6
Reproducibility limit (R) g/l	14,0	17,0	18,7	9,6	19,2

Sample types

A Advocaat, blind duplicates

B Advocaat, blind duplicates

C Advocaat, blind duplicates

D Advocaat (diluted), split levels (*)

E Advocaat, blind duplicates

▼M2

X. DETERMINATION OF THE FOLLOWING WOOD COMPOUNDS IN SPIRIT DRINKS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC): FURFURAL, 5-HYDROXYMETHYLFURFURAL, 5-METHYLFURFURAL, VANILLIN, SYRINGALDEHYDE, CONIFERALDEHYDE, SINAPALDEHYDE, GALLIC ACID, ELLAGIC ACID, VANILLIC ACID, SYRINGIC ACID AND SCOPOLETIN

1. **Scope**

The method pertains to the determination of furfural, 5-hydroxymethylfurfural, 5-methylfurfural, vanillin, syringaldehyde, coniferaldehyde, sinapaldehyde, gallic acid, ellagic acid, vanillic acid, syringic acid and scopoletin, by high-performance liquid chromatography.

2. **Normative reference**

Analytical method recognised by the General Assembly of the International Organisation of Vine and Wine (OIV) and published by OIV under the reference *OIV-MA-BS-16: R2009*.

3. **Principle**

Determination by high-performance liquid chromatography (HPLC), with detection by ultraviolet spectrophotometry at several wavelengths and by spectrofluorimetry.

4. **Reagents**

The reagents must be of analytical quality. The water used must be distilled water or water of at least equivalent purity. It is preferable to use microfiltered water with a resistivity of 18,2 M Ω.cm.

4.1. 96 % vol. alcohol.

4.2. HPLC-quality methanol (Solvent B).

4.3. Acetic acid diluted to 0,5 % vol. (Solvent A).

4.4. Mobile phases: (given as an example only).

Solvent A (0,5 % acetic acid) and solvent B (pure methanol). Filter through a membrane (porosity 0,45 μm). Degas in an ultrasonic bath, if necessary.

4.5. Reference standards of 99 % minimum purity: furfural, 5-hydroxymethyl furfural, 5-methylfurfural, vanillin, syringaldehyde, coniferaldehyde, sinapaldehyde, gallic acid, ellagic acid, vanillic acid, syringic acid and scopoletin.

4.6. Reference solution: the standard substances are dissolved in a 50 % vol. aqueous-alcoholic solution. The final concentrations in the reference solution should be of the order of:

furfural: 5 mg/l; 5-hydroxymethyl furfural: 10 mg/l; 5-methylfurfural 2 mg/l; vanillin: 5 mg/l; syringaldehyde: 10 mg/l; coniferaldehyde: 5 mg/l; sinapaldehyde: 5 mg/l; gallic acid: 10 mg/l; ellagic acid: 10 mg/l; vanillic acid: 5 mg/l; syringic acid: 5 mg/l; scopoletin: 0,5 mg/l.

5. **Apparatus**

Standard laboratory apparatus

5.1. A high-performance liquid chromatograph capable of functioning in binary gradient mode and equipped with:

5.1.1. A spectrophotometric detector capable of measuring at wavelengths from 260 to 340 nm. It is however preferable to work with a multiple wavelength detector with a diode array or similar, in order to confirm the purity of the peaks.

▼M2

5.1.2. A spectrofluorimetric detector — excitation wavelength: 354 nm, emission wavelength: 446 nm (for the trace determination of scopoletin; which is also detectable at 313 nm by spectrophotometry).

5.1.3. An injection device capable of introducing 10 or 20 µl (for example) of the test sample.

5.1.4. A high-performance liquid chromatography column, RP C18 type, 5 µm maximum particle size.

5.2. Syringes for HPLC.

5.3. Device for membrane-filtration of small volumes.

5.4. Integrator-computer or recorder with performance compatible with the entire apparatus, and in particular, it must have several acquisition channels.

