Method OIV-MA-BS-15 : R2009

Type II method

Anethole. Gas chromatography determination of trans-anethole in spirit drinks of viti-vinicultural origin

(OIV/OENO 379/2009)

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 (ca. 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 (15-35°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 transanethole (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 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 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

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: 1µL, 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 for each injection plot a graph of their mother solution concentration in g/L versus the ratio R for each.

R = trans-anethole peak area divided by the estragole peak 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 5 unknowns (8.2); insert a linearity (quality control) sample after every 5 unknown samples, to ensure analytical stability.

9. Calculation of response factor

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

9.1. Response factor (RF_i) calculation

The response factor is calculated as follows

$$RF_i = (C_i/area_i) \times (area_{is}/C_{is})$$

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 of the trans-anethole peak
- $area_{is}$ is the area of the internal standard peak
- RF_i is calculated from the 5 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:

$$C_i = C_{is} \times (area_i/area_{is}) \times RF_i$$

where:

- C_i is the unknown trans-anethole concentration
- C_{is} is the concentration of internal standard in the unknown (4.5.2)
- $area_i$ is the area of the trans-anethole peak
- $area_{is}$ is the area 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 5 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

$$C_i = (m_{is}/V) \times (area_i/area_{is}) \times RF_i$$

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 on a variety of spirit drinks 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 outlirs (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 sample
F, single sample
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
Reproducibility 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

14. Bibliography

1. Commission Regulation (EC) N° 2091/2002 of 26 November 2002 amending Regulation (EC) No 2870/2000 laying down Community reference methods for the analysis of spirits drinks, *OJEC of 27 November 2002, L322/11*
2. P. Brereton, S. Hasnip, A. Bertrand, R. Wittkowski, C. Guillou, Analytical methods

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