COMMISSION REGULATION (EC) No 2091/2002

of 26 November 2002

amending Regulation (EC) No 2870/2000 laying down Community reference methods for the analysis of spirits drinks

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Regulation (EEC) No 1576/89 of 29 May 1989 laying down general rules on the definition, description and presentation of spirit drinks [1], as amended by the Act of Accession of Austria, Finland and Sweden, and in particular Article 4(8) thereof,

Whereas:

(1) Commission Regulation (EC) No 2870/2000 of 19 December 2000 laying down Community reference methods for the analysis of spirits drinks [2] describes these methods in an Annex.

(2) Four methods of analysis for the determination of trans-anethole in aniseed-flavoured spirit drinks, glycyrrhizic acid and chalcones in pastis and egg yolk in egg liqueur and liqueur with egg have been validated according to internationally recognised procedures as part of a research project supported by the Commission.

(3) These four methods may be recognised as Community reference methods and must be added to the Annex to Regulation (EC) No 2870/2000.

(4) The measures provided for in this Regulation are in accordance with the opinion of the Implementation Committee for Spirit Drinks,

HAS ADOPTED THIS REGULATION:

Article 1

The Annex to Regulation (EC) No 2870/2000 is hereby amended as follows:

1. In the summary of the Annex, the term '(p.m.)' under points V, VI, VII and IX shall be deleted.

2. Chapters V, VI, VII and IX in the Annex to this Regulation shall be added after Chapter III.

Article 2

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

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

Done at Brussels, 26 November 2002.

For the Commission
Franz FISCHLER
Member of the Commission

[1] OJ L 160, 12.6.1989, p. 1.
[2] OJ L 333, 29.12.2000, p. 20.

ANNEX

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.

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:

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.

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
$\text{Area or height}_{is}$	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.

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.

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

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.

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).

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).

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
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

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.

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,

— 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).

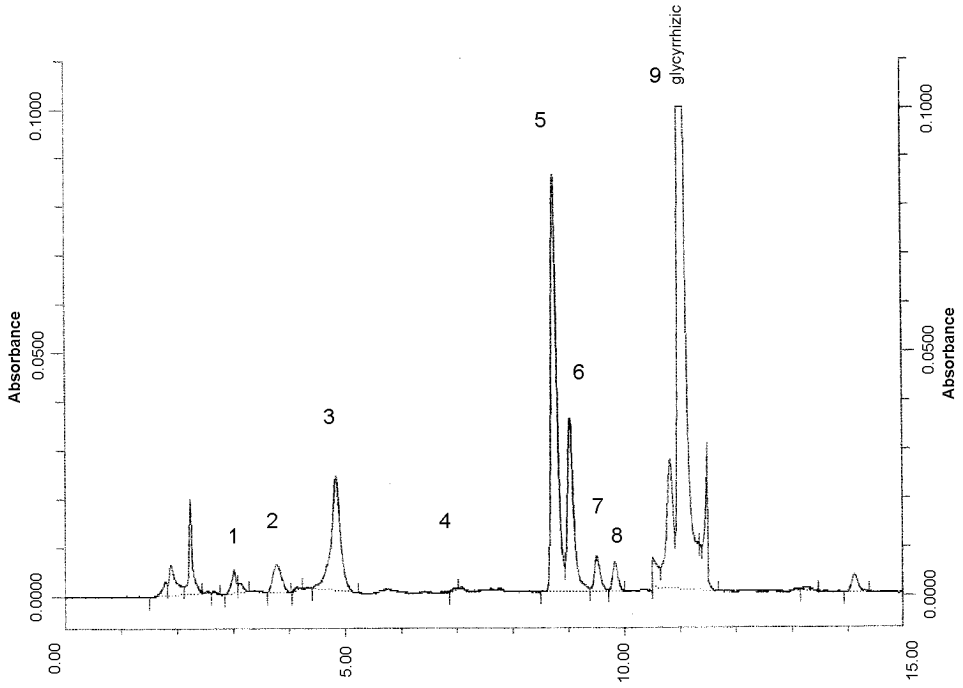
7.3.2. Inject 20 µl of the sample (7.1) (aniseed-flavoured spirit sample). Perform the analysis using the chromatography conditions described above (6.5).

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.



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

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.

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.

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).

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

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