6. **Procedure**

6.1. Preparation of the solution to be injected

The reference solution and the spirit drink are filtered, if necessary, through a membrane with a maximum pore diameter of 0,45 µm.

6.2. Chromatographic operating conditions: carry out the analysis at ambient temperature by means of the equipment described in (5.1) and using the mobile phases (4.4) with a flow of approximately 0,6 ml per minute following the gradient below (given as an example only)

Time: 0 min 50 min 70 min 90 min

solvent A (water-acid): 100 % 60 % 100 % 100 %

solvent B (methanol): 0 % 40 % 0 % 0 %

Note that in certain cases this gradient should be modified to avoid co-elutions.

6.3. Determination

6.3.1. Inject the reference standards separately, then mixed.

Adapt the operating conditions so that the resolution factors of the peaks of all the compounds are equal to at least 1.

6.3.2. Inject the sample as prepared in 6.1.

6.3.3. Measure the area of the peaks in the reference solution and the spirit drink and calculate the concentrations.

7. **Expression of results**

Express the concentration of each constituent in mg/l.

8. **Performance characteristics of the method (precision)**

The following data were obtained in 2009 from an international method-performance study on a variety of spirit drinks, carried out following internationally-agreed procedures (1) (2).

8.1. Furfural

Analyte	Furfural					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	15	15	15	15	15	15
No of results accepted (laboratories)	14	12	13	14	13	13

▼**M2**

Analyte	Furfural					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
Mean value (mg/l)	2,9	1,2	1,7	10,6	15,3	13,9
Repeatability standard deviation, s_r (mg/l)	0,04	0,05	0,04	0,18	0,23	0,20
Repeatability relative standard deviation, RSD_r (%)	1,4	4,5	2,3	1,7	1,5	1,5
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,1	0,2	0,1	0,5	0,6	0,6
Reproducibility standard deviation, s_R (mg/l)	0,24	0,18	0,09	1,4	0,49	0,69
Reproducibility relative standard deviation, RSD_R (%)	8	15	5	13	3	5
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,7	0,5	0,3	3,8	1,4	1,9

8.2. 5-Hydroxymethylfurfural

Analyte	5-Hydroxymethylfurfural					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	16	16	16	16	16	16
No of results accepted (laboratories)	14	14	14	14	14	14
Mean value (mg/l)	5,0	11,1	9,4	33,7	5,8	17,5
Repeatability standard deviation, s_r (mg/l)	0,09	0,09	0,09	0,42	0,07	0,13
Repeatability relative standard deviation, RSD_r (%)	1,7	0,8	1,0	1,3	1,2	0,8
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,2	0,3	0,3	1,2	0,2	0,4
Reproducibility standard deviation, s_R (mg/l)	0,39	1,01	0,50	4,5	0,4	1,6
Reproducibility relative standard deviation, RSD_R (%)	8	9	5	13	7	9
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	1,1	2,8	1,4	12,5	1,1	4,6

8.3. 5-Methylfurfural

Analyte	5-Methylfurfural					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	11	11	11	11	11	11
No of results accepted (laboratories)	11	11	8	11	10	11

▼M2

Analyte	5-Methylfurfural					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
Mean value (mg/l)	0,1	0,2	0,1	0,5	1,7	0,8
Repeatability standard deviation, s_r (mg/l)	0,01	0,01	0,02	0,02	0,03	0,07
Repeatability relative standard deviation, RSD_r (%)	10,7	6,1	13,6	4,7	2,0	10,0
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,0	0,0	0,1	0,1	0,1	0,2
Reproducibility standard deviation, s_R (mg/l)	0,03	0,04	0,03	0,18	0,20	0,26
Reproducibility relative standard deviation, RSD_R (%)	35	18	22	39	12	35
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,1	0,1	0,1	0,5	0,6	0,7

