Use of compound specific isotope analysis approach to monitor the aging process of Italian balsamic vinegars

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ABSTRACT

Stable isotope analysis has become a valuable tool for studying food chain processes and verifying the authenticity and geographical origin of typical products. The analysis is particularly important for those foods with geographical indications, such as Aceto Balsamico Tradizionale di Modena labelled with the protected designation of origin mark (ABTM PDO) and Aceto Balsamico di Modena with the protected geographical indication (ABM PGI). Understanding how the aging process affects the isotopic composition of specific compounds in ABTM is important for distinguishing between traditional and non-traditional products, as well as for verifying their authenticity. Previous studies have explored isotopic variations in balsamic vinegars, but challenges remain in fully understanding how aging influences isotopic ratios and fractionation phenomena particularly for individual compounds such as glucose, fructose, and acetic acid. This study investigated the impact of aging on the isotopic ratios in Italian balsamic vinegar, focusing on $\delta^{18}O$ of water and $\delta^{13}C$ of glucose, fructose, and acetic acid. Bulk variables such as water content, density, total acidity, refractive index, and glucose and fructose concentration were also evaluated. The findings revealed that $\delta^{18}O$ values of water progressively increased with aging inside the casks' series for ABTM, allowing a clear differentiation between traditional and non-traditional balsamic vinegars. In contrast, the $\delta^{13}C$ values of glucose, fructose, and acetic acid were also influenced by the conditions of production and origins of the starting raw materials. Further research is needed to better understand the effects of the individual factors that influence the $\delta^{13}C$ values for enhancing the ability to authenticate and differentiate balsamic vinegar products.

1. Introduction

In recent decades, the food quality sector has seen significant expansion, driven by consumers' growing awareness of their dietary choices and a rising demand for authentic and high-quality products (Krystallis, 2017). Nowadays, consumption patterns favor foods with attributes such as organic certification, locally sourced origin (termed 'zero km'), absence of additives, and Geographical Indications (GI), such as Protected Designation of Origin or Protected Geographical Indication. These preferences reflect a lifestyle choice focused on enhancing psycho-physical well-being rather than merely patriotic interests. The European Union's quality policy aims to preserve the names of specific products, emphasizing their unique characteristics, including geographical origin and traditional craftsmanship (Green Paper on Agricultural Product, 2008; Green Paper on Promotion Measures, 2011). The products bearing 'geographical indication' establish a distinct connection to their place of production, which provides consumers with confidence in product authenticity while assisting producers in their marketing strategies. Although many consumers often lack a clear understanding of traceability systems, they associate traceability with the ability to verify the origin of products (Giraud and Halawany, 2006; Chryssochoidis et al., 2006), equating it with higher-quality goods, and, consequently, higher prices (Chryssochoidis et al., 2006).

In this context, Italian products continue to represent excellence and are envied by many countries. Aceto Balsamico Tradizionale di Modena PDO (ABTM) is a symbolic product of the Emilia-Romagna region, specifically the Modena district, which is known and appreciated worldwide. Unique aspects of its production process, such as the type of

starting raw material, the refinement-aging process, and the extended aging period (Cocchi et al., 2002; ABTM Production Rule), distinguish it in the agri-food sector. Despite this renown, the authenticity of ABTM faces challenges from fraudulent imitation and industrial variants, making reliable authentication methods essential. Although several analytical approaches have been employed to verify the authenticity, characterization, and geographical origin of ABTM (Durante et al., 2021; Masino et al., 2008; Papotti et al., 2015; Caligiani et al., 2007; Gullo et al., 2006), challenges remain, particularly regarding the differentiation of ABTM from industrially produced substitutes like Aceto Balsamico di Modena PGI (ABM). The complexity of the ABTM production chain requires a more detailed analytical approach to understand and track the chemical transformations occurring within the wooden barrels of the battery during the aging period.

The use of both direct (Durante et al., 2021; Sighinolfi et al., 2018; Ndung'u et al., 2011) and indirect (Cocchi et al., 2006; Cirlini et al., 2009; Masino et al., 2005; Papotti et al., 2013) indicators has provided interesting insights into the transformations occurring during the production and aging of balsamic vinegar. Among the direct indicators, the determination of isotopic ratios of light elements such as C, N, H, O, and S constitutes a reliable approach for investigating the origin and authenticity of both animal- and plant-based foods (Bontempo et al., 2019; Erasmus et al., 2016; Simsek et al., 2012; Goitom et al., 2011). Isotope compositions within organisms are influenced by biological metabolism, environmental conditions (such as lithology and hydrology), and climate, resulting in distinct isotopic signatures that can differentiate products from various origins (Dou et al., 2023). Recent technological advancements have enabled direct monitoring of stable light element isotopic ratios in various food compounds. These advancements allowed for: *i)* increased accuracy of determination by avoiding lengthy and complex sample preparations (Compendium of International Methods of Analysis for Vinegars - OIVa), *ii)* direct measurement of the molecules of interest, *iii)* important insights into the production process and product transformation, and *iv)* enhanced analytical productivity via robust statistical analysis. Compound-specific isotope analysis (CSIA) has become feasible for non-volatile analytes by developing a wet combustion interface with liquid chromatography coupled with isotope ratio mass spectrometry (LC-IRMS). However, challenges persist in carbon isotope analysis of non-polar and moderately polar compounds due to the limitations of LC-IRMS, which relies on water as the eluent phase. Indeed, analytes must be converted to CO_2 to accurately measure their carbon isotope ratio. The LC effluent must pass through an interface where it is non-selectively converted to carbon dioxide by peroxodisulfate in a heated capillary reactor, after which the CO_2 is extracted from the aqueous phase into a carrier gas stream and transported to the IRMS inlet. Consequently, the presence of additional carbon-containing compounds (such as buffers or solvents) in the eluent can reduce the sensitivity and accuracy of the determination of carbon isotope ratio. Additionally, the isotope ratio mass spectrometer signal is saturated, thereby limiting the effectiveness of the LC-IRMS separation technique. Several ion exchange chromatography methods have been developed for determining the carbon isotope ratios of carbohydrates, amino sugars, and amino acids (Godin et al., 2005; Krummen et al., 2004).

