Rapid Determination of the Stable Oxygen Isotope Ratio of Ethanol in Aqueous Samples

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Abstract: During the last years, it has been demonstrated that $\delta^{18}O$ isotope analysis of ethanol in juices and alcoholic beverages provides powerful information to assess their adulteration by addition of water, or to authenticate their geographical origin. However, it is difficult to accurately and conveniently quantify it due to the measurement principle and its water miscibility. To eliminate the impact of water on ethanol $\delta^{18}O$ analysis, a porous-polymer-bonded GC column was used to achieve a baseline separation of ethanol and water, then the water was vented by backflush function and the ethanol was converted and analyzed. This is the first manuscript presenting a systematic evaluation and characterization of a method for $\delta^{18}O$ isotope analysis of ethanol in aqueous samples by direct injection of the sample (diluted with acetone) into a GC-TC-IRMS system, with the consequent benefits of eliminating sample pre-treatment, minimum sample amounts required and minimum sample throughput. This method has the following advantages: only 70-200 μL of the sample was required for up to 300 possible injections, and high throughput was observed, caused by short analysis time (18 min for each run), the influences of oxygen-containing compounds on ethanol $\delta^{18}O$ analysis were eliminated by the use of a capillary-column bonded porous polymer to obtain a baseline separation prior to high-temperature conversion. Precision was determined to be less than 0.5‰ (1σ), and accuracy was validated by spiked samples and proficiency test samples.

Key words: ethanol; stable oxygen isotope ratio; aqueous samples; extraction
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快速测定含水溶液中乙醇的氧同位素比值

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摘要:近年来,乙醇的氧同位素比值($\delta^{18}O$)在果汁和饮料酒真实性鉴别中起着重要作用,利用该指标可检测产品中的外源水、追溯产品的产地。本工作采用多孔聚合物气相色谱柱实现了水与乙醇的在线快速分离,建立了溶剂稀释后直接用气相色谱-裂解-稳定同位素比值质谱(GC-TC-IRMS)测定溶液中乙醇$\delta^{18}O$值的方法。实验结果表明,该方法可排除水对乙醇$\delta^{18}O$分析的干扰,乙醇浓度在1%~100%时测定稳定性良好,在不同水溶液中乙醇$\delta^{18}O$的测定值保持一致;乙醇$\delta^{18}O$值重复性和再现性的标准偏差均优于0.5‰,并通过欧盟实验室间能力验证(FIT-PTS)证明了方法的准确性。该方法具有样品用量少(仅需70~200 μL)、分析速度快(约18 min)、操作简单方便等特点,可为乙醇$\delta^{18}O$在果汁和饮料酒真实性领域的研究与应用提供方法参考。

关键词:乙醇;稳定氧同位素比值;含水溶液;提取

Stable isotope ratios are useful tools for fighting against the adulteration of food products[1]. In particular, oxygen isotopic proxies are effective for the detection of fruit juices and authentication of alcoholic beverages, because they can provide valuable data on the amount of added water[2-3]. $\delta^{18}O$ of ethanol is now considered as a reliable internal reference to improve the detection of the watering in wine and fruit juices[4-7]. A major challenge in implementing the use of stable oxygen isotope proxies is the analysis of ethanol $\delta^{18}O$ in aqueous samples. In isotope ratio mass spectrometry (IRMS) analysis, ethanol is required to be converted into carbon monoxide (CO) by the unterzaucher reaction, however, in the same reaction conditions, H_2O is also converted into CO[8-11]. Considering that H_2O can potentially affect the accurate analysis of $\delta^{18}O$ of ethanol in aqueous samples, it is imperative to develop efficient techniques for the extraction and purification of ethanol for the accurate determination of ethanol $\delta^{18}O$.

A distillation procedure for the $\delta^{18}O$ analysis of ethanol in alcoholic beverages and fruit juices has been established[12-13]. However, this procedure is time-consuming (more than 4 h) and requires careful laboratory practice to avoid isotopic fractionation during ethanol extraction. The ethanol content in the distillate is merely greater than 95% (volume fraction), so molecular sieves are additional required to trap the co-extracted water, for this reason, an extended time of 24 h is needed. Moreover, although only a few micromoles of pure ethanol is injected for conversion, sample volumes of greater than 350 mL is utilised for distillation.

