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^{2}H NMR and ^{13}C-IRMS analyses of acetic acid from vinegar, ^{18}O-IRMS analysis of water in vinegar: International collaborative study report

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ABSTRACT

An international collaborative study of isotopic methods applied to control the authenticity of vinegar was organized in order to support the recognition of these procedures as official methods. The determination of the $^{2}H/^{1}H$ ratio of the methyl site of acetic acid by SNIF–NMR (site-specific natural isotopic fractionation–nuclear magnetic resonance) and the determination of the $^{13}C/^{12}C$ ratio, by IRMS (isotope ratio mass spectrometry) provide complementary information to characterize the botanical origin of acetic acid and to detect adulterations of vinegar using synthetic acetic acid. Both methods use the same initial steps to recover pure acetic acid from vinegar. In the case of wine vinegar, the determination of the $^{18}O/^{16}O$ ratio of water by IRMS allows to differentiate wine vinegar from vinegars made from dried grapes. The same set of vinegar samples was used to validate these three determinations.

The precision parameters of the method for measuring $\delta^{13}C$ (carbon isotopic deviation) were found to be similar to the values previously obtained for similar methods applied to wine ethanol or sugars extracted from fruit juices: the average repeatability (r) was 0.45 ‰, and the average reproducibility (R) was 0.91‰. As expected from previous in-house study of the uncertainties, the precision parameters of the method for measuring the $^{2}H/^{1}H$ ratio of the methyl site were found to be slightly higher than the values previously obtained for similar methods applied to wine ethanol or fermentation ethanol in fruit juices: the average repeatability was 1.34 ppm, and the average reproducibility was 1.62 ppm. This precision is still significantly smaller than the differences between various acetic acid sources ($\delta^{13}C$ and $\delta^{18}O$) and allows a satisfactory discrimination of vinegar types. The precision parameters of the method for measuring $\delta^{18}O$ were found to be similar to the values previously obtained for other methods applied to wine and fruit juices: the average repeatability was 0.15‰, and the average reproducibility was 0.59‰. The above values are proposed as repeatability and reproducibility limits in the current state of the art.

On the basis of this satisfactory inter-laboratory precision and on the accuracy demonstrated by a spiking experiment, the authors recommend the adoption of the three isotopic determinations included in this study as official methods for controlling the authenticity of vinegar.

1. Introduction

Vinegar is defined as the acetic acid solution resulting from a double fermentation: (i) transformation of sugars to ethanol and (ii) transformation of ethanol to acetic acid. Both the ethanol and acetic acid should be obtained by a biotechnological process. Synthetic acetic acids obtained from either petroleum derivatives or the pyrolysis of wood is potential adulterants. The use of cheaper materials is illegal, creates economic distortions in the market, and could potentially lead to health risks when harmful adulterants would be used. The control of the authenticity of vinegar is therefore a very important issue for consumer protection and fair trade. Hence the need for official methods to enforce the legal definition of vinegar.

Due to their unique ability to trace back the origin of chemically identical molecules such as water, and ethanol, isotopic methods have been implemented as regulations for the analytical control of wine in the European market [1–3]. The isotopic analysis of acetic acid extracted from vinegar by SNIF–NMR and IRMS enables the distinction of grape origin from other sources, such as beet, cane, malt, apple and synthesis [4,5] (see Fig. 1). In addition the $^{18}O/^{16}O$ ratio of water in wine vinegar also allows differentiating wine vinegar from vinegars made from raisins [6].

The method for acetic acid extraction from vinegar and ^{2}H NMR analysis had been originally described in the OIV (Organisation Internationale de la Vigne et du Vin) Resolution 71/2000 [7], and its application to canned fish in vinegar was described in an AFNOR (Agence Française de Normalisation) method [8]. However in both

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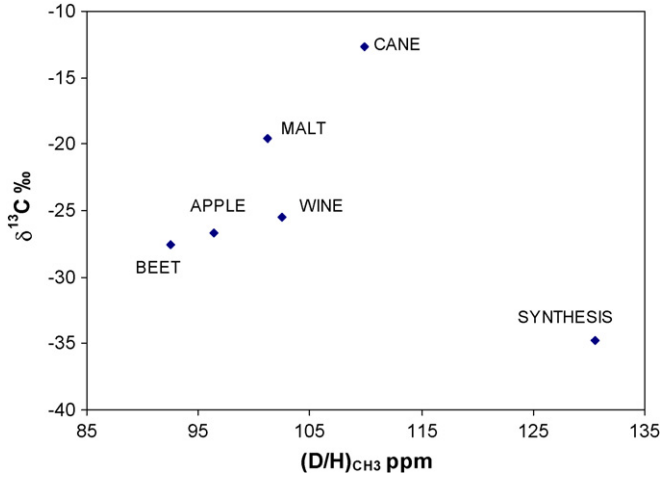

Fig. 1. $\delta^{13}C$ and $\left(\frac{D}{H}\right)_{CH_3}$ of acetic acid from various origin (average values from [5]).

cases the norms were only a description of the SNIF–NMR application (from the authors laboratory), and were not backed up by collaborative studies, as required nowadays by all official methods standardisation bodies. The protocol used in this collaborative study has been updated to take into account the current state of the art. Moreover, it includes the two complementary measurements described above. The ^{13}C measurement can be performed on the same extract, using a similar procedure for the IRMS measurement as described for ethanol in the EC regulation for wine [2]. The ^{18}O measurement can be performed using the so-called equilibration method also described in an EC regulation for wine [3].