The ability to monitor the carbon isotopic ratios of specific chemical species during the aging process of balsamic vinegar within the production battery represents a powerful tool for understanding the transformations that occur within the product. Key steps of the process, such as the cooking of grape juice to produce the concentrated cooked must followed by alcoholic fermentation and acetic bio-oxidation, result in isotopic fractionation phenomena which involve various molecules. In particular, fractionation occurs in the $^{18}O/^{16}O$ ratio during water evaporation, while changes in the $^{13}C/^{12}C$ ratio occur for ethyl alcohol during the transformation of glucose, fructose, and acetic acid during the battery aging process. The $^{13}C/^{12}C$ ratio is crucial for assessing authenticity since the carbon isotopic ratio of acetic acid and sugars can

indicate whether their sources are truly grape-derived or from other agricultural products (*e.g.* beet sugar, cane sugar, or starch from cereal or potato) (Werner and Roβmann, 2015). In the case of musts and wine, the addition of water and exogenous sugars has been detected by analyzing the isotopic ratios of carbon in ethanol and of oxygen in water (Perini et al., 2014). Methods established by the International Organization of Vine and Wine (OIV) are currently used for such analysis: OIV-MA-AS312-06 for measuring the $^{13}C/^{12}C$ ratio and OIV-MA-AS2-12 for the $^{18}O/^{16}O$ ratio. Detection of adulteration is achieved by comparing these results against an appropriate database, such as the official databank established in 1991 (EU Regulations 2347 and 2348/91) by the European Union for all wine-producing countries within its territory. This isotope database provides reference data yearly, allowing the establishment of legal limits based on isotopic data for each country, subregion, and protected denomination (PDO). Even general limits can be established when the origin and year of production are not specified (Dordevic et al., 2013). However, the absence of a comprehensive isotopic databank specific to ABM and ABTM underlines the need for a deeper understanding of the production processes and their impact on isotopic composition. The complexity of ABM and ABTM production processes, particularly the extended and complex aging of ABTM, poses challenges for developing a robust analytical method. For ABTM, the prolonged aging process and the traditional topping-up procedure (Cocchi et al., 2002) can significantly alter the isotopic composition over time. The detection of adulteration and quality variations are further complicated if the characteristics of the must change during the lengthy aging process. Improving analytical precision and developing region-specific isotopic data for ABM and ABTM are crucial steps towards ensuring the authenticity of these geographically protected products. This study is unique in its scope, particularly regarding the number of samples analyzed and the analytical approach adopted. The isotopic characterization of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ in vinegar samples from six ABTM production batteries provides a novel perspective on the effects of aging on isotopic composition. The samples were sourced from companies with documented and long-standing experience in ABTM production. Additionally, to provide a more detailed understanding of the processes occurring along the production chain, several bulk variables were also measured, such as water content, density, total acidity, refractive index, and concentrations of glucose and fructose. For comparative purposes, these variables were also determined in some samples of Aceto Balsamico di Modena PGI, a commercial substitute product for ABTM PDO (ABM Production Rule). The significance of this research lies in its ability to provide insights into the chemical transformations occurring during the aging process of balsamic vinegar. Also, it offers a robust method for verifying the authenticity and geographical origin of traditional products like ABTM. By combining compound-specific isotope analysis with bulk variable measurements and data analysis through Principal Component Analysis (PCA), this study contributes to the broader goal of a deeper understanding of the ABTM production process.

2. Materials and methods

2.1. Collection of ABTM and ABM samples

Regarding the ABTM production, the focus was on samples coming from six *batterie,* each consisting of a variable number of wooden casks, ranging from 5 to 8 (Table 1). These samples were provided directly by local producers. As concern producer F, the *batteria* was also sampled after 15 months to account for the variability of the chemical-physical parameters and the isotopic data after the topping up procedure. For the ABM samples, five vinegars were purchased from a local retail store.

An aliquot of 100 mL of ABTM samples, taken from each barrel of each battery, and ABM samples were collected and stored in sterile Pyrex bottles at 4 °C in the dark. Each sample was homogenized before taking a sub-sample for analysis.

Table 1
Type and number of samples for the ABTM and ABM products.

Sample acronym	N samples
Producer A; *Batteria* **A1** (youngest) to **A8** (oldest)	8
Producer B; *Batteria* **B1** (youngest) to **B5** (oldest)	5
Producer C; *Batteria* **C1** (youngest) to **C6** (oldest)	6
Producer D; *Batteria* **D1** (youngest) to **D8** (oldest)	8
Producer E; *Batteria* **E1** (youngest) to **E6** (oldest)	6
Producer F; *Batteria* **F1** (youngest) to **F5** (oldest)	5
Producer F; *Batteria* **F15_1** (youngest) to **F15_5** (oldest)	5
ABM	5

2.2. Control sample

A control sample was employed to assess the uncertainty associated with the experimental measurements, particularly the isotope ratio determinations. This approach is essential for ensuring consistent performance of the overall experimental procedure. A concentrated must and a wine vinegar were selected and used as control samples for both the ABTM and ABM samples. The concentrated must is used for the isotopic ratio analysis of $^{13}C/^{12}C$ of glucose and fructose, while wine vinegar was employed for the isotopic ratio analysis of $^{13}C/^{12}C$ of acetic acid and the isotopic ratio analysis of $^{18}O/^{16}O$ of water.

