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DETERMINATION OF CHITOSAN ISOTOPIC RATIOS ^{13}C/^{12}C AND ^{15}N/^{14}N BY ISOTOPE RATIO MASS SPECTROMETRY

THE GENERAL ASSEMBLY,

IN VIEW OF Article 2, paragraph 2 iv of the Agreement of 3 April 2001 establishing the International Organization of Vine and Wine,

CONSIDERING the work of the "Specifications of Oenological Products" Expert Group,

DECIDES, at the proposal of Commission II "Oenology", amend the monograph COEI-1-CHITOS of Chapter I of the *International Oenological Codex* by adding the following sentence in italic under part 5.1.2 Determination of the source: *"The method described in Appendix VIII and the limits given for the different isotope ratios can be used to determine the origin of the chitosan (crustacean or fungal)."*

DECIDES, at the proposal of Commission II "Oenology", to add the following method as Appendix VIII to the monograph COEI-1-CHITOS of Chapter I of the *International Oenological Codex*:

APPENDIX VIII

DETERMINATION OF CHITOSAN ISOTOPIC RATIOS ^{13}C/^{12}C AND ^{15}N/^{14}N BY ISOTOPE RATIO MASS SPECTROMETRY

INTRODUCTION

Chitosan is a linear polysaccharide composed of □(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, with a number of commercial uses. In oenology, it is used as an antioxidant, fining agent, metal chelator, and for microbiological control, reducing the use of sulphites.

Chitosan is produced by the deacetylation of chitin. It may derive from the exoskeleton of crustaceans or from fungal mycelium. Only chitosan obtained from fungal chitin may be used in oenology.

The data in the literature demonstrate that the chitosan isotopic ratios ^{13}C/^{12}C and

$^{15}N/^{14}N$ are able to differentiate products obtained from the exoskeleton of crustaceans from those obtained from fungal mycelium.

The method described here and the limits given for the different isotope ratios can be used to determine the origin of the chitosan (crustacean or fungal).

WARNING: Some reagents used in this procedure are dangerous: take particular care when using them. The operator is advised to observe the instructions on the product container label and to consult the safety data sheets for specific information on the danger of the reagents used and on how to dispose of them.

1. PURPOSE AND SCOPE OF APPLICATION

The objective of the method is to analyse the chitosan isotopic ratios $^{13}C/^{12}C$ and $^{15}N/^{14}N$ by isotope ratio mass spectrometry techniques (IRMS).

2. TERMS AND DEFINITIONS

For the purposes of this document, the following definitions apply:

$^{13}C/^{12}C$: Ratio of carbon-13 to carbon-12 isotopes for a given sample.

$\delta^{13}C$: Ratio between carbon-13 (^{13}C) and carbon-12 (^{12}C) isotopes expressed in delta permil (‰).

$^{15}N/^{14}N$: Ratio of nitrogen-15 to nitrogen-14 isotopes for a given sample.

$\delta^{15}N$: Ratio between nitrogen-15 and nitrogen-14 isotopes expressed in delta permil (‰).

V-PDB (Vienna-Pee Dee Belemnite): International reference standard for the calculation of $\delta^{13}C$.

V-AIR (Air): International reference standard for the calculation of $\delta^{15}N$.

3. PRINCIPLE

The chitosan is analysed. The stable isotope ratios of C and N are analysed using an isotope ratio mass spectrometer (IRMS) interfaced with an elemental analyser (EA) starting from the ionic currents:

- m/z = 44 ($^{12}C^{16}O^{16}O$) and m/z = 45 ($^{13}C^{16}O^{16}O$) produced by the carbon dioxide obtained from the combustion of the sample in the elemental analyser,

- m/z = 28 ($^{14}N^2$) and m/z = 29 ($^{15}N^{14}N$) produced by the nitrogen obtained from the combustion of the sample in the elemental analyser.

4. REAGENTS AND MATERIALS

The reagents and consumables depend on the equipment used by the laboratory. Elemental analysers are generally used for sample combustion. These systems can be equipped with automatic systems for the introduction of samples placed in sealed metal capsules.