A collaborative study was needed to further validate the application of these procedures to the vinegar matrix, taking into account the inter-laboratory variability. This study was coordinated by Eurofins laboratory in Nantes (referred to as "the coordinator" in the following text), and sponsored by the European vinegar producers syndicate (CPIV, Permanent International Vinegar Committee, Brussels).

The official control of wine within EU member states involves several official laboratories, equipped with SNIF–NMR and/or IRMS, which are now well-experienced in the applications of isotopic methods, usually under ISO 17025 accreditation. This network of laboratories is coordinated by the BEVABS (Bureau Européen des Vins, Alcools et Boissons Spiritueuses), laboratory of the Joint Research Center of the European Union in Ispra (Italy), and one of the commitments they have to follow is to take part in a Proficiency Testing Scheme called FIT-PTS (Food analysis using Isotopic Techniques – Proficiency Testing Scheme), run jointly by Eurofins Nantes laboratory and BEVABS.

Fourteen volunteer laboratories have been initially recruited by the coordinator to participate in the inter-comparison study of vinegar: they are listed in Table 1. The analyst in charge of the analyses at Eurofins laboratory was not one of the authors but person who had no more information about the composition of samples than the other participants.

2. Experimental

The raw materials used to prepare the collaborative study samples are described in Table 2. Because of the very large volumes needed for the study, commercial products have been bought and homogenized in the laboratory. The acetic acid contents determined by titration ranged from 5 to 7% (w/v).

Table 1
list of participants in the vinegar collaborative study.

Laboratory	Contacts
Bundesinstitut für Risikobewertung, Berlin – Germany	Carsten Fauhl-Hassek
Central Science Laboratory, York – UK	Adrian Charlton
Custom Technical Laboratory, Prag – Czech Republic	Jiri Mazac, Adam Méhes;
Chemical Institute of the Hungarian Customs and Finance Guard, Budapest – Hungary	Csilla Benedek, Rita Kapiller-Dezsőfi
Eurofins, Nantes–France	Melinda Retif
IASMA, San Michele all'Adige – Italy	Federica Camin
Joint Reasearch Center, Ispra – EU	Claude Guillou
Arbitral Agroalimentario Del MAPA, Madrid – Spain	Mercedes Rupérez
Landesuntersuchungsamt, Speyer – Germany	Armin Hermann
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Wurzburg – Germany	Norbert Christoph, Sandra Heil
Service Commun des Laboratoires, Bordeaux – France	Francois Guyon
Service Commun des Laboratoires, Montpellier – France	Sylvie Giraudon
Service Commun des Laboratoires, Paris – France	Catherine Lamoureux, Patrice Janvion
Unione Italiana Vini, Verona – Italy	Paolo Bendazzoli

Table 2
Bulk materials used to prepare the samples.

Material code	Description	Acetic acid content (%, m/v)
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

2.1. Samples

The 12 samples listed in Table 3 have been sent to participating laboratories by Eurofins. All samples have been analysed as blind duplicates. Samples 4 and 8 were prepared by mixing the two materials B and C which have the same acetic acid content (therefore the acetic acid and water proportions are equal to the proportions of the raw materials in the blend: 80% C and 20% B). Following the IUPAC internal harmonized protocol for collaborative studies [9], this experimental design intends to have more than five « materials », i.e. different matrix/sample pairs.

Samples have been homogenised and divided into sealed bottles of 500 mL. 50% of the prepared samples were stored to allow subsequent examination. Homogeneity tests have been performed by the coordinator before shipment of samples by analysing 10 aliquots in duplicates of each test material randomly selected and comparing

Table 3
description of samples sent to the participants.

Sample number	Material used	Duplicates
1	A	
2	D	
3	B	
4	C+20%B	
5	A	Blind duplicate of 1
6	C	
7	D	Blind duplicate of 2
8	C+20%B	Blind duplicate of 4
9	B	Blind duplicate of 3
10	C	Blind duplicate of 6
11	E	Blind duplicate of 12
12	E	Blind duplicate of 11

the corresponding values to the within laboratory reproducibility (^{13}C-IRMS measurement on the whole vinegar). The statistical tests performed according to [10] confirmed a sufficient homogeneity for the six sample pairs.

2.2. Method

The SOPs (standard operation procedures) coded A, B, C and D presented in the appendix of this article have been distributed to all participants. The main steps are described below.

The acetic acid from vinegar is first extracted with diethyl oxide, using a liquid–liquid extractor, during at least 5 h. It is then purified by distillation (to eliminate the diethyl oxide). The water content of the residue is determined by the Karl Fischer method.

The isotopic ratio of hydrogen atoms at the methyl site of acetic acid, $\left(\frac{D}{H}\right)_{CH_3}$, is determined by nuclear magnetic resonance analysis of the deuterium in the acetic acid extracted from the vinegar. The method is similar to the official one applied to ethanol for wines [1].