These samples were prepared and measured multiple times, following the same analytical procedure used for the investigated vinegars, to monitor the variability of the analytical procedure, without replicating each sample. Since the samples were generally measured once, the uncertainty associated with the final $\delta^{13}C$ and $\delta^{18}O$ values was expressed as two standard deviations of the mean values of the control sample (with a confidence interval of 95%). The vinegar samples from the different *batteries* were measured during 12 sessions. In the same sessions, the control samples were also evaluated obtaining the following values for the $\delta^{13}C^0/_{00}$: 25.5 ± 0.4, −25.3 ± 0.5, −27.5 ± 0.5 (n = 86, mean ± 2sd), for glucose, fructose, and acetic acid respectively. The oxygen isotope ratio values of the water were collected during 10 measurement sessions in which the control wine vinegar was always included. The determined isotope ratio value $\delta^{18}O^0/_{00}$ was equal to 0.04 ± 0.14 (n = 90, mean ± 2sd). This level of uncertainty is consistent with the standard deviation of reproducibility reported in the official method OIV-MA-AS2-12. These uncertainties were associated with the $\delta^{13}C$ and $\delta^{18}O$ data measured for the sampled vinegars.

2.3. Determination of total acidity, density, water, fructose, and glucose

A detailed characterization of the vinegar samples collected from each *batteria* is essential before proceeding with the isotopic determination. Different physical and chemical variables were measured to describe the composition of vinegar. Total acidity for balsamic vinegar corresponds to the sum of the not-volatile (mainly tartaric and malic acids) and volatile (acetic and lactic acids) fractions. Total acidity was determined using a potentiometric method by constructing the acid-base titration curve. The pH-meter (ORION model 420A) was calibrated using standard buffer solutions at pH = 4.00 and pH = 10.00 (T = 20 °C). One g of the sample was diluted with ultrapure water to cover the pH-meter electrode. Titration was then performed with a 0.1 N NaOH standard solution. For each sample, the equivalent point calculation was carried out using the derivative method, both first derivative and second derivative, based on the pH vs. mL NaOH titration curve. Total acidity data are provided in Table S1 (*available as Supplementary Material*).

The density of the vinegar samples was measured using a weighing method with an analytical balance with a sensitivity of 0.1 mg (Mettler Toledo AE 240 model). A one mL automatic positive displacement pipette, previously calibrated with ultrapure water at a temperature of 20 °C, was used to draw the same volume successively for each vinegar sample. The ratio between the masses of vinegar and water provides the density of the sample. The density values, obtained from the average of

at least five replicates for each sample, are reported in Table S1. The water content was also determined and expressed as percent (w/w) using a Karl Fischer automated titration system (Mettler Toledo DL31 model). The Karl Fischer titrant solution was standardized daily by performing at least five titrations, with repetitions at the beginning and end of the measuring session, using a weighted mass of ultrapure Milli Q water. Each sample was analyzed in triplicate. Data are reported in Table S1. The refractive index values were determined using an Index Instrument, GPR 11-37X model, automatic refractometer, operating based on the limit angle principle (λ = 589 nm), with an accuracy of n_λ^{20} ± 0.00005 up to n_λ^{20} = 1.45 and n_λ^{20} ± 0.0001 above. Temperature control of the refractive index cell was maintained by a Haake F3-C recirculating thermostatic bath maintained to 20.00 ± 0.02 °C during all the measurement sessions.

The monosaccharide content, including glucose and fructose, was determined using high-performance liquid chromatography (HPLC) technique (Waters 2690 model, USA). HPLC separations were performed using an Aminex HPX-87C column, (300 × 7.8 mm) with a 9 μm particle size and 8% crosslinked (Bio-Rad Laboratories), thermostated at 85 °C. Ultrapure water was used as the mobile phase at a flow rate of 0.8 mL min^{-1}. The eluted monosaccharides were detected by a refractive-index detector (Waters 2410 model, USA) with the detector cell set at 30 °C. The external standard method was used for the quantification of sugars. For each monosaccharide, a four-point calibration curve was constructed with the sugar concentration ranging from 100 to 1000 mg kg^{-1}.

2.4. Isotopic ratio analysis of $^{18}O/^{16}O$ of water

The determination of the $^{18}O/^{16}O$ ratio of vinegar water was performed using an Isotope Ratio Mass Spectrometer, IRMS Isoprime PrecisION (Elementar, Langenselbold, Germany) connected to a water/CO_2 equilibration system, Isoflow (Elementar, Langenselbold, Germany). The instrumental setup is reported in Table S2 (*available as Supplementary Material*).

Measurements were conducted following the procedure described in the OIV-MA-VI-23: R2013 method (Compendium of International Methods of Analysis for Vinegars - OIVb). For ABTM samples, it was necessary to adapt the procedure using the isotopic dilution method, which involves diluting the sample with water of known isotopic composition before measurement.

In accordance with the International Union of Pure and Applied Chemistry (IUPAC) guidelines (Brand et al., 2014), the isotope ratios were expressed in δE ($^0/_{00}$) versus Vienna Pee Dee Belemnite (V-PDB) for $\delta^{13}C$ and Vienna Standard Mean Ocean Water, (VSMOW) for $\delta^{18}O$ respectively, determined by the following relation, equation (1):

$$\delta E^0/_{00} = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000 \tag{1}$$

Where R_{sample} represents the $^{18}O/^{16}O$ ratio or $^{13}C/^{12}C$ ratio in the sample, and $R_{standard}$ is the isotope ratio of the international standard.

The calibration procedure was performed using three international reference materials and five working in-house standards. All standards and samples were processed in triplicate.