The laboratory may use any certified international reference material in addition to those indicated in the table in 4.3.

4.1. Consumables

4.1.1. Absolute ethyl alcohol (CAS 64-17-5);

4.1.2. Helium for analysis (CAS 07440-59-7);

4.1.3. Oxygen for analysis (CAS 07782-44-7);

4.1.4. Oxidation and reduction reagents for the furnace and the combustion system such as copper oxide (II) for elemental analysis (CAS 1317-38-0);

4.1.5. Desiccant to eliminate the water produced by the combustion. For example: anhydrone for elemental analysis (magnesium perchlorate) (CAS 10034-81-8). Not necessary in the case of equipment equipped with a water elimination system through a cryogenic or capillary trap that is selectively permeable.

4.1.6. Disposable tin capsules.

4.2. Working standards

4.2.1. Carbon dioxide (CO_2) (CAS 00124-38-9) with a purity of no less than 99.998% and nitrogen (N_2) with a purity of no less than 99.999% as reference gas for measurement.

4.2.2. Working and control standards with values of $\delta^{13}C$ and $\delta^{15}N$ calibrated with respect to international reference materials.

4.3. Most commonly used reference materials with certified $\delta^{13}C$ and $\delta^{15}N$ values

Name	Material	δ^{13}C vs V-PDB ‰	δ^{15}N vs AIR ‰
USGS40	Glutamic acid	-26.39 ± 0.04	-4.52 ± 0.06
USGS42	Tibetan human hair powder	-21.09 ± 0.10	+8.05 ± 0.10
USGS43	Indian human hair powder	-21.28 ± 0.10	+8.44 ± 0.10
USGS54	Canadian lodgepole pine wood powder	-24.43 ± 0.02	-2.42 ± 0.32
USGS55	Mexican ziricote wood powder	-27.13 ± 0.02	-0.3 ± 0.4
USGS56	South African red ivorywood powder	-24.34 ± 0.01	+1.8 ± 0.4
USGS90	Millet flour from Tuscany, Italy	-13.75 ± 0.06	+8.84 ± 0.17
IAEA-600	Caffeine	-27.771 ± 0.043	+1.0 ± 0.2

5. EQUIPMENT

Normal laboratory equipment, and in particular the following:

5.1. Isotope ratio mass spectrometer (IRMS)

The isotope ratio mass spectrometer allows the determination of the relative contents of the heavy isotope compared to the light isotope of the carbon dioxide and nitrogen obtained from combustion, with an internal precision of 0.1‰ for C and 0.2‰ for N. Internal precision is defined here as the difference between two measurements of the same gas sample.

The mass spectrometer used is generally equipped with a series of collectors to measure the ionic currents m/z = 44, 45, 46 or m/z = 28, 29, 30 at the same time.

By measuring the corresponding intensities, the $^{13}C/^{12}C$ isotope ratio is, for example, determined by the intensity ratio of m/z = 45 and m/z = 44 after corrections for the isobaric species $^{12}C^{17}O^{16}O$, whose contribution can be calculated as a function of the intensity of the current measured for m/z = 46 and of the relative abundance of ^{18}O and ^{17}O (Craig's correction).

The mass spectrometer for the determination of isotope ratios must be equipped with:

- A double introduction system (double inlet system) to alternately measure the sample and a reference standard,
- Or a continuous flow system that quantitatively transfers the gases resulting from the combustion of samples and working standards into the mass spectrometer.

5.2. Elemental analyser (EA)

Combustion equipment capable of quantitatively converting the sample into carbon dioxide (in case of combustion) and capable of separating gases and eliminating water without any isotope fractionation. The equipment can be either a continuous flow system integrated into the mass spectrometer or an autonomous combustion system. In the latter case, the gases are collected in special containers, which are then interfaced with the IRMS.

5.3. Microanalytical balance (min. range 0-100 mg and min. precision 0.01 mg)

5.4. Centrifuge

5.5. Freeze-drier or drying system

6. PROCEDURE

6.1. Preparation of the sample for analysis

10 g of chitosan are washed three times with a 10% v/v aqueous solution of high purity ethanol. The sample is then centrifuged and freeze-dried.