The $\left(\frac{D}{H}\right)_{CH_3}$ (in parts per million, ppm) is obtained as follows:

$$\left(\frac{D}{H}\right)CH_3 = \frac{P_{st}}{P_{aa}} \times \frac{M_{aa}}{M_{st}} \times \frac{m_{st}}{m_{aa}} \times \frac{S_{aa}}{S_{st}} \times \left(\frac{D}{H}\right)st, \quad \text{where}$$

aa: acetic acid,

st: internal standard TMU,

P: number of equivalent deuterium positions for the considered molecular site,

M: molecular weight,

m: weighted mass (corrected for moisture in the case of aa), S: NMR signal area, (D/H)st (ppm): certified deuterium content of TMU provided by the supplier.

The $^{13}C/^{12}C$ ratio of acetic acid from vinegar can be determined on CO_2 gas after complete combustion at high temperature. The method is similar to the official one applied to ethanol for wines [2]

The $^{13}C/^{12}C$ isotope ratio can be expressed by its deviation from a working reference. The isotopic deviation of carbon 13 ($\delta^{13}C$) is then calculated on a delta scale per thousand (‰) by comparing the results obtained for the sample to be measured with those for a working reference previously calibrated on the basis of the primary international reference (V-PDB). The $\delta^{13}C$ values are expressed in relation to the working reference as follows:

$$\delta^{13}C\ [‰] = \left[\frac{R_{sample}}{R standard} - 1\right] \times 1000$$

where R_{sample} and $R_{standard}$ are respectively the $^{13}C/^{12}C$ isotope ratios of the sample and of the standard calibrated against V-PDB.

The $^{18}O/^{16}O$ ratio of water from vinegar is determined on CO_2 gas after equilibration of reference CO_2 gas with raw vinegar. The method is similar to the official one applied to water in wines [3].

The $^{18}O/^{16}O$ isotope ratio can be expressed by its deviation from a working reference. The isotopic deviation of oxygen 18 ($\delta^{18}O$) is then calculated on a delta scale per thousand (‰) by comparing the results obtained for the sample to be measured with those for a working reference previously calibrated on the basis of the primary international reference (V.SMOW2). The $\delta^{18}O$ values are expressed in relation to the working reference as follows:

$$\delta^{18}O\ [‰] = \left[\frac{R_{sample}}{R standard} - 1\right] \times 1000$$

where R_{sample} and $R_{standard}$ are respectively the $^{18}O/^{16}O$ isotope ratios of the sample and of the standard calibrated against V.SMOW2.

3. Results and discussion

One extraction according to method A was performed for each sample, and laboratories were asked to report the final value for all parameters. The raw results received are presented in Tables 4–6. From the 14 potential laboratories who received the samples, three of them could not report any values by the deadline, mainly due to instrumental problems, and some of them reported only IRMS results.

For SNIF–NMR, nine laboratories have reported results. After examination of these data and comments from the participants, four laboratories (coded as lab 1, 2, 7 and 8) were eliminated as technical outliers because they did not follow the SOPs and present large deviations. The minimum of five laboratories supplying valid results tolerated in the IUPAC harmonised protocol for collaborative studies on complex methods [9] was still satisfied.

For ^{13}C-IRMS, 11 laboratories have reported results, and none of them was eliminated as technical outlier. However only partial results were received for samples 11 and 12.

For ^{18}O-IRMS, 8 laboratories have reported results, and none of them was eliminated as technical outlier. However only partial results were received for samples 11 and 12.

Statistical calculations have then been performed according to the ISO Standard 5725 [11] and the IUPAC protocol [9]. 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 Tables 7–9.

For carbon 13 and oxygen 18, the s_r and s_R values obtained on all samples are very similar and comparable to the values previously observed for wine and fruit juices using similar methods [2,3]. The values obtained for all materials are also comparable. Still sample E was removed for the calculation of an average value because there were fewer data points. For carbon 13 the average repeatability (r) was 0.45 ‰, and the average reproducibility (R) was 0.91‰. For oxygen 18 the average repeatability was 0.15‰, and the average reproducibility was 0.59‰

For SNIF–NMR, the r and R values observed on sample A was much higher than the values observed on the other samples, so the coordinators think there might have been a problem of stability for this sample, although the homogeneity was initially satisfactory. The ranges of r and R values obtained on all other samples are coherent, so the average repeatability of 1.34 ppm, and the average reproducibility of 1.62 ppm calculated from the five remaining materials are reasonable estimates of the method performance. As expected from the coordinator's own experience, both carbon 13 and deuterium values for acetic acid tend to have slightly larger fidelity values than those observed on ethanol from wine. This can be explained by the difficulty to remove 100% the diethyl oxide used for extraction and the potential risk of isotopic fractionation at this stage. Moreover for SNIF–NMR the use of surfaces instead of heights increases the uncertainty. However the observed reproducibility is still sufficiently small for a proper discrimination of acetic acid sources, as can be observed from the distance of the average values of the groups in Fig. 1 (see [5] for the ranges of variation of each group).

