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Vinegar — Isotopic analysis of acetic acid and water

Part 1: ^{2}H-NMR analysis of acetic acid

National foreword

This British Standard is the UK implementation of EN 16466-1:2013.

The UK participation in its preparation was entrusted to Technical Committee AW/-/2, Food Technical Committee Chairmen.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Foreword

This document (EN 16466-1:2013) has been based on an international collaborative study of the methods published in Analytica Chimica Acta 649 (2009) 98-105, and organised under the auspices of the Permanent International Vinegar Committee (CPIV, Brussels).

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2013, and conflicting national standards shall be withdrawn at the latest by July 2013.

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The European standard, *Vinegar — Isotopic analysis of acetic acid and water*, consists of the following parts:

— *Part 1: ^{2}H-NMR analysis of acetic acid*;

— *Part 2: ^{13}C-IRMS analysis of acetic acid*;

— *Part 3: ^{18}O-IRMS analysis of water*.

According to the CEN/CENELEC Internal Regulations, the national standards organisations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

Vinegar is defined in EN 13188 as the acetic acid solution resulting from a double fermentation:

a) transformation of sugars to ethanol and

b) transformation of ethanol to acetic acid.

Conversely, EN 13189 defines acetic acid as "Product made from materials of non-agricultural origin".

Wine vinegar is defined by the European Regulations 479/2008 and 491/2009 as the product obtained exclusively from the acetous fermentation of wine, which is in turn defined as the product exclusively obtained from the alcoholic fermentation of fresh grapes, whether crushed or not, or of grape must.

In all types of vinegar, both the ethanol and the acetic acid should be obtained by a biotechnological process, and the use of acetic acids obtained from either petroleum derivatives or the pyrolysis of wood is not permitted according to the above definitions.

The isotopic analysis of acetic acid extracted from vinegar by ^{2}H-SNIF-NMR and ^{13}C-IRMS enables the distinction of grape origin from other sources, such as beet, cane, malt, apple and synthesis [1].

1 Scope

This European Standard specifies an isotopic method to control the authenticity of vinegar. This method is applicable on acetic acid of vinegar (from wine, cider, agricultural alcohol, etc.) in order to characterise the botanical origin of acetic acid and to detect adulterations of vinegar using synthetic acetic acid or acetic acid from a non-allowed origin (together with the method described in EN 16466-2).

The isotopic analysis of the extracted acetic acid by ^{2}H-NMR is based on a similar method already normalised for wine analysis [2].

This European Standard is not applicable to complex matrices made with vinegar as an ingredient, such as balsamic vinegar.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

Not applicable

3 Principle

The acetic acid from vinegar is first extracted with diethyl ether (or alternatively another solvent with similar properties such as tert-butyl methyl ether), using a liquid-liquid extractor, during at least 5 h. The solvent is then eliminated by distillation. The water content of the residue can be determined by the Karl Fischer method, or alternatively the acetic acid content may be determined by ^{1}H-NMR [3, 4]. The presence of organic impurities in the residue shall be checked e.g. on the basis of ^{1}H-NMR analysis or by GC analysis. The isotopic ratio of hydrogen atoms at the methyl site of acetic acid, $(D/H)_{CH3}$, is then determined by Nuclear Magnetic Resonance analysis of the Deuterium in the acetic acid extracted from the vinegar.

In case a correction is applied to the $(D/H)_{CH3}$ result to correct for organic impurities, this should be stated in the analytical report.

4 Reagents

All reagents and consumables used shall meet stated requirements of the used method/apparatus (as specified by the manufacturer). However, all reagents and consumables can be replaced by items with similar performance.

4.1 Diethyl ether

For analysis.

4.2 Standard N,N-tetramethylurea (TMU)

Standard TMU with a calibrated isotope ratio D/H.

4.3 Hexafluorobenzene (C_6F_6)

Used as field-frequency stabilisation substance (lock).

5 Apparatus

All materials listed below are commercially available and used in food control laboratories.