6.2. Analysis of isotope ratios

For instrumental measurement, the procedure indicated in the instrument manual and recommended by the manufacturer's technicians is followed.

The following description refers to the procedure generally used for the combustion or pyrolysis of samples by means of commercial automated combustion systems. It is possible to use other methods that ensure the quantitative conversion of the samples into carbon dioxide and nitrogen without loss by evaporation.

6.2.1. Inserting the samples into the capsules and analysis

- Use clean capsules, tweezers and a prep shelf.
- Use tin capsules for combustion.
- Take a capsule of a suitable size using tweezers.
- Introduce the required amount of sample into the capsule using the specific spatula.

Note:
It is necessary to set the correct amount of sample to be weighted, such that the amount of CO_2 *produced by the sample and the working standard (or reference material) do not differ for more than 50%. To fall within this range of acceptability, it is necessary to perform a preliminary measurement revealing the amount of sample to be weighed (if unknown).*

- Weigh using microanalytical balance.
- Close the capsule with tweezers.
- Prepare at least two capsules for each sample.
- Properly arrange the capsules in the sample drum of the elemental analyser (if available). Each capsule should be carefully identified in numerical order.
- Systematically arrange the capsules containing the working standard at the beginning and at the end of the series of samples.
- Regularly insert control samples into the sample set (e.g., a commercial chitosan previously calibrated, see 4.2) in order to build a control chart.

6.2.2. Control and adjustment of the instrumentation for isotope analysis

- For an optimal sample combustion, adjust the temperature of the furnaces of the elemental analyser and the gaseous carbon dioxide, helium and oxygen flows following the manufacturer's instructions.

- Verify the absence of leaks in the system for elemental analysis/pyrolysis and mass spectrometry (for example, by checking the ionic current for the ratio m/z = 40, which corresponds to argon).
- Adjust the mass spectrometer following the manufacturer's instructions.
- Before starting to perform measurements on samples, check the system using working standard samples.

6.2.3. Performing the measurements

- The samples are introduced successively into the elemental analyser autosampler. The gases produced by the combustion of each sample are transported in succession to the mass spectrometer, which measures the ionic currents. The computer, interfaced with the instrumentation, records the intensity of the ionic currents and calculates the δ values for each sample.

7. CALCULATION

7.1. Correction and expression of the isotope data

According to the IUPAC protocol, the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ values are expressed on the delta scale (□‰) with respect to the international standard V-PDB (Vienna-Pee Dee Belemnite) and AIR (air) according to equation 1:

$$(1)\delta_{ref}({}^{i}E/{}^{j}E,sample) = \left[\frac{R({}^{i}E/{}^{j}E,sample)}{R({}^{i}E/{}^{j}E,ref)}\right] - 1$$

where *ref* is the international measuring standard; *sample* is the sample analysed; $^{i}E/^{j}E$ is the isotope ratio between heavier and lighter isotopes. The delta values □□are then multiplied by 1000 and expressed in units per thousand (‰).

The data are usually provided by the instrument and refer to the standard gases used for analysis (CO_2 and N_2). The data obtained should be corrected and normalised. At least two international reference materials (see table 4.3) or working standards (previously calibrated) should be placed at the beginning and end of the analytical sequence and have certified values at the extremes of the measurement range, both for the carbon analysis and for the nitrogen analysis. The two points provided by the reference materials (or working standards) are used to create an interpolation line and calculate the relevant equation, which is then used to correct all the data obtained.

7.2. Quality control of the analyses

- The mean value obtained for the working or international standards used by the laboratory should fall within the validity range established by the laboratory during calibration or reported in the certificate.
- The difference between the two repeated measurements of the same sample should be less than 0.3‰ for the carbon and the nitrogen analysis.

7.3. Estimation of the repeatability and reproducibility of the method

The interlaboratory study took place from June to September 2024 and, through the participation of nine different international laboratories, made it possible to estimate the repeatability and reproducibility of the method. Repeatability and reproducibility studies were conducted on six double-blind chitosan samples (total of 12 samples), 4 from fungi and 2 from crustacean exoskeleton with different $\delta^{13}C$ and $\delta^{15}N$ values. Each laboratory was also provided with two international standards with certified values (USGS40 L-glutamic acid with certified values $\delta^{13}C$ =-26.39‰ and $\delta^{15}N$ =-4.52‰ and USGS 89 porcine collagen with certified values $\delta^{13}C$ =-18, 13‰ and $\delta^{15}N$ = + 6.25‰) for a two-point line data correction.