Finally the accuracy of the methods was evaluated from the results of a sample which was prepared from material C (red wine

Table 4
raw data received from the participants for SNIF–NMR analyses (*technical outliers).

Material	A		B		C		D		C+20%B		E	
Sample	1	5	3	9	6	10	2	7	4	8	11	12
	(D/H) of CH_3 position in acetic acid (ppm)											
Lab 1*	93.3	94.2	90.1	89.9	99.6	100.1	97.9	97.2	98.0	98.5	97.9	98.1
Lab 2*	93.4	92.8	91.2	91.9	99.0	99.6	98.7	98.3	97.9	98.1	100.7	101.0
Lab 3	95.9	94.5	91.3	91.9	99.9	100.9	99.3	100.7	97.8	98.7	98.4	98.2
Lab 4	96.0	94.3	91.6	92.0	101.5	102.0	100.9	100.2	99.5	99.4	99.6	98.9
Lab 5	94.6	95.8	91.6	92.3	100.6	101.4	100.4	99.9	100.0	99.9	98.7	99.0
Lab 6	95.7	95.0	91.3	92.2	100.8	101.7	102.3	100.8	99.7	99.0	98.8	98.8
Lab 7*	92.5	92.7	90.3	90.2	98.0	98.9	97.8	96.2	96.9	98.1	96.2	n.d.
Lab 8*	96.9	97.8	92.2	92.0	103.2	103.7	104.3	103.5	102.0	102.3	102.1	101.6
Lab 9	96.7	93.6	91.8	92.2	101.2	101.8	100.7	101.2	99.4	99.1	99.2	98.9

Table 5
raw data received from the participants for ^{13}C-IRMS analyses.

Material	A		B		C		D		C+20%B		E	
Sample	1	5	3	9	6	10	2	7	4	8	11	12
	$\delta^{13}C$ (‰)											
Lab 1	−29.35	−28.95	−29.55	−29.8	−26.64	−26.35	−27.37	−27.19	−26.89	−26.99	−26.95	−27.52
Lab 2	−29.1	−29.16	−29.73	−29.38	−26.57	−26.52	−27.45	−27.47	−27.38	−26.99	−27.28	−27.33
Lab 3	−30.13	−29.45	−30.04	−29.71	−27.06	−26.88	−28.11	−27.59	−27.73	−27.54	−28.1	−27.64
Lab 4	−28.75	−29.2	−29.25	−29.21	−26.38	−26.44	−27.3	−27.15	−27.05	−27.1	−26.51	−26.58
Lab 5	−28.94	−28.65	−29.01	−29.17	−26.53	−26.2	−27.35	−27.13	−26.82	−26.84	−26.96	−27.03
Lab 6	−29.6	−29.7	−30.1	−30	−27	−27	−28.1	−27.8	−27.5	−27.5	−26.6	−27.6
Lab 7	−28.71	−28.6	−29.32	−29.29	−26.44	−26.44	−27.63	−27.08	−26.84	−26.85	−27.08	
Lab 8	−29.4	−29.35	−29.5	−29.63	−26.92	−26.87	−27.99	−27.76	−27.56	−27.1	−27.33	−27.28
Lab 9	−29.29	−29.25	−29.7	−29.8	−26.93	−26.88	−27.64	−27.78	−27.34	−27.14	−27.72	
Lab 10	−29.3	−29.3	−30.3	−29.2	−26.7	−26.6	−28.5	−27.4	−27.2	−27.2	−28.04	
Lab 11	−28.6	−28.63	−29.17	−29.09	−26.67	−26.53	−27.2	−27.18	−26.83	−26.85		

Table 6
raw data received from the participants for ^{18}O-IRMS analyses.

Material	A		B		C		D		C+20%B		E	
Sample	1	5	3	9	6	10	2	7	4	8	11	12
	$\delta^{18}O$ (‰)											
Lab 2	−4.65	−4.64	−7	−6.99	−0.79	−0.76	−1.37	−1.33	−1.69	−1.72	−1.27	−1.27
Lab 3	−4.88	−4.83	−7.3	−7.38	−1.11	−1.13	−1.66	−1.63	−2.08	−2.09		
Lab 4	−4.2	−4.2	−6.7	−6.7	−0.5	−0.5	−1	−1	−1.5	−1.4	−1	−1.1
Lab 5	−4.5	−4.47	−7.03	−7.04	−0.83	−0.9	−1.4	−1.38	−1.87	−1.86	−1.37	−1.43
Lab 6	−4.3	−4.4	−6.7	−6.68	−0.8	−0.6	−1.2	−1.2	−1.7	−1.6	−1.2	−1.1
Lab 8	−4.78	−4.68	−7.17	−7.07	−0.96	−1.09	−1.57	−1.58	−1.96	−1.97	−1.28	−1.31
Lab 9	−4.52	−4.32	−6.96	−6.77	−0.9	−1.06	−1.46	−1.45	−1.87	−1.86	−1.3	
Lab 11	−4.55	−4.51	−6.89	−6.96	−0.85	−0.96	−1.37	−1.4	−1.69	−1.83		

Table 7
Summary of statistics for 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 (ppm)	95.2	91.8	101.2	100.6	99.3	98.9
s_r (ppm)	1.28	0.46	0.57	0.72	0.37	0.27
s_R (ppm)	0.91	0.36	0.66	0.81	0.66	0.40

Table 8
Summary of statistics for ^{13}C-IRMS results.