8.4. Vanillin

Analyte	Vanillin					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	16	15	16	16	16	16
No of results accepted (laboratories)	16	15	16	16	16	16
Mean value (mg/l)	0,5	0,2	1,2	1,2	3,2	3,9
Repeatability standard deviation, s_r (mg/l)	0,03	0,02	0,06	0,11	0,11	0,09
Repeatability relative standard deviation, RSD_r (%)	6,8	9,6	4,6	8,9	3,5	2,3
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,1	0,1	0,2	0,3	0,3	0,3
Reproducibility standard deviation, s_R (mg/l)	0,09	0,06	0,18	0,27	0,41	0,62
Reproducibility relative standard deviation, RSD_R (%)	19	25	15	22	13	16
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,3	0,2	0,5	0,8	1,2	1,7

8.5. Syringaldehyde

Analyte	Syringaldehyde					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	16	15	16	16	16	16
No of results accepted (laboratories)	13	13	13	12	14	13

▼**M2**

Analyte	Syringaldehyde					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
Mean value (mg/l)	1,0	0,2	4,8	3,2	10,5	9,7
Repeatability standard deviation, s_r (mg/l)	0,03	0,02	0,04	0,08	0,10	0,09
Repeatability relative standard deviation, RSD_r (%)	2,6	8,1	0,8	2,6	0,9	0,9
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,1	0,1	0,1	0,2	0,3	0,3
Reproducibility standard deviation, s_R (mg/l)	0,08	0,07	0,23	0,19	0,39	0,43
Reproducibility relative standard deviation, RSD_R (%)	8	33	5	6	4	4
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,2	0,2	0,7	0,5	1,1	1,2

8.6. Coniferaldehyde

Analyte	Coniferaldehyde					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	13	12	13	12	13	13
No of results accepted (laboratories)	12	12	13	12	13	13
Mean value (mg/l)	0,2	0,2	0,6	0,8	4,6	1,3
Repeatability standard deviation, s_r (mg/l)	0,02	0,02	0,03	0,03	0,09	0,06
Repeatability relative standard deviation, RSD_r (%)	9,2	9,8	4,6	4,3	1,9	4,5
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,04	0,04	0,07	0,09	0,24	0,16
Reproducibility standard deviation, s_R (mg/l)	0,04	0,04	0,11	0,18	0,38	0,25
Reproducibility relative standard deviation, RSD_R (%)	23	27	21	23	8	19
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,1	0,1	0,3	0,5	1,1	0,7

8.7. Sinapaldehyde

Analyte	Sinapaldehyde					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	14	14	14	14	15	14
No of results accepted (laboratories)	14	13	12	13	13	12

▼M2

Analyte	Sinapaldehyde					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
Mean value (mg/l)	0,3	0,2	0,2	1,6	8,3	0,3
Repeatability standard deviation, s_r (mg/l)	0,02	0,01	0,02	0,06	0,14	0,03
Repeatability relative standard deviation, RSD_r (%)	7,5	4,6	11,2	3,7	1,6	11,4
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,06	0,03	0,06	0,17	0,38	0,08
Reproducibility standard deviation, s_R (mg/l)	0,09	0,05	0,08	0,20	0,81	0,18
Reproducibility relative standard deviation, RSD_R (%)	31	27	46	13	10	73
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,2	0,2	0,2	0,6	2,3	0,5

8.8. Gallic acid

Analyte	Gallic acid					
Sample	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	16	15	16	16	16	16
No of results accepted (laboratories)	15	14	16	16	16	16
Mean value (mg/l)	1,2	0,4	2,0	6,1	7,3	21,8
Repeatability standard deviation, s_r (mg/l)	0,07	0,04	0,06	0,18	0,18	0,60
Repeatability relative standard deviation, RSD_r (%)	6,1	8,1	2,9	3,0	2,4	2,8
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,2	0,1	0,2	0,5	0,5	1,7
Reproducibility standard deviation, s_R (mg/l)	0,43	0,20	0,62	3,3	2,2	7,7
Reproducibility relative standard deviation, RSD_R (%)	36	47	31	53	30	35
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	1,2	0,6	1,7	9,1	6,2	21,7