On the other hand, during recent years, gas chromatography-isotope ratio mass spectrometry (GC-IRMS) coupled with direct sample injection has become an important tool for examining the stable isotopic composition of food ingredients and for assessing the authenticity of food[14-20]. Typically, for ethanol $\delta^{13}C$ analysis, capillary GC columns filled with high-polarity stationary phases, such as polyethylene glycol, are employed for separating

ethanol from other polar compounds. Ethanol $\delta^{13}C$ analysis exhibits advantages of a small sample size, in addition, the pre-extraction of ethanol from other organic compounds is not required. However, a rapid and accurate method for ethanol $\delta^{18}O$ analysis by GC-IRMS is still a specialised endeavour to some extent, which is only practiced by a few laboratories worldwide. There are few studies about ^{18}O analysis by GC-IRMS reported in the literature, one attempt was reported for the determination of $\delta^{18}O$ of ethanol from aqueous samples by the direct injection mode combined with GC-IRMS, where a DB-FFAP column was used, but the result reported is unfortunately a suspected outlier[6]. Although co-injected water is suggested to not affect $\delta^{13}C$ measurements, it is uncertain whether the outlier is attributed to the incomplete separation of water and ethanol[21-22]. Hence, an alternative route of injection without the co-injection of a large amount of water has been established: ethanol in aqueous samples is extracted prior to injection by solid-phase microextraction (SPME). In this case, samples volume greater than 4 mL and extraction time of 60 min are needed[23-24]. However, this methodology has not been widely applied to the analysis of ethanol $\delta^{18}O$ in alcoholic beverages and fruit juices. There are several reasons: variation in isotopic fractionation according to the SPME conditions; this methodology is difficult to control[5,21]; the adsorption efficiency of SPME fibres directly depends on the concentration and inversely depends on the aqueous solubility of the analyte, and the dissolved organic species that compete for SPME-active sites will also affects efficiency[24-25]. For these reasons, there is a demand for the development of advanced techniques that can rapidly determine the $\delta^{18}O$ of ethanol in aqueous samples from the viewpoints of simplified procedures and marginal sample consumption.

Recently, a rapid method for the determination of water $\delta^{18}O$ in alcoholic beverages by means of GC-IRMS coupled with direct sample injection was reported[26]. On this basis, in order to achieve the online isotope ratio analysis for ethanol without the off-line or SPME pretreatment procedures, this experiment intended to establish a simpler and more rapid method for measuring the oxygen isotope ratios of ethanol in aqueous samples using a porous-polymer-bonded GC column combined with a commercially available gas chromatography-high temperature conversion-isotope ratio mass spectrometry (GC-TC-IRMS).

1 Materials and Methods

1.1 Instrumentation

TC/EA-IRMS system: an isotope ratio mass spectrometry (IRMS, Delta V Advantage) coupled with a high-temperature conversion elemental analyzer; Gas Bench II-IRMS system: IRMS connected to a water-CO_2 equilibration system (Gas Bench II equipment); GC-TC-IRMS system: a Trace GC Ultra system coupled to an IRMS system via a GC-IsoLink and ConFlo IV universal interface, the GC system was equipped with a TriPlus autosampler and a deactivated guard column (2 m×0.25 mm) and a CP-PoraBOND Q column (50 m×0.32 mm×5.0 μm); The GC-Isolink was equipped with a high temperatrue conversion reactor that an alumina (Al_2O_3) tube (1.5 mm o.d., 320 mm length) packed with Pt and Ni wires, and with auxiliary ('magic-mix') gas (1.8% hydrogen in helium, flow rate of 0.5 mL/min) was used; Data were collected using the Isodat 3.0 software. All these above mentioned components were purchased from Thermo Fisher Scientific (Bremen) GmbH (Bremen, Germany), except the CP-PoraBOND Q column from Varian Inc. (Lake Forest, CA, USA).

1.2 Reagents and Samples

Absolute ethanol reagent (HPLC grade), referred to as Std-1, was obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). Acetone and methanol (both HPLC grade) were purchased from Duksan Pure Chemicals Co., Ltd (Ansan, South Korea) and Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA), respectively. Deionized water and mineral water were purchased from a supermarket in China.

Three proficiency test samples were obtained from Eurofins Scientific, Nantes (France): dry wine, pure ethanol and plum spirit in 2012 R1, 2012 R2 and 2012 R3, respectively. Edible ethyl alcohol (used as Std-2), Chinese spirit and red wine were purchased from a supermarket in China.