Sample description	A	B	C	D	C+20%B	E
Number of valid results	11	10	11	10	11	7
Number of replicates	2	2	2	2	2	2
Mean $\delta^{13}C$ (‰)	−29.16	−29.52	−26.66	−27.51	−27.15	−27.19
s_r (‰)	0.21	0.14	0.11	0.20	0.14	0.33
s_R (‰)	0.41	0.34	0.25	0.33	0.29	0.46

Table 9
Summary of statistics for ^{18}O-IRMS results.

Sample description	A	B	C	D	C+20%B	E
Number of valid results	8	8	8	8	8	5
Number of replicates	2	2	2	2	2	2
Mean $\delta^{18}O$ (‰)	−4.53	−6.96	−0.86	−1.38	−1.79	−1.23
s_r (‰)	0.06	0.06	0.08	0.02	0.05	0.05
s_R (‰)	0.22	0.22	0.21	0.20	0.20	0.14

Table 10
Recovery calculations based on isotopic values for the spiked sample (20% v/v of beet vinegar B in wine vinegar C).

Parameter	Fraction	Unit	Wine vinegar C	Alcohol vinegar B	Mixture C+20%B	Calculated addition (%)	Expected additon (%)
$\left(\frac{D}{H}\right)_{CH_3}$	Acetic acid	ppm	101.2	91.8	99.3	20	22
$\delta^{13}C$	Acetic acid	‰	−26.66	−29.52	−27.15	17	22
$\delta^{18}O$	Water	‰	−0.86	−6.96	−1.79	15	20

vinegar) blended with 20% (v/v) of material B (Alcohol vinegar). The results of this spiking experiment are displayed in Table 10. The average isotopic values of acetic acid confirmed that C is an authentic wine vinegar, while B is a typical beet vinegar. A satisfactory recovery was observed for the three isotopic parameters ("calculated addition" is based on mean values from all valid results; "expected addition" is the theoretical added amount of acetic acid or water respectively, taking into account the acetic acid contents of B and C). In this case the calculation based on $\left(\frac{D}{H}\right)_{CH_3}$ values is the most accurate, due to the higher difference between wine and beet for this parameter. But carbon 13 would become more efficient in the case of a C4 plant (cane, maize) source, as shown in Table 1, and therefore both measurements are complementary.

In the case of vinegar made from grape alcohol, the carbon and hydrogen isotopic content of acetic acid are similar to those observed in wine vinegar, because the botanical source is the same. But then the oxygen 18 content of water comes into play, fairly reflecting the origin of the water in vinegar, as illustrated in Table 9 (the O18 deviation of beet vinegar is significantly lower than the values observed in the wine vinegars). A vinegar made from dried grapes would show similar figures as those observed for beet vinegar in this study, reflecting the isotopic content of the tap water used in production [6]. This parameter therefore allows differentiating wine vinegar from all types of alcohol vinegars, including those made from dried grapes.

4. Conclusions

This collaborative study demonstrates the ability of laboratories experienced in food isotopic testing to generate comparable figures for the three determinations under investigation (deuterium, carbon 13 and oxygen 18). Therefore we recommend the adoption of the standard operating procedures described in this article as official methods for the control of the vinegar market.

Moreover the generated average reproducibility standard deviation will provide a target standard deviation to be used in future proficiency testing activities. Our commitment is to incorporate the isotopic analysis of vinegar in the next FIT-PTS schemes so that laboratories can evaluate their performance along time and more easily obtain the accreditation for these determinations.

Acknowledgements

The authors would like to thank all laboratories listed in Table 1 for their participation in the study.

The authors are also grateful to the CPIV (Permanent International Vinegar Committee, Brussels) who provided financial support for the organization of this study.

Appendix A. Standard Operating Procedures used in the collaborative study

A.1. Method of extraction of acetic acid from vinegar

A.1.1. Objective

Acetic acid must be extracted from vinegar and purified in order to be analysed by isotopic techniques. At least 6 mL of pure acetic acid must be recovered at the end of the extraction.

A.1.2. Principle

The acetic acid from vinegar is first extracted with diethyl oxide. It is then purified by distillation (with a manual « Cadiot » column). The water content of the residue is finally determined by a Karl Fischer method.

A.1.3. Reagents

- Diethyl oxide, analytical grade.

A.1.4. Laboratory equipment

- Liquid–liquid extractor
- Cadiot column
- Round bottom flask
- Condenser
- Heater.

A.1.5. Experimental determinations

A.1.5.1. Liquid–liquid extraction. Put 125 mL of diethyl oxide into a 250 mL round bottom flask. Use a 400 mL or 800 mL liquid–liquid extractor, depending on the acetic acid content of the vinegar (at least 6 mL of pure acetic acid must be recovered at the end of the extraction).

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

Then, after this time, separate the aqueous and the organic solution. Add the organic solution to the extract in the round bottom flask.

A.1.5.2. Purification of the extract. The round bottom flask containing the acetic acid in solution in diethyl oxide is distilled on a « Cadiot type» column (Fig. 2).