2.5. Isotopic ratio analysis of $^{13}C/^{12}C$ of glucose, fructose and acetic acid

The 1260 Infinity II LC system (Agilent) was coupled to an IRMS Isoprime PrecisION (Elementar, Langenselbold, Germany) via a Liquiface interface (Elementar, Langenselbold, Germany) for the determination of $\delta^{13}C$ of glucose, fructose, and acetic acid. The LC system comprised a pump (Isopump G7110B), an autosampler (G7129C) with a 100 μL loop, a column thermostat (G7116A), and a Refractive Index detector (G7162A).

Chromatographic separation of sugars was conducted at 85 °C with a

Carbomix Ca-NP5 column (Sepax Technologies Company). The eluent was 100% ultrapure water with a flow rate of 0.5 mL min^{-1}. For the acetic acid δ^{13}C determination, a Hi-Plex H column (Agilent) was employed at a 65 °C, and 2.5 mM sulfuric acid served as the mobile phase at a flow rate of 0.5 mL min^{-1}. The instrumental setup is reported in Table S3 (*available as Supplementary Material).*

For the isotopic ratio determination of monosaccharides, the ABTM and ABM samples were diluted to a sugar concentration of 1000 mg kg^{-1}. The diluted samples were treated using the Solid Phase Extraction (SPE) technique using amino propyl solid-phase extraction columns (Discovery DSC-NH$_2$, Supelco), to reduce the content of organic acids. In particular, the resin was activated and washed with 5 mL of ultrapure water, followed by loading a 5 mL aliquot of the sample solution. The first 3 mL of eluate were discarded, and the subsequent eluate was collected for isotopic ratio determination.

For the determination of acetic acid δ^{13}C, vinegar samples were diluted to a total acidity of 700–800 mg kg^{-1} and filtered to 0.45 μm.

The total flow of the LC was split into two flows, with 0.3 mL min^{-1} directed to the Liquiface system. Inside the interface, the sample flow rate was blended in a mixing cross with a flow rate of 0.15 mL min^{-1} of 20 % (w/w) sodium peroxodisulfate and water, and then heated in the reactor at 105 °C. Under these conditions, the organic compounds were converted into CO_2. After cooling, the CO_2 was extracted in the gas separator where the helium flow rate was set at 4.5 mL min^{-1}. The gas was dried on the Nafion membrane and carried to the IRMS by helium flow for isotope analysis of each CO_2 peak.

2.6. Data analysis

Microsoft Excel, version 16.83, of the Microsoft 365 Premium suite was used for data processing and graph generation.

The results were analyzed by means of Principal Component Analysis (PCA). PCA was carried out by using PLS_Toolbox 8.9.2 software (Eigenvector Research Inc., Manson, WA, USA) for MATLAB ® (Matlab version: 9.13.0, R2022b, Natick, MA, USA: The Mathworks Inc., 2022).

2.7. Reagents and standards

All solutions required for isotope ratio measurements were prepared by using high-purity deionized water Type1 obtained from a Milli-Q IQ 7000 system (Millipore, Bedford MD).

For HPLC-IRMS studies, 2.5 mM sulfuric acid (Merck KGaAA, Darmstadt, Germany) and 20 % (w/w) sodium peroxodisulfate (VWR chemicals, Milan, Italy) were prepared.

For ^{18}O/^{16}O ratio analyses, three international reference materials were purchased from the International Atomic Energy Agency (IAEA): VSMOW2, GRESP, and IAEA-606. The δ^{18}O ‰ certified values of VSMOW2, GRESP, and IAEA-606 are 0 ± 0.02, -33.40 ± 0.04 and 2.43 ± 0.04 (mean ± s), respectively (Reference sheet for certified referencea; Reference sheet for certified referenceb; Reference sheet for certified referencec).

δ^{13}C values were calculated using a four-point linear calibration method with an international isotopic standard of glucose (BCR-657, δ^{13}C = $-10.76 \pm 0.04°/_{00}$) (Cert-BCR657) obtained from the IAEA and three international isotopic standards of glycine (USGS64, δ^{13}C = $-40.81 \pm 0.04°/_{00}$; USGS65, δ^{13}C = $-20.29 \pm 0.04°/_{00}$, and USGS66, δ^{13}C = $-0.67 \pm 0.04°/_{00}$) (United States Geological Survey (USGS)) obtained from the IAEA. Four internal laboratory standards (fructose, mannitol, sucrose, and erythritol), calibrated to the Vienna Pee Dee Belemnite, were used to determine the isotope ratios of the samples. The δ^{13}C$°/_{00}$ values measured for these working standards were -11.49 ± 0.16, -12.31 ± 0.17, -23.35 ± 0.21 and -26.54 ± 0.27 (mean ± s), respectively.

For the sample pre-treatment for ^{13}C/^{12}C ratio analyses, a Discovery DSC-NH$_2$ SPE with 500 mg of stationary phase and a 6 mL volume capacity (Supelco, Bellefonte, PA, USA) was used.

For the quantification of glucose and fructose, the carbohydrate standards were purchased from VWR chemicals. Sodium hydroxide normex solution 0.1 N was purchased from Merck for the determination of total acidity. The water content was determined using a Karl Fischer titrant reagent, 5 mg H$_2$O/mL, and methanol obtained from Scharlab Italia S.r.l. (Lodi, Italy). The titrant solution was standardized daily before use with ultrapure Milli Q water.

3. Result and discussion

To achieve its distinctive organoleptic parameters ABTM undergoes to chemical and physical transformations within the barrels during the aging period. These changes significantly affect various factors such as total acidity, sugar content, and water content, which in turn influence physical properties such as density, viscosity, surface tension, and refractive index.