Based on the results obtained (reported in Table 1 and 2 of Appendix 1), it is possible to estimate the following validation parameters.

d^{13}C/ ‰ vs V-PDB

sample description	chitosan 1	chitosan 2	chitosan 3	chitosan 4	chitosan 5	chitosan 6

number of valid results	9	9	9	9	9	9
number of replicates	2	2	2	2	2	2
mean	-20.81	-25.36	-13.03	-25.73	-21.32	-14.04
Sr	0.04	0.07	0.09	0.07	0.07	0.08
SR	0.14	0.09	0.23	0.10	0.16	0.21

d^{15}N / ‰ vs AIR

sample description	chitosan 1	chitosan 2	chitosan 3	chitosan 4	chitosan 5	chitosan 6
number of valid results	9	9	9	9	9	9
number of replicates	2	2	2	2	2	2
mean	-5.39	5.05	-5.41	2.92	-4.55	-7.15
Sr	0.08	0.12	0.09	0.08	0.13	0.15
SR	0.11	0.19	0.11	0.14	0.23	0.15

7.4. Repeatability and reproducibility

Repeatability

The absolute difference between two individual results detected on an identical sample by an operator using the same equipment in the shortest possible time interval will exceed the repeatability limit r in no more than 5% of cases.

The accepted values of the relative standard deviation of repeatability (RSDr) are equal to 0.09‰ for the d^{13}C parameter and 0.15‰ for the d^{15}N parameter.

Reproducibility

The absolute difference between two individual results found on an identical sample reported by two laboratories will exceed the reproducibility R in no more than 5% of cases.

The accepted values of the relative standard deviation of reproducibility (RSDR) are equal to 0.23‰ for the δ^{13}C parameter and 0.23‰ for the δ^{15}N parameter.

8. DIFFERENTIATION BETWEEN CHITOSAN OF FUNGAL ORIGIN AND CRUSTACEAN EXOSKELETON ORIGIN

8.1. δ^{13}C and δ^{15}N limiting values at 95% for authentic fungal chitosan

Based on the literature (Perini et al., 2020 and Claverie et al., 2023), authentic fungal chitosan has δ^{13}C values above −14.2‰ or below −24.9‰ (data available in the reported open access journal). Chitosan with a δ^{13}C value between -24.9‰ and -25.1‰ should also be analyzed for the δ^{15}N ratio to confirm its fungal origin. In this specific case the value of δ^{15}N must be higher than +2.7‰.

8.2. Guide to the interpretation of isotopic data

Samples with an isotopic value δ^{13}C and δ^{15}N above or below the limiting value at 95% for chitosan for fungi as defined in point 8.1 plus (for the upper limit) or minus (for the lower limit) the uncertainty calculated as 2* RSDR (see section 7.4) are to be regarded as no-fungal origin.

9. BIBLIOGRAPHY

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APPENDIX VIII.1

Table 1 shows the δ^{13}C results provided by the different laboratories with an indication of the Z-score.

Lab	Sample 7	Sample 12	Z-score	Sample 6	Sample 11	Z-score	Sample 1	Sample 9	Z-score
1	-20.75	-20.69	0.63	-25.38	-25.43	-0.54	-13.04	-13.10	-0.19
2	-20.74	-20.74	0.48	-25.26	-25.30	0.86	-12.77	-12.87	0.92
3	-20.65	-20.76	0.73	-25.30	-25.32	0.52	-13.30	-13.21	-1.01
4	-20.84	-20.78	-0.02	-25.30	-25.28	0.75	-12.79	-12.92	0.76
5	-21.16	-21.05	-2.12	-25.14	-25.40	0.97	-13.12	-13.26	-0.72
6	-20.71	-20.68	0.81	-25.37	-25.50	-0.88	-13.34	-13.29	-1.27
7	-20.80	-20.82	-0.02	-25.37	-25.37	-0.15	-12.60	-12.72	1.62
8	-20.72	-20.75	0.52	-25.38	-25.40	-0.37	-13.08	-13.12	-0.32
9	-20.95	-20.95	-1.02	-25.52	-25.40	-1.16	-13.11	-12.85	0.21
Mean		-20.81			-25.36			-13.03	

