Standard Operation Procedure

DETERMINATION OF THE $^{13}C/^{12}C$ ISOTOPE RATIO OF ETHANOL IN WINE BY GAS CHROMATOGRAPHY COMBUSTION COUPLED TO ISOTOPE RATIO MASS SPECTROMETRY

(China standard QB/T 5164-2017)

1. Purpose / Field of Application

A significant difference exists between the carbon-13 content of sugars from plants following the different photosynthetic C3 (Calvin cycle) and C4 (Hatch-Slack) cycles. Most plants, such as the vine and beet, belong to the C3 group, whilst maize and cane belong to the C4 group. The carbon-13 contents of the sugar and of the corresponding metabolites obtained by fermentation (ethanol, glycerol) are correlated. The measurement of the carbon-13 content of ethanol may enable possible detection of addition of ethanol from maize or cane sugar (C4 plant) to wines or to spirit drinks.

This SOP describes a method, based on gas chromatography coupled to an isotope ratio mass spectrometer (GC-C-IRMS), permit measurements of the $^{13}C/^{12}C$ ratio of ethanol. It is a industry standard method in China to determine the $^{13}C/^{12}C$ ratio of ethanol in *Chinese Baijiu*, and can be used in wine and spirits.

2. Definitions

- $^{13}C/^{12}C$: ratio of carbon-13 (^{13}C) to carbon-12 (^{12}C) isotopes for a given sample.
- $\delta^{13}C$: carbon-13 content (^{13}C) expressed in parts per 1000 (‰, per mil).
- GC-C-IRMS: hyphenated technique of gas chromatography coupled to a combustion interface and isotope ratio mass spectrometry.
- V-PDB: Vienna-Pee-Dee-Belemnite. PDB is the primary reference material for measuring natural variations of carbon-13 isotope content, consisting of calcium carbonate from a Cretaceous belemnite rostrum from the Pee Dee Formation in South Carolina (USA). Its $^{13}C/^{12}C$ isotope ratio or RPDB is 0.0112372. PDB reserves have been exhausted for a long time, but it has remained the primary reference for expressing natural variations of carbon-13 isotope content and against which the reference material available at the IAEA (International Atomic Energy Agency) in Vienna (Austria) is calibrated. Isotopic indications of naturally occurring carbon-13 are conventionally expressed in relation to V-PDB.

3. Principles

Wines were diluted with organic solvent which fit for the gas chromatography column. The separation of ethanol from the matrix is achieved using gas chromatography. In GC-C-IRMS, after the chromatographic separation the effluent undergoes a combustion and a reduction step, passing through the oxidation and the reduction ovens of a combustion interface. Components other than the ethanol, namely the solvent, are vented with a back-flush valve during the run, to avoid oven soiling and interferences in chromatograms. The carbon-13 content is determined on the carbon dioxide gas resulting from the oxidation of the ethanol contained in the sample. Once the ethanol is oxidized, CO_2 and H_2O are produced. Water produced during the oxidation is eliminated by a water-removing trap, consisting of a Nafion® membrane. The carbon dioxide is eluted by a helium stream to the IRMS source for 13C/12C analysis.

The various possible combinations of the ^{18}O, ^{17}O, ^{16}O and ^{13}C, ^{12}C, isotopes lead to the mass 44 corresponding to the $^{12}C^{16}O_2$ isotopomer, the mass 45 corresponding to $^{13}C^{16}O_2$ and $^{12}C^{17}O^{16}O$ species and the mass 46 to the $^{12}C^{16}O^{18}O$ isotopomer ($^{13}C^{17}O^{16}O$ and $^{12}C^{17}O_2$ can be neglected due to their very low abundance). The corresponding ion currents are determined on three different collectors. The ion current m/z 45 is corrected for the contribution of $^{12}C^{17}O^{16}O$ which is computed from the current intensity measured for m/z 46 by considering the relative abundance of ^{18}O and ^{17}O (Craig correction). The comparison with a reference calibrated against the international standard V-PDB permits the calculation of the carbon-13 content on the $\delta^{13}C$ ‰ relative scale.

4. Reagents

The following reagents and working standards should be used:

4.1 HPLC grade acetone (CAS number 67-64-1).

4.2 Helium for analysis, used as carrier gas (CAS 07440-59).

4.3 Oxygen for analysis, used as regenerating gas for the oxidation reactor (CAS 07782-44-7).

4.4 Cylinder of carbon dioxide for analysis, used as a secondary reference gas for the carbon-13 content (CAS 00124-38-9).

4.5 BCR-656 (CRM-656) ,

4.6 Working standard samples of ethanol with a known $\delta^{13}C$ ‰ calibrated against international reference materials.

5. Apparatus and Equipment

5.1 Isotope ratio mass spectrometer

Isotope ratio mass spectrometer (IRMS) capable of determining the relative ^{13}C content of naturally-occurring CO_2 gas with an internal accuracy of 0.05 ‰ or better expressed as a relative value (point 8. Calculation). Internal accuracy here is defined as the difference between two measurements of the same sample of CO_2. The mass spectrometer used to measure isotope ratios is equipped with a triple collector to simultaneously measure intensities for m/z = 44, 45 and 46. The IRMS is equipped with software for running the analysis, acquisition of data and processing of analytical results for computation of isotope ratios.

5.2 Gas chromatograph

Gas chromatograph (GC) coupled through a oxidation interface to an isotope ratio mass spectrometer (5.1).

The gas chromatograph must be equipped with a polar capillary column enabling the chromatographic separation of ethanol from the other sample matrix components (e.g. Chrompack WCOT fused silica capillary column filled with bonded polyethylene glycol CP-Wax-57 CB, 50 m, 0.25 mm id, 0.20 μm film thickness).