8.9. Ellagic acid

Analyte	Ellagic acid					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	7	7	7	7	7	7
No of results accepted (laboratories)	7	7	7	7	7	6

▼M2

Analyte	Ellagic acid					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
Mean value (mg/l)	3,2	1,0	9,5	13	13	36
Repeatability standard deviation, s_r (mg/l)	0,20	0,16	0,30	0,41	0,95	0,34
Repeatability relative standard deviation, RSD_r (%)	6,3	16	3,2	3,2	7,4	1,0
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,6	0,4	0,9	1,1	2,7	1,0
Reproducibility standard deviation, s_R (mg/l)	1,41	0,42	4,0	5,0	4,9	14
Reproducibility relative standard deviation, RSD_R (%)	44	43	42	39	39	40
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	4,0	1,2	11	14	14	40

8.10. Vanillic acid

Analyte	Vanillic acid					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	15	15	15	15	15	15
No of results accepted (laboratories)	12	11	14	14	15	14
Mean value (mg/l)	0,2	0,2	1,5	0,8	2,4	2,7
Repeatability standard deviation, s_r (mg/l)	0,03	0,04	0,03	0,10	0,13	0,21
Repeatability relative standard deviation, RSD_r (%)	14,2	16,5	2,3	12,6	5,3	7,7
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,1	0,1	0,1	0,3	0,4	0,6
Reproducibility standard deviation, s_R (mg/l)	0,06	0,05	0,51	0,2	1,22	0,70
Reproducibility relative standard deviation, RSD_R (%)	28	20	35	31	51	26
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,2	0,1	1,4	0,7	3,4	2,0

8.11. Syringic acid

Analyte	Syringic acid					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	16	15	16	16	16	16
No of results accepted (laboratories)	16	15	15	15	16	15

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Analyte	Syringic acid					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
Mean value (mg/l)	0,4	0,2	2,5	1,4	3,4	4,8
Repeatability standard deviation, s_r (mg/l)	0,03	0,02	0,06	0,13	0,08	0,11
Repeatability relative standard deviation, RSD_r (%)	6,7	12,6	2,3	9,0	2,3	2,3
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,1	0,1	0,2	0,4	0,2	0,3
Reproducibility standard deviation, s_R (mg/l)	0,08	0,05	0,29	0,26	0,43	0,67
Reproducibility relative standard deviation, RSD_R (%)	19	29	11	18	13	14
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,2	0,1	0,8	0,7	1,2	1,9

8.12. Scopoletin

Analyte	Scopoletin					
Samples	Whisky	Brandy	Rum	Cognac 1	Bourbon	Cognac 2
No of laboratories participating	10	10	10	10	10	10
No of results accepted (laboratories)	9	8	9	8	8	8
Mean value (mg/l)	0,09	0,04	0,11	0,04	0,65	0,15
Repeatability standard deviation, s_r (mg/l)	0,0024	0,0008	0,0018	0,0014	0,0054	0,0040
Repeatability relative standard deviation, RSD_r (%)	2,6	2,2	1,6	3,3	0,8	2,7
Repeatability limit, r (mg/l) ($r = 2,8 \times s_r$)	0,007	0,002	0,005	0,004	0,015	0,011
Reproducibility standard deviation, s_R (mg/l)	0,01	0,01	0,03	0,01	0,09	0,02
Reproducibility relative standard deviation, RSD_R (%)	15	16	23	17	15	15
Reproducibility limit, R (g/l) ($R = 2,8 \times s_R$)	0,04	0,02	0,07	0,02	0,26	0,06

(1) ‘Protocol for the design, conduct and interpretation of method-performance studies’, Horwitz, W. (1995) *Pure and Applied Chemistry*, 67, 332-343.

(2) Horwitz, W. (1982) *Analytical Chemistry*, 54, 67A-76A.

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XI. DETERMINATION OF ^{14}C CONTENT IN ETHANOL

1. Introduction

Determination of the ^{14}C content in ethanol permits a distinction to be made between alcohol from fossil fuels (synthesis alcohol) and alcohol from recent raw materials (fermentation alcohol).