An appropriate 250 mL vial is used to collect the distillate.

Open the water for the condenser and switch the heater on. Be careful, the heating must be weak during the distillation

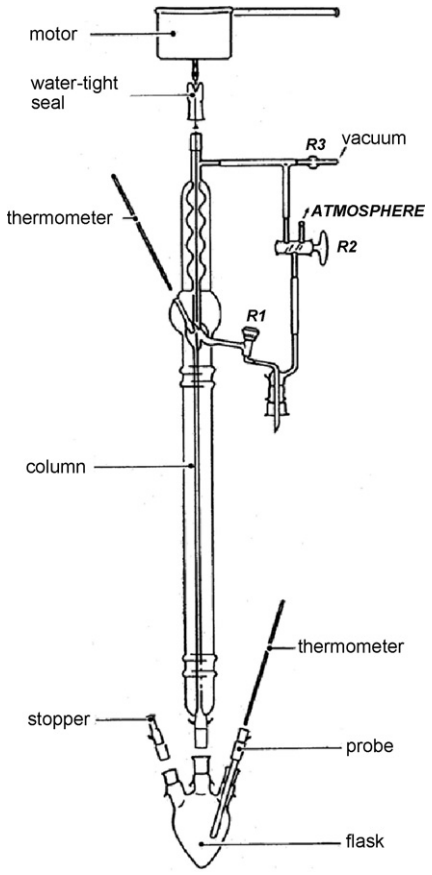

Fig. 2. scheme of the distillation device used in standard operating procedure A.

of diethyl oxide (boiling point: 34 °C), which must last at least 1.5 h.

When the main part of the diethyl oxide has been distilled (no more vapours at the head of the column), increase the heating.

The distillation is completed when the temperature is stable at about 98 °C (pure acetic acid distils at 116–117 °C).

A.1.5.3. Determination of the purity. First, the traces of diethyl oxide in the acetic acid are removed by blowing dry N_2 on the cold residue for 10 min.

Then the water content is determined with the Karl Fischer method (less than 25% (w/w) are allowed for the NMR measurement).

A.2. Method for SNIF–NMR determination of acetic acid from vinegar

Adapted from EC Regulation 2676/90 [1].

A.2.1. Objective

The deuterium contained in acetic acid is distributed in isotopomers I and II of the molecule:

(I) CH_2DCO_2H and (II) CH_3CO_2D

The second isotopomer is affected by water and atmospheric conditions whereas the first isotopomer is related to the botanical origin of vinegar.

$\left(\frac{D}{H}\right)_{CH_3}$ is the isotope ratio associated with molecule I.

Only the parameter $\left(\frac{D}{H}\right)_{CH_3}$ will be used for authenticity testing.

A.2.2. Principle

The parameter defined above $\left(\frac{D}{H}\right)_{CH_3}$ is determined by nuclear magnetic resonance of the deuterium in the acetic acid from the vinegar. The principle is the same as the one used for wines (EC 2676/90).

A.2.3. Reagents

- N,N-tetramethylurea (TMU); Sold as internal standard for SNIF–NMR (STA003k) by Institute of Reference Material and Measurements (IRMM), Geel, Belgium.
- Hexafluorobenzene, used as field-frequency stabilisation substance (lock).

A.2.4. Laboratory equipment

- Analytical balance, precision 0.1 mg
- Filter 0.45 μm
- NMR spectrometer fitted with a specific "deuterium" probe tuned to a frequency νo, characteristic of channel B_0 (e.g. $B_0 = 7.05$ T, $\nu_0 = 46.05$ MHz and for $B_0 = 9.4$ T, $\nu_0 = 61.4$ MHz) having a decoupling channel (B2) and a field-frequency stabilization 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 must be less than 0.5 Hz. The sensitivity (signal-to-noise ratio), measured with an exponential multiplying factor LB equal to 2 must be greater than or equal to 150 for the methyl signal of acetic acid containing less than 25% of water. For example, using a 11.4 T NMR spectrometer, 400 scans are necessary to reach this value.
- Automatic sample changer (optional)
- Data-processing software
- 10 mm sample tubes
- Fume hood.

A.2.5. Experimental determinations

A.2.5.1. 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 TMU as internal standard to the nearest 0.1 mg. Add 150 μl of 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.

A.2.5.2. Acquisition of ^2H NMR spectra. Spectrometer must be checked for sensitivity and resolution according to specifications given above.

Place a sample of acetic acid prepared as in 5.1 in a 10 mm tube and introduce it into the probe.

The conditions for obtaining NMR spectra are as follows:

- a constant probe temperature (e.g. 303 K)
- acquisition time of at least 5.5 s for 1200 Hz spectral width (16 Kb memory)
- (i.e. about 20 ppm at 61.4 MHz or 27 ppm at 46.1 MHz)
- 90° pulse
- adjustment of acquisition time: its value must be of the same order as the dwell time
- parabolic detection: fix the offset O1 between the OD and CH_2D reference signals for acetic acid
- determine the value of the decoupling offset O2 from the proton 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 CH_3 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 4 and repeat this set of NS accumulations NE = 5 times. The values of NS depend on the types of spectrometer and probe used.