5.1 For the extraction of acetic acid from vinegar

5.1.1 Liquid-liquid extractor of 400 ml or 800 ml.

5.1.2 Spinning band or Vigreux distillation column.

5.1.3 Round bottom flask of 500 ml.

5.1.4 Erlenmeyer of 250 ml.

5.1.5 Condenser.

5.1.6 Heater.

5.2 For ^{2}H-SNIF-NMR determination of acetic acid from vinegar

5.2.1 Analytical balance, precision 0,1 mg.

5.2.2 Filter 0,45 µm.

5.2.3 NMR spectrometer fitted with a specific "deuterium" probe tuned to a frequency *v*o, characteristic of channel Bo (e.g. Bo = 7,05 T, *v*o = 46,05 MHz and for Bo = 9,4 T, *v*o = 61,4 MHz) having a decoupling channel (B2) and a field-frequency stabilisation channel (lock) at the fluorine frequency. The resolution measured on the spectrum, transformed without exponential multiplication (i.e. LB = 0) and expressed by the width at the half-height of the methyl signals of acetic acid and the methyl signal of TMU shall be less than 0,5 Hz. The sensitivity (signal-to-noise ratio), measured with an exponential multiplying factor LB equal to 2 shall be greater than or equal to 150 for the methyl signal of acetic acid containing less than 25 % of water. For example, using a NMR spectrometer of field Bo = 7,05 T, 400 scans are necessary to reach this value.

5.2.4 Automatic sample changer (optional).

5.2.5 Data-processing software enabling lorentzian integration.

5.2.6 10 mm sample tubes of sufficient quality for NMR spectrometer 400 MHz.

5.2.7 Fume hood.

6 Procedure

6.1 Extraction of acetic acid from vinegar

6.1.1 Liquid-liquid extraction

Put 125 ml of diethyl ether into a 250 ml round bottom flask. Use a 400 ml or a 800 ml liquid-liquid extractor, depending on the acetic acid content of the vinegar (at least 6 ml of pure acetic acid shall be recovered at the end of the extraction).

Pour the vinegar into the extractor and complete with diethyl ether. Adapt the round bottom flask, open the water for the condenser and switch the heater on. The extraction shall last at least 5 h.

Then, after this time, separate the aqueous and the organic solution. Recover the organic solution from the extractor and add it to the extract in the round bottom flask.

6.1.2 Purification of the extract

The round bottom flask containing the acetic acid in solution in diethyl ether is distilled on spinning band or Vigreux column.

An appropriate 250 ml Erlenmeyer is used to collect the distillate.

Open the water for the condenser and switch the heater on. The heating shall be weak during the distillation of the solvent (boiling point of diethyl ether: 34 °C). When the main part of the solvent has been distilled (no more vapours at the head of the column), increase the heating.

The distillation is completed when the temperature at the top of the column is at least 90° (pure acetic acid distils at 116 °C – 117 °C).

6.1.3 Determination of the residual water content

First, the traces of solvent in the acetic acid are removed by blowing dry N_2 on the cold residue for 10 min.

The water content is determined by the Karl Fischer method. In case more than 25 % (w/w) residual water is found, the extraction should be performed again.

6.2 ^{2}H-SNIF-NMR determination of acetic acid from vinegar

6.2.1 NMR preparation

Weigh approximately 3,25 g of acetic acid (solution obtained from the extraction) to the nearest 0,1 mg into a previously weighed bottle. Add approximately 1,1 g of N,N-tetramethylurea (TMU) as internal standard to the nearest 0,1 mg. Add 150 µl of hexafluorobenzene (C_6F_6) as lock substance. Homogenise by shaking.

The samples should be filtered on 0,45 µm syringe filters while transferring into 10 mm NMR tube. Cap on the tube tightly to avoid evaporation during measurement.

CAUTION — It is strongly recommended to perform the NMR tube preparation under a fume hood, wearing safety glasses and gloves.

6.2.2 Acquisition of ^{2}H-SNIF-NMR spectra

Spectrometer shall be checked for sensitivity and resolution according to specifications given above (5.2).

Place a sample of acetic acid prepared as in 6.1 in a 10 mm tube and introduce it into the probe.

Typical conditions for obtaining ^{2}H-SNIF-NMR spectra are as follows:

- a constant probe temperature (e.g. 303 K)
- acquisition time of at least 5,5 s for 1 200 Hz spectral width (16 Kb memory) (i.e. about 20 x 10^{-6} at 61,4 MHz or 27 x 10^{-6} at 46,1 MHz)
- 90° pulse
- adjustment of acquisition time: its value shall be of the same order as the dwell time
- parabolic detection: fix the offset O1 between the OD and CH2D reference signals for acetic acid
- determine the value of the decoupling offset O2 from the ^{1}H-NMR spectrum measured by the decoupling coil on the same tube. Good decoupling is obtained when O2 is located in the middle of the frequency interval existing between the CH3 and TMU groups. Use the wide band-decoupling mode.

For each spectrum, carry out a number of accumulations NS sufficient to obtain the signal-to-noise ratio given in 5.2 and repeat this set of NS accumulations NE = 5 times. The values of NS depend on the types of spectrometer and probe used.