International reference material from the International Atomic Energy Agency (IAEA, Vienna, Austria): VSMOW (water, 0‰), SLAP (water, −55.5‰) and IAEA-601 (benzoic acid, +23.3‰). Molecular sieves (2 mm beads, UOP type 3Å) were purchased from Fluka Chemie GmbH, Buchs, Switzerland.

1.3 Methods

1.3.1 Determination of $\delta^{18}O$ Values for Laboratory Ethanol Working Standards

Std-1 and Std-2 were used as working standards for oxygen isotope ratio analysis, and trace water in the working standards was trapped by storage for at least 24 h on molecular sieves; the $\delta^{18}O$ value [calibrated *vs* Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation (VSMOW-SLAP)][27] was determined by TC/EA-IRMS system [13].

1.3.2 Determination of $\delta^{18}O$ Values for Water

$\delta^{18}O$ values of deionized water and mineral water were determined by GasBench Ⅱ-IRMS sysytem. All procedures have been described in the OIV-MA-AS2-12-MOU18 method (2009)[28]. The $\delta^{18}O_{VSMOW}$ values of deionized water and mineral water were (−9.85±0.05)‰ and (−19.33±0.12)‰, respectively.

1.3.3 Determination of $\delta^{18}O$ Values for Ethanol in Aqueous Samples

$\delta^{18}O$ values of ethanol in aqueous samples were determined by GC-TC-IRMS sysytem. The temperature program started at 100 ℃ and was maintained for 2 min, then increased at a rate of 10 ℃/min to 150 ℃ and maintained for 5 min, and then at 20 ℃/min to 200 ℃ and maintained for 2 min. Once the compounds were separated on the GC column and eluted, ethanol was transferred into the conversion furnace of oxygen analysis. The carrier gas helium was set at 1.2 mL/min.

The reactor was set at 1 280 ℃. Elemental carbon was used to provide a reactive layer, which should be well distributed, and this was done after 80 runs by flushing the reactor with high methane gas concentrations.

The ethanol concentration in the working standards and samples were diluted with acetone to give approximately the same peak amplitude (m/z 28) between 4 000 and 8 000 mV. For samples containing undissolved compounds, such as wine and a fermented matrix, after dilution, the solutions were filtered using 0.20 μm or 0.45 μm syringe filters[24-25]. Sample solutions (1 μL) were injected (10 μL syringe) in the split mode (1∶20). The injector equipped with a straight-bore inlet sleeve containing a plug of quartz wool (Thermo Scientific, Bremen, Germany) was set to 200 ℃, and the inlet sleeve was cleaned. The quartz wool plug was replaced after 200 injections; meanwhile the GC column was baked at 280 ℃ to remove any retained substances.

The impact of isotopic fractionation caused by injection, chromatography, pyrolysis and gas transfer was minimised following the principle of identical treatment[29-30]. Here, Std-1 was used as a standard and analyzed along with the unknown samples.

1.4 Isotopic Standardisation

All results were calculated according to the equation $\delta^{18}O[‰]=[R_{sample}/R_{standard}-1]$, where R is ratio of the heavy-to-light stable isotope in the sample (R_{sample}) and in the working standard ($R_{reference}$). The isotopic values were calculated and normalised against the working standards: Std-1 and Std-2[27].

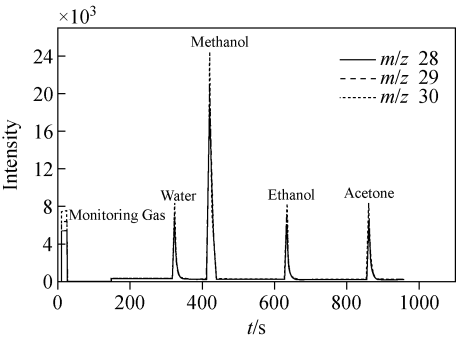
1.5 Data Analysis

The $\delta^{18}O$ values obtained from the GC-TC-IRMS analyses are reported as means (±standard deviation) of triplicate analyses. Statistical analysis was performed using t-tests to compare two means. A value of $p<0.05$ was considered to indicate statistical significance.