For instance, Fig. 1 shows the trends of water content, density, refractive index, and main sugars, glucose and fructose) evaluated in the product of the casks from *batteria* **D**.

These trends were also representative of batteries **A**, **B**, **C**, **E**, and **F**, with only slight variations due to the different production methods employed by the producers. Specifically, the reduction in water content, caused by slow evaporation, led to an increase in the concentration of fixed constituents in the liquid phase, as shown in Fig. 1. Among these factors, total acidity was the most complex trend to interpret. This parameter results from the combination of fixed acidity, mainly tartaric, malic, succinic, and citric acids, along with volatile acids, predominantly acetic acid. Notably, total acidity did not always increase with aging. Generally, depending on the management of the *batterie* and the characteristics of the fermented must used as raw material, an initial increase in acidity was observed owing to the activity of acetic bacteria, followed by a subsequent decrease in the final ABTM product due to the high volatility of acetic acid (Cocchi et al., 2002).

The situation differed for ABM samples. Industrially produced vinegars are characterized by more uniform data. Apart from one sample with a higher density (indicating higher sugar content), the samples exhibited a consistent range for acidity (5.5 ± 0.1, g acetic acid/100 g), water content (77 ± 3, % w/w), refractive index (1.365 ± 0.003), and density (1.080 ± 0.007 g mL^{-1}) (data are reported as mean ± sd).

3.1. Isotopic ratio $^{18}O/^{16}O$ of water in ABTM e ABM

It is essential to consider the distinct processes involved in ABTM and ABM production to accurately study the δ^{18}O of water across different batteries. In the case of ABTM, the only raw material is grape must, which undergoes a direct flame cooking process. Subsequently, the concentrated cooked must undergoes both alcoholic and acetic fermentation before being transferred to barrels. Conversely, ABM production regulation allows the addition of wine vinegar and water to the concentrated must, affecting the water oxygen isotopic ratio in the final product. Fig. 2 shows the oxygen isotopic ratios of water measured in the vinegars sampled from the ABTM *batterie* (experimental data are reported in Table S4, *available as Supplementary Material).*

The δ^{18}O values were determined for each series of casks within the five investigated *batterie*, with aging increasing from left to right within each set. Generally, the values in each *batteria* exhibited a slight increase in the initial barrels, with δ^{18}O ranging between 5 and 12 ‰. Subsequently, as aging progressed, δ^{18}O rapidly decreased, up to $-17.8°/_{00}$.

The observed behaviors and negative values measured for the aged products could not be only explained by the water evaporation process occurring during aging in the barrel and along the *batteria*. According to isotopic fractionation principles, the heavier isotope, ^{18}O, should have become progressively enriched compared to the lighter isotope, ^{16}O, during evaporation. Therefore, we should have observed a trend starting from a specific value of δ^{18}O in the younger barrels, with subsequent enrichment of the heavier isotope as aging progresses, resulting in

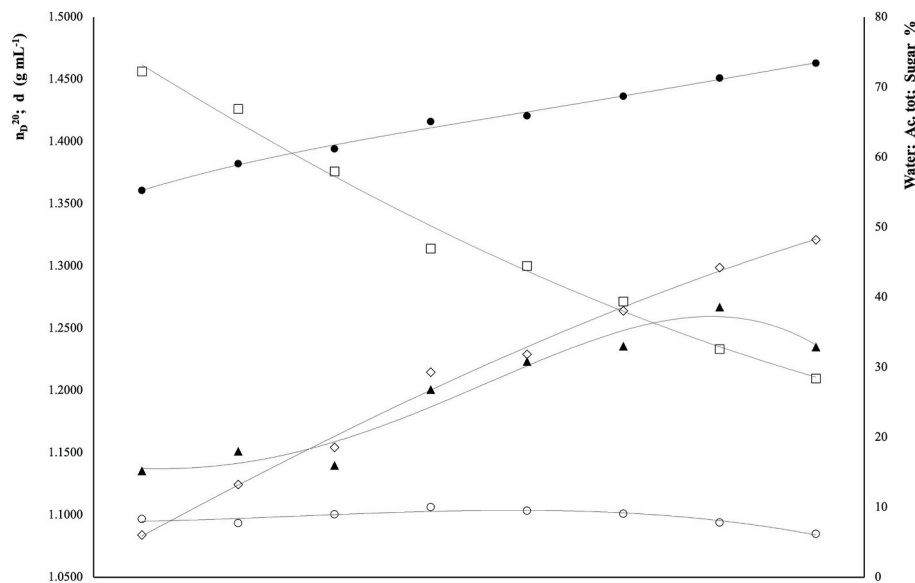**Fig. 1.** Trend of different parameters determined along the casks' series, from the youngest (left) to the ABTM (right) for *batteria* **D**. Symbols are as follow: (□) water content (% w/w); (▲) total sugars, glucose and fructose (% w/w)); (●) refractive index (n_D^{20}); (◇) measured density (g/mL) and, (○) total acidity (g acetic acid/100 g). Lines are used to depict the trend of the variables.
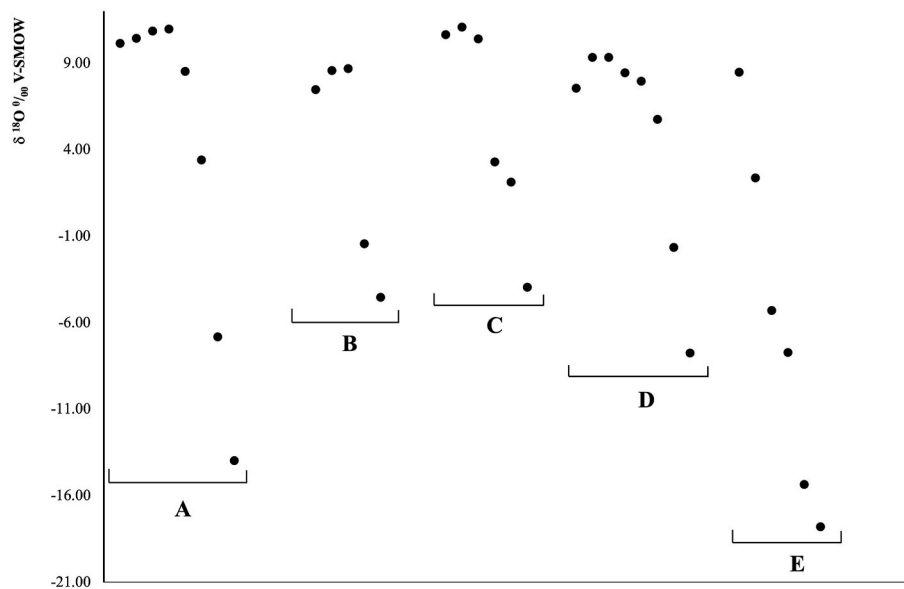