A.2.6. 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) (For example: EUROSPEC software, Eurofins, Nantes, France).

Calculate for each spectrum the $\left(\frac{D}{H}\right)_{CH_3}$ (ppm) as follows:

$$\left(\frac{D}{H}\right) CH_3 = \frac{P_{st}}{P_{aa}} \times \frac{M_{aa}}{M_{st}} \times \frac{m_{st}}{m_{aa}} \times \frac{S_{aa}}{S_{st}} \times \left(\frac{D}{H}\right) st, \quad \text{where}$$

aa: acetic acid,

st: internal standard TMU,

P: number of equivalent deuterium positions for the considered molecular site,

M: molecular weight,

m: weighted mass,

S: NMR signal area,

(D/H)st (ppm): certified deuterium content of TMU provided by the supplier.

Calculate average of five determinations and standard deviation.

A.3. Method to determine of the isotopic ratio $^{13}C/^{12}C$ of acetic acid from vinegar

Adapted from EC regulation 440/2003 [2].

A.3.1. Objective

The $^{13}C/^{12}C$ ratio of acetic acid from vinegar can be determined on CO_2 gas after complete combustion at high temperature.

A.3.2. Principle

The isotopic ratio $^{13}C/^{12}C$ is determined by isotopic ratios mass spectrometry from ion currents *m*/*z* 45 ($^{13}C^{16}O_2$) and *m*/*z* 44 ($^{12}C^{16}O_2$) produced by carbon dioxide obtained after complete combustion in an elemental analyser. Corrections are made to delete the contribution of $^{12}C^{16}O^{17}O$ in current *m*/*z* 45 (Craig correction).

A.3.3. Reagents

- Carbon dioxide for analysis, used as secondary reference gas
- Helium for analysis
- Oxygen for analysis
- Oxidation reagent for the furnace of the combustion system
- Desiccant to eliminate water produced in combustion if necessary.
- International standards from International Agency of Atomic Energy (IAEA), Vienna, Austria: NBS-22, IAEA-CH-6, IAEA-CH-7 or USGS-40 or equivalent, or from Institute of Reference Material and Measurements (IRMM), Geel, Belgium: BCR-656, BCR-657, BCR-660
- Reference sample specific to the laboratory carefully standardized in relation to the reference sample of the IAEA.

A.3.4. Laboratory equipment

- Isotope Ratio Mass spectrometer with an internal repeatability of 0.05‰
- Triple collector for simultaneous recording of ions *m*/*z* 44, 45 and 46
- Dual Inlet or Conflo to introduce alternatively reference CO_2 gas and CO_2 produced by sample combustion
- Elemental Analyser to carry out the complete combustion of organic products into CO_2 gas and equipped with a water trap
- Tin capsules for liquide samples
- Tweezers for encapsulation
- Eppendorf pipette with plastic disposable tip.

A.3.5. Experimental determinations

Acetic acid must be extracted from vinegar following the method described above for SNIF–NMR.

Place the samples in capsules (the appropriate quantity of acetic acid must be calculated according to the quantity of carbon necessary given the sensitivity of the mass spectrometry apparatus). Each capsule must be completely sealed. At least 2 capsules must be prepared for every sample. Place the capsules in the appropriate place on the tray of the automatic sampler of the elemental analyser. Place systematically capsules containing working references at the beginning and at the end of the sample series, and insert regularly control samples.

Check the IRMS instrument and adjust it for optimal combustion: furnace temperature, helium and oxygen flows. Check the system for leaks. Adjust the IRMS to measure the ionic currents *m*/*z* = 44, 45 and 46. Check the accuracy of the system using known control samples before starting to measure the samples.

The samples placed on the auto sampler of the elemental analyser are introduced in turn. The CO_2 from each sample combustion is eluted towards the mass spectrometer which measures the ionic currents. The software records the ionic currents and calculates the δ value for each sample.

A.3.6. Calculation and expression of the results

The purpose of the method is to measure the $^{13}C/^{12}C$ ratio of acetic acid extracted from vinegar. The $^{13}C/^{12}C$ isotope ratio can be expressed by its deviation from a working reference. The isotopic deviation of carbon 13 ($\delta^{13}C$) is then calculated on a delta scale per thousand (‰) by comparing the results obtained for the sample to be measured with those for a working reference previously calibrated on the basis of the primary international reference (V-PDB). The $\delta^{13}C$ values are expressed in relation to the working reference as follows:

$$\delta^{13}C\,[‰] = \left[\frac{R_{sample}}{R standard} - 1\right] \times 1000$$

where R_{sample} and $R_{standard}$ are respectively the $^{13}C/^{12}C$ isotope ratios of the sample and of the standard calibrated against V-PDB.

Between two measurements of the standard working sample, the variation, and therefore the correction to be applied to the results obtained from the samples, may be assumed to be linear. The standard working sample must be measured at the beginning and at the end of all sample series. A correction can then be calculated for each sample using linear interpolation.

A.4. Method to determine the isotopic ratio $^{18}O/^{16}O$ of water in vinegar

Adapted from EC regulation 822/97 [3].