6.2.3 Calculations and expression of the result

Appropriate software based on a complex least square curve fitting algorithm should be used to determine the signal area (phasing and baseline correction are sensitive parameters to be correctly adjusted).

Calculate for each spectrum the D/H_{CH3} (x 10^{-6}) as follows:

$$(D/H)CH3 = \frac{Pst}{Paa} x \frac{Maa}{Mst} x \frac{mst}{maa * purity} x \frac{Saa}{Sst} x (D/H)st$$

where

aa is the acetic acid;

st is the internal standard TMU;

P is the number of equivalent deuterium positions for the considered molecular site;

M is the molecular weight, in $g.mol^{-1}$;

m is the weighted mass in g, weight to the nearest 0,1 mg;

S is the NMR signal area, integrated by data processing software;

(D/H)st (x 10^{-6}) is the certified deuterium content of TMU provided by the supplier of the reference product.

Calculate average of 5 determinations and standard deviation.

Optional softwares enable such calculations to be carried out on line.

7 Precision

7.1 General

The values derived from the inter-laboratory study may not be applicable to concentration ranges and matrices other than those given in Annex A.

7.2 Repeatability

In the collaborative study organised in 2009 (see Annex A: Results of the collaborative study (2009)), the average repeatability limit ($r = 2,8 \times s_r$) of the D/H_{CH3} of acetic acid was 1,34 x 10^{-6}.

7.3 Reproducibility

In the collaborative study organized in 2009 (see Annex A: Results of the collaborative study (2009)), the average reproducibility limit ($R = 2,8 \times s_R$) of the D/H_{CH3} of acetic acid was 1,62 x 10^{-6}.

8 Test report

The test report shall contain at least the following:

a) all information necessary for the identification of the sample;

b) a reference to this European Standard or to the method used;

c) the results and the units in which the results have been expressed;

d) date and type of sampling procedure (if known);

e) date of receipt;

f) date of test;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional which might have affected the results.

Annex A
(informative)

Results of the collaborative study (2009)

The samples have been prepared using the materials listed in Table A.1, plus a mixture of samples B and C. All samples have been analysed as blind duplicates, so in total twelve samples have been sent to each participating laboratories. Following the IUPAC internal harmonised protocol for collaborative studies [5], this experimental design intends to have more than five "materials", i.e. different matrix / sample pairs.

Homogeneity tests have been performed by the coordinator before shipment of samples. The statistical tests performed according to [6] confirmed a sufficient homogeneity for the six sample pairs.

Statistical calculations have then been performed according to ISO 5725-4 [7] and the IUPAC protocol [6]. Outliers have been removed in the following way: a loop of Cochran tests for removal of laboratories with highest variance, single and pair value Grubbs tests for individual or paired individual outliers, then back to Cochran test, etc., keeping a proportion of outliers <2/9. Then the standard deviations of repeatability (s_r) and of reproducibility (s_R) for each material have been computed from valid results pairs on the blind duplicates. A summary of these calculations are presented in Table A.2.

Table A.1 — Bulk materials used to prepare the samples

Material code	Description	Acetic acid content %
A	Vinegar from cider	4,9
B	Alcohol vinegar	7,8
C	Red wine vinegar n° 1	7,0
D	White wine vinegar	6,0
E	Red wine vinegar n°2	7,0

Table A.2 — Summary of statistics for 2H-SNIF-NMR results

Sample description	A	B	C	D	C+20%B	E
Number of valid results	5	5	5	5	5	5
Number of replicates	2	2	2	2	2	2
Mean $(D/H)_{CH3}$ (x 10^{-6})	95,2	91,8	101,2	100,6	99,3	98,9
s_r (x 10^{-6})	1,28	0,46	0,57	0,72	0,37	0,27
s_R (x 10^{-6})	0,91	0,36	0,66	0,81	0,66	0,40

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[9] EN 13189, *Acetic acid food grade — Product made from materials of non-agricultural origin — Definitions, requirements, marking*

[10] COUNCIL REGULATION (EC) No 479/2008 of 29 April 2008 on the common organisation of the market in wine, amending Regulations (EC) No 1493/1999, (EC) No 1782/2003, (EC) No 1290/2005, (EC) No 3/2008 and repealing Regulations (EEC) No 2392/86 and (EC) No 1493/1999, L 148/1 –61

[11] COUNCIL REGULATION (EC) No 491/2009 of 25 May 2009 amending Regulation (EC) No 1234/2007 establishing a common organisation of agricultural markets and on specific provisions for certain agricultural products (Single CMO Regulation), L154/1-56

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