2. Definition

The ^{14}C content of ethanol shall be considered as the ^{14}C content determined using the method described here or the method described in standard EN 16640 Method C.

The natural ^{14}C content in the atmosphere (the reference value), which is absorbed by living vegetation by assimilation, is not a constant value. The reference value is therefore determined on ethanol from raw materials of the most recent vegetation period. This annual reference value is determined according to standard EN 16640. However, another reference value can be accepted where it is certified by an accredited body.

3. Principle

The ^{14}C content of samples containing alcohol with at least 85 % mass ethanol is determined directly by liquid scintillation count.

4. Reagents

4.1. Toluene scintillator

5,0 g 2,5-diphenyloxazole (PPO)

0,5 g p-bis-[4-methyl-5-phenyloxazolyl(2)]-benzene (dimethyl-POPOP) in 1 litre analytical grade toluene.

Commercial, ready-to-use toluene scintillators of this composition may also be used.

4.2. ^{14}C standard

n-Hexadecane ^{14}C with an activity of about 1×10^6 dpm/g (approximately $1{,}67 \times 10^6$ cBq/g) and a guaranteed accuracy of determined activity of ± 2 % rel.

4.3. ^{14}C-free ethanol

Synthesis alcohol from raw materials of fossil origin with at least 85 % mass ethanol, to determine the background.

4.4. Alcohol from recent raw materials of the most recent vegetation period with at least 85 % mass ethanol as reference material.

5. Apparatus

5.1. Multi-channel liquid scintillation spectrometer with processor and automatic external standardisation and display of the external standard/channel ratio (usual design: three meter channels and two external standard channels).

5.2. Low-potassium counter tubes suitable for the spectrometer, with dark screw-tops containing a polyethylene insert.

5.3. Volumetric pipettes, 10 ml.

5.4. Automatic dosing device 10 ml.

5.5. 250 ml round-bottom flask with ground-glass stopper.

5.6. Alcohol distillation apparatus with heating mantle, e.g. type Micko.

5.7. Microliter syringe 50 µl.

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5.8. Pycnometer funnel, pycnometers, 25 ml and 50 ml. As an alternative, equivalent equipment, such as electronic densimetry, should be allowed.

5.9. Thermostat with a temperature stability of ± 0,01 °C.

6. **Procedure**

6.1. Adjusting the equipment

The equipment shall be adjusted according to the manufacturer's instructions. Measuring conditions are optimal when the value E_2/B, the quality index, is at its maximum.

E = efficiency

B = background

Only two meter channels are optimised. The third is left fully open for control purposes.

6.2. Selection of counter tubes

A larger number of counter tubes than will later be needed are each filled with 10 ml of ^{14}C-free synthesis ethanol and 10 ml toluene scintillator. Each is measured for at least 4 cycles × 100 minutes. Tubes whose backgrounds vary by more than ± 1 % rel. from the mean are discarded. Only tubes new from the factory and from the same batch may be used.

6.3. Determination of the external standard/channel ratio (ESCR).

During the process of setting the channels (point 6.1) the ESCR is determined using the appropriate computer program when the efficiency is determined. The external standard used is ^{137}caesium, which is already built-in by the manufacturer.

6.4. Preparation of sample

Samples having an ethanol content of at least 85 % mass and free from impurities, which absorb at wavelengths below 450 nm may be measured. The low residue of esters and aldehydes has no disruptive effect. The alcohol content of the sample is previously determined with an approximation of 0,1 %.

7. **Measurement of samples using external standard**

7.1. The low absorbance samples as described in point 6.4 with an ESCR value of approximately 1,8 may be measured through the ESCR, which provides a measure of the efficiency ratio.