A.4.1. Objective

The $^{18}O/^{16}O$ ratio of water from vinegar can be determined on CO_2 gas after equilibration of reference CO_2 gas with raw vinegar.

A.4.2. Principle

The isotopic ratio $^{18}O/^{16}O$ is determined by isotopic ratios mass spectrometry from ion currents m/z 46 ($^{12}C^{16}O^{18}O$) and m/z 44 ($^{12}C^{16}O_2$) produced by carbon dioxide obtained after an exchange with the water in wine according to the reaction:

$$C^{16}O_2 + H_2{}^{18}O \leftrightarrow C^{16}O^{18}O + H_2{}^{16}O$$

Carbon dioxide in the gaseous phase is used for analysis.

A.4.3. Reagents

- Carbon dioxide for analysis
- International standards from international agency of atomic energy (IAEA), Vienna, Austria: V.SMOW2 (Vienna standard mean ocean water), GISP (Greenland ice sheet precipitation), SLAP 2 (standard light arctic precipitation)
- Reference water specific to the laboratory carefully standardized in relation to the reference sample of the IAEA.

A.4.4. Laboratory equipment

- Isotope Ratio Mass spectrometer with an internal repeatability of 0.05 ‰.
- Triple collector for simultaneous recording of ions m/z 44, 45 and 46 or, by default, a double collector for measuring ions m/z 44 and 46.
- Temperature controlled system (± 0.5 °C) to carry out the equilibration between CO_2 and the water content in wine.
- Vacuum pump able to reach an internal pressure of 0.13 Pa.
- Vials for samples having 15 mL volume and a capillary annex tube with an interior diameter of about 0.015 mm.
- Eppendorf pipette with plastic disposable tip.

A.4.5. Experimental determinations

A.4.5.1. Manual method.

- Introduction of the sample:
  Take the Eppendorf pipette at a fixed volume of 1.5 mL, attach a tip and pipette the liquid to be analyzed in a round flask. Then place silicon grease around the neck of the flask and attach the flask to the valve while verifying that it is tightly shut.Repeat the operation for each flask on the manifold while introducing the laboratory's reference water into one of the flasks.
- Degassing of the ramp:
  The two manifolds are cooled down with liquid nitrogen, then the whole system is purged to 0.1 mm Hg by opening the valves. Then the valves are shut off and the system is allowed to heat. The degassing cycle is repeated until there is no more pressure variation.
- Equilibration of the water and the CO_2:
  Cool the two manifolds to −70 °C (liquid nitrogen and alcohol mixture) to freeze the water and put it all under a vacuum. After stabilization of the vacuum, isolate the manifold by activating the valve and purge the CO_2 inlet system. Insert the gaseous CO_2 into the work manifold and, after having isolated it from the rest of the system, place the manifold in a thermostated bath at 25 °C (± 0.5 °C) for 12 h (overnight). To optimize the necessary time for equilibration, it is advised to prepare the samples at the end of the day and let the equilibrium establish itself during the night.
- Transfer of the CO_2 exchanged in the measuring cells:
  A sample holder which supports as many measuring cells as flasks containing exchanged CO_2 is attached to the empty line next to the work manifold. The empty cells are carefully purged and the exchanged gases contained in the flasks are transferred one after the other, into the measuring cells that have been cooled by liquid nitrogen. Then the measuring cells are allowed to come to room temperature.

A.4.5.2. Use of an automatic exchange apparatus. In order to carry out the equilibration, sample vials are filled with, either 2 mL of vinegar or 2 mL of water (laboratory work reference) and cooled down to −18 °C. The sample vials containing the frozen products are attached to the equilibration system and, after the system is placed under vacuum, carbon dioxide is introduced at a pressure of 800 hPa.

Equilibrium is reached at a temperature of 25 ± 0.5 °C after a minimum period of 5 h and with moderate agitation. Since the equilibration duration depends on the vial's geometry, the optimum duration should be determined first for the system used.

Carbon dioxide contained in the vials is then transferred into the introduction chamber of the mass spectrometer by a capillary tube and the measurement is carried out according to a specific protocol for each kind of equipment.

A.4.6. Calculation and expression of the results

The purpose of the method is to measure the $^{18}O/^{16}O$ ratio of water extracted from vinegar. The $^{18}O/^{16}O$ isotope ratio can be expressed by its deviation from a working reference. The isotopic deviation of oxygen 18 ($\delta^{18}O$) is then calculated on a delta scale per thousand (‰) by comparing the results obtained for the sample to be measured with those for a working reference previously calibrated on the basis of the primary international reference (V.SMOW2). The $\delta^{18}O$ values are expressed in relation to the working reference as follows:

$$\delta^{18}O\,[‰] = \left[\frac{R_{sample}}{R\text{standard}} - 1\right] \times 1000$$

where R_{sample} and $R_{standard}$ are respectively the $^{18}O/^{16}O$ isotope ratios of the sample and of the standard calibrated against V.SMOW2.

Between two measurements of the standard working sample, the variation, and therefore the correction to be applied to the results obtained from the samples, may be assumed to be linear. The standard working sample must be measured at the beginning and at the end of all sample series. A correction can then be calculated for each sample using linear interpolation.

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