Lab	Sample 5	Sample 8	Z-score	Sample 3	Sample 4	Z-score	Sample 2	Sample 10	Z-score
1	-25.77	-25.69	-0.02	-21.10	-21.19	1.07	-14.12	-14.13	-0.41
2	-25.61	-25.60	1.27	-21.49	-21.39	-0.72	-13.73	-13.86	1.17
3	-25.68	-25.68	0.49	-21.24	-21.30	0.31	-14.26	-14.30	-1.16
4	-25.65	-25.61	1.01	-21.33	-21.24	0.22	-13.89	-13.83	0.86

5	-25.81	-25.77	-0.64	-21.51	-21.72	-1.78	-14.19	-14.11	-0.53
6	-25.79	-25.88	-1.11	-21.17	-21.03	1.34	-14.20	-14.25	-0.89
7	-25.69	-25.67	0.49	-21.29	-21.25	0.31	-13.68	-13.75	1.55
8	-25.77	-25.81	-0.64	-21.39	-21.42	-0.51	-14.11	-14.19	-0.53
9	-25.68	-25.94	-0.85	-21.38	-21.35	-0.26	-13.91	-14.19	-0.05
Mean		-25.73			-21.32			-14.04	

Table 2 shows the δ^{15}N results provided by the different laboratories with an indication of the Z-score.

Lab	Sample 7	Sample 12	Z-score	Sample 6	Sample 11	Z-score	Sample 1	Sample 9	Z-score
1	-5.52	-5.49	-1.08	4.94	4.94	-0.59	-5.56	-5.50	-1.15
2	-5.30	-5.25	1.06	5.07	5.09	0.16	-5.36	-5.20	1.23
3	-5.40	-5.38	-0.01	5.01	5.01	-0.22	-5.41	-5.46	-0.24
4	-5.23	-5.41	0.64	5.11	4.79	-0.54	-5.29	-5.34	0.90
5	-5.43	-5.28	0.32	5.05	5.41	0.97	-5.33	-5.53	-0.20
6	-5.32	-5.19	1.24	4.73	4.79	-1.57	-5.39	-5.38	0.23
7	-5.55	-5.44	-0.99	5.12	5.01	0.08	-5.30	-5.57	-0.24
8	-5.50	-5.48	-0.94	5.08	5.10	0.22	-5.52	-5.50	-0.96
9	-5.39	-5.44	-0.24	5.30	5.35	1.49	-5.33	-5.40	0.42
Mean		-5.39			5.05			-5.41	
Lab	Sample 5	Sample 8	Z-score	Sample 3	Sample 4	Z-score	Sample 2	Sample 10	Z-score
1	2.77	2.85	-0.73	-4.70	-4.49	-0.21	-7.23	-7.21	-0.47

2	3.02	2.92	0.37	-4.46	-4.63	0.01	-7.04	-6.87	1.25
3	2.95	2.93	0.17	-4.50	-4.46	0.29	-7.13	-7.11	0.18
4	2.76	2.64	-1.49	-5.07	-4.68	-1.41	-6.91	-7.39	-0.02
5	3.15	3.15	1.62	-4.46	-4.32	0.68	-7.19	-7.42	-1.03
6	2.73	2.92	-0.63	-4.85	-4.66	-0.90	-6.99	-6.98	1.06
7	2.85	2.88	-0.35	-4.65	-4.81	-0.79	-7.04	-7.28	-0.08
8	2.94	2.96	0.23	-4.31	-4.28	1.09	-7.22	-7.21	-0.44
9	3.14	2.93	0.82	-4.28	-4.24	1.24	-7.16	-7.27	-0.44
Mean		2.92			-4.55			-7.15	