7.2. Measurement

10 ml each of the samples prepared according to point 6.4 is pipettes into a selected counter tube checked for background and 10 ml of toluene scintillator is added via an automatic dosing device. The samples in the tubes are homogenised by suitable rotary movements; the liquid shall not be allowed to wet the polyethylene insert in the screw-top. A tube containing ^{14}C-free fossil ethanol is prepared in the same way to measure the background. To check the relevant annual ^{14}C value a duplicate of recent ethanol from the latest vegetation period is prepared, a tube being mixed with internal standard, see point 8.

The control and background samples are placed at the beginning of the measurement series, which shall contain no more than 10 samples for analysis. Total measuring time per sample is at least 2 × 100 minutes, with the individual samples being measured in part stages of 100 minutes so that any equipment drift or other defect can be detected. (One cycle therefore corresponds to a measuring interval of 100 minutes per sample).

Background and control samples shall be freshly prepared every four weeks.

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In the case of slightly extinguished samples (ESCR circa 1,8) the efficiency is only negligibly affected by the change in this value. If the change is within ± 5 % rel. the same efficiency can be expected. For more greatly extinguished samples, such as denatured alcohols, the efficiency may be established via the extinction correction graph. If an appropriate computer program is not available the internal standard shall be used, and this gives an unambiguous result.

8. **Measuring samples using internal standard hexadecane^{14}C**

8.1. Procedure

Control and background samples (recent and fossil ethanol) and the unknown material are each measured as duplicates. One sample of the duplicate is prepared in a non-selected tube and an accurately dosed quantity (30 µl) of hexadecane^{14}C is added as internal standard (added activity around 26 269 dpm/gC approximately 43 782 cBq/gC). For the sample preparation and measuring time of the other samples see point 7.2, but the measuring time for the samples with the internal standard may be reduced to about five minutes by presetting at 10^5 pulses. One duplicate each of background and control samples is used per measuring series; these are placed at the beginning of the measuring series.

8.2. Handling the internal standard and counter tubes

To prevent contamination when measuring with the internal standard these shall be stored and handled well away from the area where the samples for analysis are prepared and measured. After measurement the tubes checked for background may be re-used. The screw-tops and tubes containing the internal standard shall be disposed of.

9. **Expression of the results**

9.1. The unit of activity of a radio-active substance is the becquerel; 1 Bq = 1 decay/sec.

Indication of specific radio-activity is expressed as becquerels relative to one gram carbon = Bq/gC.

To obtain more practical results, these shall be expressed in centi-bequerels = cBq/gC.

The descriptions and formulae used in the literature, based on dpm, may also be used. To obtain corresponding figures in cBq merely multiply the dpm figure by 100/60.

9.2. Expression of results with external standard

$$\text{cBq/g C} = \frac{(\text{cpm}_{\text{pr}} - \text{cpm}_{\text{NE}}) \cdot 1{,}918 \cdot 100}{\text{V} \cdot \text{F} \cdot \text{Z} \cdot 60}$$

9.3. Expression of results with internal standard

$$\text{cBq/g C} = \frac{(\text{cpm}_{\text{pr}} - \text{cpm}_{\text{NE}}) \cdot \text{dpm}_{\text{IS}} \cdot 1{,}918 \cdot 100}{(\text{cpm}_{\text{IS}} - \text{cpm}_{\text{pr}}) \cdot \text{V} \cdot \text{F} \cdot 60}$$

9.4. Abbreviations

cpm_{pr} = the mean sample count rate over the total measuring time.

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cpm_{NE} = the mean background pulse rate calculated in the same way.

cpm_{IS} = count rate of samples, with an internal standard.

dpm_{IS} = the quantity of internal standard added (calibration radioactivity dpm).

V = the volume of the samples used in ml.

F = the content in grammes pure alcohol per ml corresponding to its concentration.

Z = the efficiency corresponding to the ESCR value.

1,918 = the number of grammes alcohol per gramme carbon.

10. **Reliability of the method**

10.1. Repeatability (r)

r = 0,632 cBq/g C; $S_{(r)}$ = ± 0,223 cBq/g C

10.2. Reproducibility (R)

R = 0,821 cBq/g C; $S_{(R)}$ = ± 0,290 cBq/g C.