2 Results and Discussion

2.1 Separation of Major Oxygen-Containing Compounds

For accurate compound specific stable isotope analysis by GC-IRMS, some crucial aspects should be highlighted: 1) suitable ion currents for analysis[20]; 2) baseline separation of target analytes with other compounds[15]. Suitable ion currents will help to avoid source-linearity effects caused by dose-dependent isotope fractionation, thus, it is important to adjust the concentration of the analytes in the sample to similar peak heights. As only 6 nmol CO is required for organic oxygen isotopic measurement, and the alcoholic strength is usually higher than 5% in alcoholic beverage samples, it is very convenient to dilute the sample with a common organic solvent—acetone and methanol, which have been used as diluents for the analysis of ethanol $\delta^{13}C$ in whisky and brandy samples[31-32]. Owing to its miscibility with water and organic reagents, the baseline separation of ethanol and other oxygen-containing compounds are absolutely required for $\delta^{18}O$ analysis, and then the effluents enter a post-column splitter at the end of the GC capillary. From here, the ethanol peak will be diverted to the pyrolysis reactor and converted into CO gas, and other effluents are vented. To check the capacity of the CP-Pora-BOND Q column for the separation of ethanol from water and select one proper diluent, equal volumes of water, ethanol, methanol and acetone were mixed. For this test, 0.02 μL mixture containing equal volumes of water, ethanol and acetone was injected into the injection port using a 0.1 μL liquid sampling syringe and then monitored by GC-TC-IRMS. The four oxygen-containing compounds were well separated with a stable background, and the result was shown in Fig. 1.



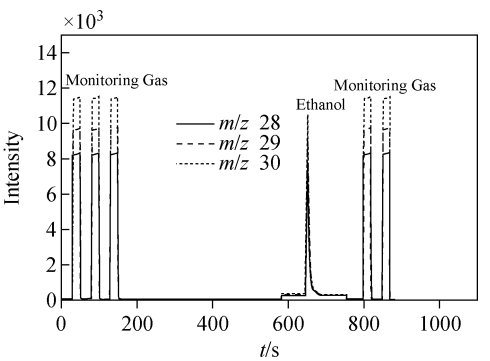
Note: The flat peak is due to monitoring gas injections introduced by the ConFlo Ⅳ interface, and the other peaks are due to the sample gas generated from water, methanol, ethanol and acetone, respectively

Fig. 1 Chromatographic of the ion currents at m/z 28, 29, 30

The effluents were observed in the following order by comparison with the retention time of their reagent: water, methanol, ethanol and acetone, with the retention times of 326, 435, 641 and 861 s, respectively. However, the CO peak heights were not similar to each other, albeit with the relative intensities of 1.17 : 3.21 : 1.21 : 1. The peak area signals of these four derived CO peak heights were observed to exhibit the relative peak areas of 1.35 : 2.00 : 1.10 : 1; discrepancy in these values are attributed to the different vapour

pressures of these four compounds in the injection port as well as the different column temperatures, while the target ingredient eluted from the GC capillary column. This test aimed to calculate the retention times of these four main oxygen-containing compounds; hence, further studies and detailed data to account for this discrepancy are not presented here.

Both methanol and acetone can be utilised for sample dilution; however, acetone was selected to distribute column load and to successfully eliminate water. Thus, we set the backflush function of GC-TC-IRMS starting at 1 s and 720 s of the run to vent water and acetone, respectively, and switched it off at 550 s to enable the access of ethanol molecules into the pyrolysis furnace. Fig. 2 shows the ion chromatogram.



Note: The flat peaks are due to monitoring gas injections introduced by the ConFlo Ⅳ interface; the second and the fifth flat peak serve as the reference points; the other peaks are for control; the chromatogram peak is generated from ethanol

Fig. 2 Chromatographic of the ion currents at *m/z* 28, 29, 30

Next, the GC column was subsequently baked at 200 ℃ with the aim of eluting higher alcohols, although these oxygen-containing substances can be ignored because of their minute quantity[26] in alcoholic beverages; in particular, the samples had been already diluted with acetone. One of the advantages of this method is that extraction and purification prior to sample injection are not required anymore, which enables the integration of the separation and pyrolysis of ethanol; this integration significantly reduces time and labour consumption. In addition, because of the liquid injection mode and the high efficiency of GC-TC-IRMS, a very small amount of the test portion is adequate for analysis.