Fig. 2. Trend of the $\delta^{18}O^0/_{00}$, data reported in the VSMOW scale, of water determined in the ABTM samples obtained from *batterie* **A** to **E**. Product ageing increases from left to right.

increasingly positive delta values as aging advances.

The fundamental principle for determining $\delta^{18}O$ relies on an efficient exchange between the oxygen in the sample water and the carbon dioxide added to the headspace of the vial (Compendium of International Methods of Analysis for Vinegars - OIVb). However, in complex matrices such as those found in balsamic vinegar production chains, the physical and chemical characteristics of the product can hinder this exchange, causing errors in $\delta^{18}O$ determination (Iacumin et al., 2018).

As an example, Fig. 3 shows the trend of water content, density, and $\delta^{18}O$ value of water for samples of *batteria* **A**, offering insight into how their physical characteristics influence the determination of the oxygen isotopic ratio.

In particular, a decrease in $\delta^{18}O$ was observed for casks with a water content below $35 \div 40$ %, and/or a density exceeding $1.20 \div 1.25$ g mL^{-1}. This trend is consistent across all batteries (data for batteries from

A to **F** and **ABM** are provided in Table S4). As reported in previous literature studies (Perini et al., 2024), $\delta^{18}O$ values obtained directly from vinegar following the official analysis method can be considered inaccurate and not representative of the aging process of ABTM when the density exceeds 1.20 g mL^{-1} or when the water content is below 40 % w/w.

This suggested that inaccuracies in $\delta^{18}O$ measurements are likely due to the limited capacity of water in the sample to exchange with the reference gas. Indeed, as the product ages, the residual water is committed to maintain the high concentration of dissolved components such as sugars, organic acid, cations and anions in solution, making it unavailable or minimally available for exchange with the gas phase. Moreover, the high density of the product reduces the liquid-gas exchange process, preventing CO_2 diffusion inside the medium, as confirmed by earlier studies (Iacumin et al., 2018; Perini et al., 2024).
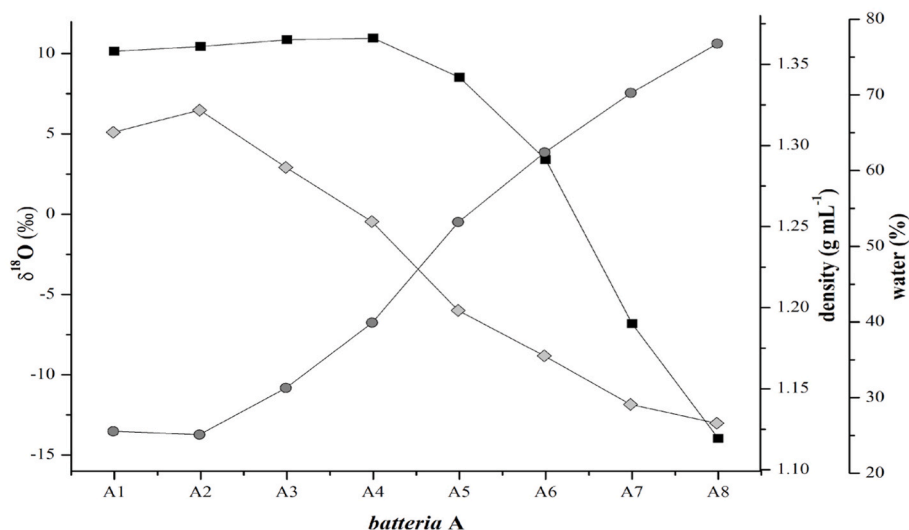
Fig. 3. Trend of the $\delta^{18}O^0/_{00}$ of water (data reported in the VSMOW scale), density (g mL^{-1}) and water content (%) determined for the samples of *batteria* **A**. Symbols are as follow: (■) $\delta^{18}O$ ‰, (●) density, g mL^{-1} and (◆) water content, % w/w.