Oxidation interface generally made up of an oxidation reactor (a ceramic tube containing nickel, platinum and copper wires) and of a reduction reactor (ceramic tube containing copper wires).

5.3 Equipment

Usual laboratory equipment and in particular the following:

- Sample injection syringes or autosampler
- Volumetric flasks, 0.2 μm filters, chromatographic vials and 10 μL syringe for liquids.

The laboratory equipment indicated in the above list is an example and may be replaced by other equipment of equivalent performance.

6. Preparation of test samples

Each wine sample is filtered on a 0.2 μm filter and then an aliquot is diluted to ca. 8g/L level with acetone (the ratio range from 1:10 to 1:14 according to the ethanol content in wine). BCR 656 and working standard are diluted in ratio 1:120.

Each sample is then transferred to an appropriate chromatographic vial which is then tightly closed and stored at $T \leq 4$ °C until analysis.

7. Procedure

The following description refers to the procedures generally used for ethanol $^{13}C/^{12}C$ isotope-ratio determination using commercial automated GC-C-IRMS systems.

Procedures may be adapted according to changes introduced by the manufacturers. Note: volumes, temperature, flows and times are indicative. The correct values should be optimized according to the manufacturer's instructions.

7.1 Working conditions

Using the column and oxidation interface described as an example in 5.2 the following parameters can be applied:

A. The injector temperature is set to180 °C.

B. The temperature program is set as follows: initial column temperature of 40 °C; a holding time of 5 min; then a temperature increase at a rate of 1 °C min^{-1}, up to the value of 50 °C, with a holding time of 1 min; then temperature increase at a rate of 15 °C min^{-1}, up to the final value of 200 °C, with a final holding time of 2 min.

C. He is used as the carrier gas at 1.0 L min^{-1}.

D. The temperatures of the oxidation reactors of the GC oxidation interface are set to 1000°C.

E. In each injection 1.0 μL of sample solution is introduced into the column using a split mode (split flow 20 mL min^{-1}).

At regular intervals (e.g. once a week or 500 runs) re-oxidation of the oxidation reactor with O2 is required (the intervals depend on the total amount of substances that has passed through the reactor).

7.2 Retention time of ethanol

Equal volume of ethanol and acetone were mixed, and 3 μL of the mixture was injected to the air, then 3 μL of air was injected into the injection port using a 10 μL liquid syringe at high-split mode (split flow 100 mL min^{-1}).

The back-flush valve was manually closed at 50s of the run, the ion peak of acetone and ethanol will appeared in fixed order. The retention time of acetone +30s, will be the closed time of the back-flush valve in routinely analysis, which enables the organic solvent to be vented and ethanol to be transfer into oxidation reactor for $^{13}C/^{12}C$ analysis.

7.3 $^{13}C/^{12}C$ ratio of ethanol

During each $^{13}C/^{12}C$ analysis, at least six pulses of reference CO_2 gas (4.4) from the cylinder are introduced (three pulses at the beginning and three before the ending of the run). This CO_2 is previously calibrated against BCR 656. The reference CO_2 gas may also be calibrated against in-house standards. Each wine sample matrix is injected 2 times and the repeatability limit (*r*) is 0.2‰.

7.4 Quality assurance and control procedures

Suitable control references must be included in each batch. BCR 656 should be arranged at the beginning of the batch, following a wine as QC sample and 8 samples (16 runs); then BCR 656 , and then following 9 samples (18 runs),……, and BCR 656 was required at the ending of the batch.

No.	content	No.	content
1	BCR 656	31	BCR 656
2	QC wine sample	32-40	Sample 26 to sample 34
3-10	Sample 1 to sample 8	41	BCR 656
11	BCR 656	42	QC wine sample
12-20	Sample 9 to sample 17	33-40	Sample 34 to sample 41
21	BCR 656	……	……
22	QC wine sample		
23-30	Sample 18 to sample25	The last	BCR 656

8. Calibration

8.1 Calculation

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 (δ/1000 or δ ‰) 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).

During 13C/12C analyses, a reference CO_2 gas is introduced, which is calibrated against other PDB-calibrated international standards. The $\delta^{13}C$ values are expressed in relation to the working reference as follows:

$$\delta^{13}C_{\text{sample/ref}}\ ‰ = (R_{\text{sample}}/R_{\text{ref}} - 1) \times 1000$$

where R_{sample} and R_{ref} are respectively the $^{13}C/^{12}C$ isotope ratios of the sample and of the carbon dioxide used as the reference gas.

The $\delta^{13}C$ values are expressed in relation to V-PDB as follows:

$$\delta^{13}C_{\text{sample/V-PDB}}\ ‰ = \delta^{13}C_{\text{sample/ref}} + \delta^{13}C_{\text{ref/V-PDB}} + (\delta^{13}C_{\text{sample/ref}} \times \delta^{13}C_{\text{ref/V-PDB}})/1000$$

8.2 Offset correction

BCR 656 was used to correct the carbon isotope fractionation during GC-C-IRMS analysis.

If the measured value of BCR 656 was drifted, for example the values of no.21 and no.31 were different, the values of sample 18 to sample 25 should be lineary corrected.

If the the measured value of BCR 656 was same (difference lower than 0.2‰), for example the values of no.1 and no. 11 were -25.81‰ and -25.91‰, respectively, the values of sample 18 to sample 25 should be corrected as following:

$\delta^{13}C$ sample=$\delta^{13}C_{BCR\text{-}656}$+ Δ cut-off value.

$\delta^{13}C_{BCR\text{-}656}$ is -26.93‰, Δ is the cut-off value of BCR 656, here it is -1.08‰ (-1.08=-26.93-(-25.81-25.91)/2).

9. Precision

The repeatability limit (*r*) is 0.20‰.

The reproductability limit (*R*) is 0.45‰.