2.2 Water Removal Efficiency

The carbon reaction is assumed to convert water into CO during ethanol conversion if the baseline separation of ethanol from water cannot be achieved, and the more water contained in the analyte, the more CO will be generated from water; moreover, the more negative the $\delta^{18}O$ value of water, the more depleted the $\delta^{18}O$ value of ethanol. In fact, the tailing of the water peak on the chromatogram has been reported to affect $^{18}O/^{16}O$ measurements because of the carbon reduction of water[21]; hence, it is imperative to successfully remove water from the target analyte (ethanol) for accurate quantative $\delta^{18}O$ of ethanol. In this study, the water removal efficiency was tested by two approaches: Firstly, Std-1 and deionized water were used to prepare a series of ethanol solutions at different ratios (details shown in Table 1), and then a consistent ethanol concentration of these solutions was achieved by the dilution of acetone before GC-TC-IRMS analysis; Secondly, Std-1 was added into deionized water and mineral water to achieve the same alcohol strength (both of 5%), followed by dilution with acetone as well as the determination of ethanol $\delta^{18}O$.

In the first verification, the effect of water by the carbon reaction on ethanol $\delta^{18}O$ analysis was not observed: Table 1 shows the values obtained from the $\delta^{18}O$ values of ethanol solutions, the mean value of seven aqueous samples is 18.32‰ (±0.31‰), similar to the value measured from pure ethanol

(18.51‰), and the maximum difference for the mean $\delta^{18}O$ values of ethanol in spiked samples is only 0.37‰, where the water content in the solutions vary from 0 to 99%.

Table 1　$\delta^{18}O$ values of ethanol calculated for Std-1 mixed with different percentages of water

Std-1 content	Deionized water content	$\delta^{18}O$ of ethanol/‰
1	99	18.14±0.11
5	95	18.23±0.40
10	90	18.30±0.23
20	80	18.39±0.11
40	60	18.48±0.23
60	40	18.36±0.29
80	20	18.31±0.20
100	0	18.51±0.20

Secondly, despite the fact that there is a difference of 9.48‰ in the $\delta^{18}O$ value of the water used, the difference of the ethanol $\delta^{18}O$ value in the two aqueous samples is only 0.14‰ (results shown in Table 2), and these variations (0.37‰ and 0.14‰) are lower than the measurement precision (1σ).

Table 2　$\delta^{18}O$ values of Std-1 calculated for 5% ethanol mixed using two different water samples

Sample	$\delta^{18}O$/‰ of water	$\delta^{18}O$/‰ of ethanol
Deionized water	−19.33	18.37±0.33
Mineral water	−9.85	18.23±0.40

All these results demonstrate that the experimental value of ethanol $\delta^{18}O$ varies neither with the water content of the samples nor with the $\delta^{18}O$ value of water in the samples; therefore, this PoraBond Q column and the relevant GC conditions are concluded to allow for the baseline separation of ethanol with water and acetone, and the effect of water on ethanol $\delta^{18}O$ analysis is successfully eliminated. Hence, this method can be applied to analysis ethanol $\delta^{18}O$ for any alcoholic beverage sample.

2.3　Application to Samples

The precision of the $^{18}O/^{16}O$ measurement for ethanol was determined using edible ethyl alcohol, wine and Chinese spirit samples. Each sample is replicated five times, and good repeatability is obtained (results shown in Table 3).

Table 3　Repeatability and reproducibility of $\delta^{18}O$ for several types of ethanol-containing samples

Sample	Repetition	$\delta^{18}O$/‰ of ethanol
Edible ethyl alcohol	5	22.52±0.17
Chinese spirit (five different days)	5	17.44±0.31
Red wine	5	26.33±0.25
Edible ethyl alcohol (five different days)	3	22.71±0.22
Chinese spirit (five different days)	3	17.18±0.15
Red wine (five different days)	3	26.45±0.38

To remove the insoluble matter in wine, syringe filters were used; however, the soluble but non-volatile compounds are also injected with ethanol, which are retained in the GC injection port; hence, a deactivated guard column (retention gap) is utilized. To evaluate the long-term effect on the analytical performance attributed to those non-volatile compounds in the samples, three independent analyses on the same sample were tested at five different days (during this period, more than 150 wine samples were continuously ana-

lyzed). Data were obtained under the same instrument conditions with the usage of an identical inlet sleeve and quartz wool, and reproducibility is shown in Table 3 with SD less than 0.5‰, which confirms the method for the ethanol $\delta^{18}O$ analysis of spirits and wines.