Therefore, the isotopic dilution method was employed to improve the accuracy of the measured data while avoiding time-consuming distillation. This method was employed to reduce the sample's density, thereby mitigating the issues associated with CO_2 diffusion and liquid-gas exchange. This method is based on the principle of additivity of contributions and enables the determination of the isotopic ratio of individual components within a mixture. Instead of measuring $\delta^{18}O$ directly on the original sample, the sample is diluted with isotopically characterized water ($\delta^{18}O = -8.73°/_{00} \pm 0.04°/_{00}$). Specifically, 90 : 10 mixtures (standard water: sample) were used, as this ratio provided the most reproducible and repeatable results. For each ABTM sample, batches composed of 5 samples were prepared and analyzed in triplicate. The mixing equation (**Equation (2)**) is used to calculate the $\delta^{18}O$ of the original sample, considering its water content (Mixing, 2006).

$$\delta^{18}O_{diluted\ ABTM} = \frac{(\delta^{18}O_{ABTM} \bullet m_{ABTM} \bullet \%_{H_2O\ ABTM}) + (\delta^{18}O_{std} \bullet m_{std} \bullet 100)}{(m_{ABTM} \bullet \%_{H_2O\ ABTM}) + (m_{std} \bullet 100)}$$

(2)

Where $\delta^{18}O_{dil\ ABTM}$, $\delta^{18}O_{ABTM}$, and $\delta^{18}O_{std}$ are the $\delta^{18}O$ values of the diluted ABTM, original ABTM, and the isotopically known water, respectively. While m_{ABTM} and m_{std} are the mass of the sample and the isotopically known water in the mixture, and $\%_{H2O\ ABTM}$ is the sample water content determined with the Karl Fisher method, respectively. From Eq. (2), it is clear that the isotopic value of the sample reflects the mass-weighted average of the two sources (m_{STD} and m_{ABTM}). Therefore, if m_{ABTM} contributes a larger proportion of the total mass, the sample will have a δ value closer to that of m_{ABTM}. Conversely, if m_{STD} contributes a larger proportion of the total mass, the isotopic signature will shift toward the δ value of m_{STD}.

Fig. 4 shows the oxygen isotopic ratios of water measured for vinegar sampled from casks in batteries A to F, using the isotopic dilution method, along with ABM data.

Using the isotopic dilution method, the results generally aligned with the proposed fractionation model, exhibiting increasingly positive values with product aging in the casks. Unlike other food matrices such
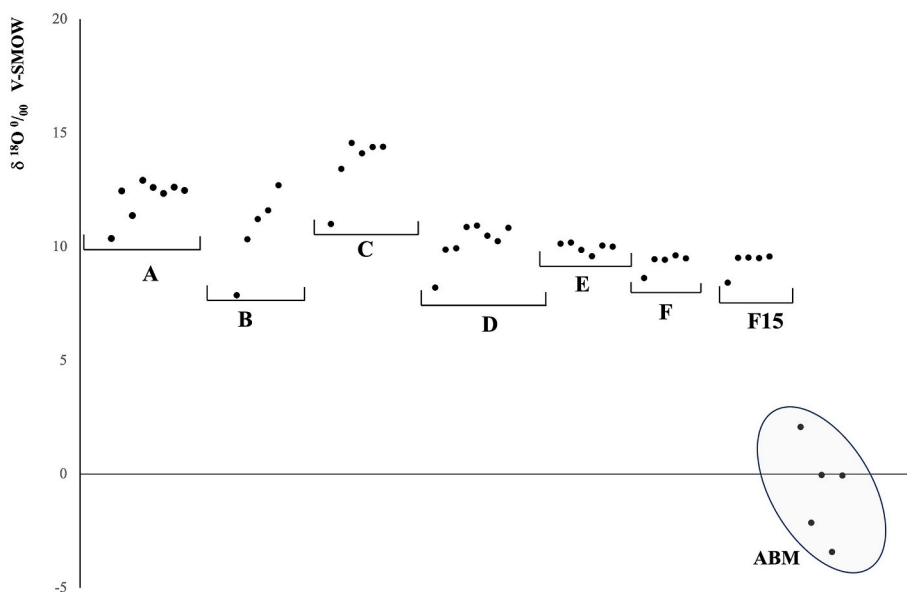

Fig. 4. Trend of the $\delta^{18}O^0/_{00}$ of water, data reported in the VSMOW scale, determined in the ABTM samples, obtained from *batterie* **A** to **F** (**F15** data are obtained from vinegars of *batteria* **F** after topping up procedure), and **ABM** samples with *isotopic dilution method*. **ABTM** product ageing increases from left to right.

as wine, there are currently no databases that enable the determination of product authenticity based only on $\delta^{18}O$ for ABTM. However, it is possible to differentiate traditional balsamic vinegar from non-traditional ones, ABM, by comparing their $\delta^{18}O$ values. Indeed, industrially produced ABM typically showed $\delta^{18}O$ values ranging from $2°/_{00}$ to $-4°/_{00}$ (Perini et al., 2024; Camin et al., 2013), owing to the addition of spring water, while ABTMs data consistently exceed $5°/_{00}$. The results obtained for ABTM samples were also consistent with the values obtained from Iacumin et al. (2018).

Regarding ABMs, it is possible to refer to (Camin et al., 2013), which demonstrated that wine vinegar exhibits a minimum $\delta^{18}O$ threshold of $-5°/_{00}$ with lower values indicating significant water addition to the starting matrix with a sugar concentration much higher than that of fresh grapes. The ABM samples analyzed in this study showed values above this threshold and thus can be considered compliant.

No significant differences were observed in values or trends after topping up procedure F15, indicating the procedure did not impact significantly $\delta^{18}O^0/_{00}$ of water.

3.2. Isotopic ratio $^{13}C/^{12}C$ of glucose, fructose and acetic acid in ABTM

Fig. 5 shows the $\delta^{13}C$ values for glucose and fructose determined in the samples of the six *batterie,* from **A** to **F**, and ABM.

The isotopic ratio values for the ABTM samples fell within a narrow range, between $-28°/_{00}$ and $-25.5°/_{00}$ for glucose and between $-27.5°/_{00}$ and $-24.5°/_{00}$ for fructose, with the latter being more positive than the former (Chartrand and Mester, 2019). In contrast, the isotopic ratios of sugars in the ABM samples were even narrower, ranging between $-26°/_{00}$ and $-24°/_{00}$ for glucose and $-23.5°/_{00}$ and $-22.5°/_{00}$ for fructose.