2.4 Method Validation

As international ethanol or alcoholic beverage reference material was not available for ethanol $\delta^{18}O$ analysis, it was difficult to validate the described method as done by Werner[8]. The optimal solution was to measure a working standard (as similar as possible to the analyte of interest) and a sample of known isotopic composition for each organic structure to be studied[32]. The standard was mixed with the sample (with known $\delta^{18}O$ values) prior to the injection, and the theoretical and experimental values of ethanol in the mixture were compared[17,33]. Here, Std-1, the red wine sample (Table 3) and their mixtures (ethanol volume from Std-1 were 25%, 50% and 75%, respectively) were prepared and injected into the GC column under identical conditions. Triplicate analyses were performed and were repeated if the SD exceeded 0.5‰. The theoretical values of ethanol (24.19‰, 22.30‰ and 20.41‰) are significantly ($p<0.01$, Pearson's correlation test) correlated with those in the spiked samples ((23.71 ± 0.41)‰, (22.01 ± 0.23)‰ and (20.95 ± 0.43)‰, respectively). And as can be seen in Fig. 3, the $\delta^{18}O$ values of ethanol obtained by this method and the added ethanol proportion in spiked samples are strongly correlated ($R^2 = 0.98$). Hence, it can be inferred that the developed method is free of any isotopic fractionation, which is suitable for aqueous samples ethanol $^{18}O/^{16}O$ isotope ratio determination.

To further evaluate the accuracy of this method, a number of proficiency tests for oxygen isotopic measurements were performed[34-35]. As shown by the data in Table 4, several laboratory reported ethanol $\delta^{13}C$ values exist for each proficiency test sample; however, only a limited number of participants given the ethanol $\delta^{18}O$ value; hence, it may be concluded that the procedures for the ethanol $\delta^{13}C$ analysis of aqueous samples cannot be directly utilized for ethanol $\delta^{18}O$ analysis (except for combustion interface replaced by pyrolysis interface), even with the reported GC/IRMS method[16-18, 20]. The SD ($\delta^{18}O$) value of a non-pure ethanol sample is higher than 2‰, which indicates technical difficulties for ethanol $\delta^{18}O$ analysis in aqueous samples.

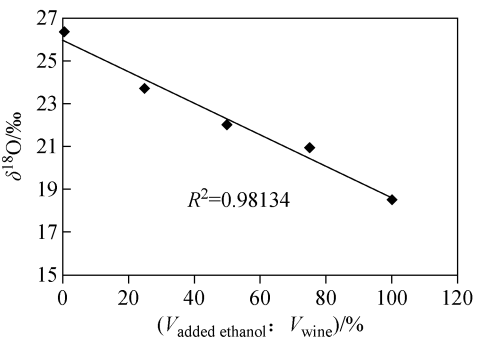


Fig. 3 Correlation of determined $\delta^{18}O$ value of ethanol *vs* the proportation of std-1

Proficiency test samples were analyzed using the developed method, the maximum deviation (less than 0.71‰) between the measured $\delta^{18}O$ value and the mean value from the proficiency test is acceptable according to the tolerance of reproducibility SD (1.0‰) given by a inter-laboratory study[13]. Furthermore, *t*-test indicated that the results from the presented method are identical within the mean values of the proficiency test ($p<0.05$). *Z*-score values demonstrate the validity of the proposed method, which can be used for routine analysis, and is suitable for the authenticity control of juices and alcoholic beverages.

Table 4　$\delta^{18}O$ results of the proficiency test samples

Sample number	Type	Participants (n/N)[a]	$\delta^{18}O$ value measured[b]	Proficiency test result ($\pm\sigma$)	Deviation	Z-score[c]
1	Dry wine	6/25	28.01	27.86±2.71	0.15	0.08
2	Pure ethanol	5/15	27.79	27.08±0.63	0.71	0.35
3	Plum spirit	5/22	25.40	25.77±2.44	0.33	−0.09

Note: a. n: number of laboratories that submitted the ethanol $\delta^{18}O$ value; N: number of laboratories that submitted the ethanol $\delta^{13}C$ value; b. $\delta^{18}O$ value determined by the developed method; c. deviation of the results of a laboratory from the mean value divided by the target standard deviation obtained from an intercomparison test, Z-score $=(\chi-\mu)/\sigma$; $Z=0$, ideal; $Z<2$, satisfactorily; $2<Z<3$, doubtful quality of results; $Z>3$, results not sufficient

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