Each ABTM *batteria* exhibited distinct trends, reflecting the unique production conditions of each one. The minor differences in $\delta^{13}C$ values between glucose and fructose could be attributed to physiological characteristics of fresh and cooked musts, which are influenced by the CO_2 fixation processes of plants, driven by the C-3, C-4, and CAM metabolic pathways (Gilbert et al., 2011). However, the observed differences may also reflect the specific production conditions of cooked musts or the effect of alcoholic fermentation by yeast. Additionally, the "topping up" process, which compensates for volume losses during aging, might influence the $\delta^{13}C$ values due to variations in the vintage and geographical origin of the musts. While water evaporation during

the cooking of fresh grape juice is a key transformation, various chemical reactions, particularly during prolonged cooking, can alter the glucose/fructose ratio and, consequently, their isotopic ratios (Cocchi et al., 2007). This is particularly true when musts are concentrated to a high degree, as the thermal instability of fructose may further affect these ratios. Following cooking, partial alcoholic fermentation occurs, which is then followed by the bio-oxidation of ethanol into acetic acid.

Trends in $\delta^{13}C$ values considered optimal or expected were observed in *batteria* B. Despite an initial 1–2 ‰ difference in the $\delta^{13}C$ values of fructose and glucose in the cooked must, this difference remained almost unchanged in subsequent samples. Unfortunately, the complex nature of the matrix, its production process, and the numerous variables affecting the composition of product, make it challenging to attribute this trend to a single factor.

Although carbon isotope ratios alone are used to characterize the production chain and vinegar samples, measuring the compound-specific isotope ratio for monosaccharides, rather than sugars as a whole, enhances the ability to discriminate between authentic and adulterated products.

In particular, for samples from batterie **A** to **F**, the ratio between the $\delta^{13}C$ of glucose and fructose was close to unity, ranging from 1.04 ± 0.02 to 1.01 ± 0.01 range (see Table S4). As highlighted by other authors (Guyon et al., 2011), a near-unity ratio between these precursor sugars is characteristic of authentic products, with deviations indicating potential adulteration. When comparing the $\delta^{13}C$ values of derived substances, such as ethyl alcohol, glycerol, or acetic acid, with their precursor glucose and fructose, the results differ from wine, where the non-equivalence of carbon atoms in the precursor sugars shifts the final $\delta^{13}C$ values (Rossmann et al., 1991). For ABTM, there was no evidence that the cooking process significantly altered the relationship between glucose and fructose. However, the $\delta^{13}C$ of acetic acid, resulting from the complete bio-oxidation of ethyl alcohol, tended to gradually become more positive within the battery as the product ages, probably due to the evaporation process. The depletion of ^{13}C in the C1 position leads to more negative $\delta^{13}C$ values for both ethyl alcohol and acetic acid. Over time, the evaporation of acetic acid enriched the heavier isotopologues, partially offsetting the $\delta^{13}C$ changes, as shown in the data reported in Table S4.

A difference in $\delta^{13}C$ values between glucose and fructose was also observed in the ABM samples. The $\delta^{13}C$ values for acetic acid and sugars were less meaningful in ABM, as it results from a mixture of

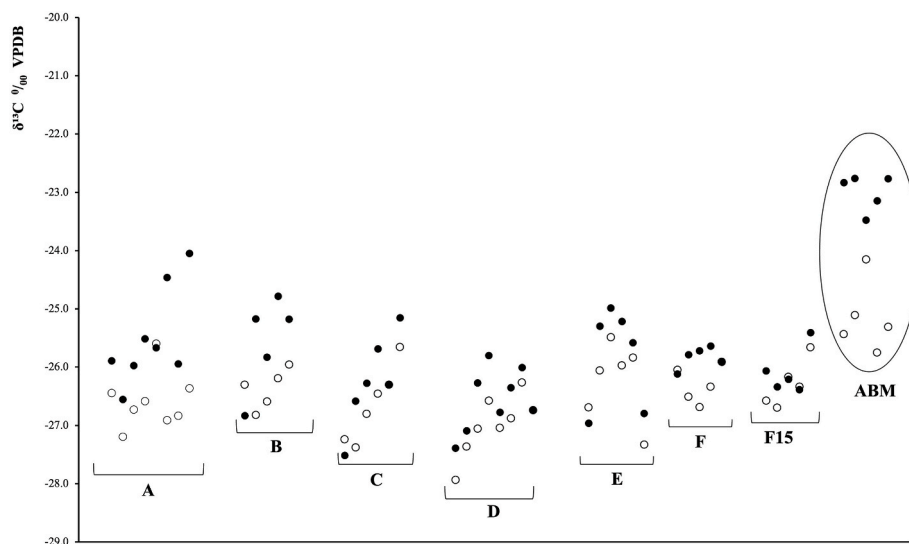**Fig. 5.** Trend of the $\delta^{13}C^0/_{00}$, data reported in the VPDB scale, of glucose and fructose determined in the **ABTM** vinegar samples obtained from *batterie* **A** to **F** (**F15** data are obtained from vinegars of *batteria* **F** after topping up procedure) and **ABM** samples. Symbols are as follow: (○) $\delta^{13}C$ glucose and (●) $\delta^{13}C$ fructose. **ABTM** ageing increases from left to right.

concentrated must and wine vinegar from different geographical regions and grape varieties.

Regarding the impact of the topping up procedure, Fig. 6 shows a comparison of the $\delta^{13}C$ values obtained from vinegar samples of *bacteria* **F** measured before and after topping up, with a 15-months interval between the two measurements.

The data presented in Fig. 6 show an interesting trend. The average $\delta^{13}C^0/_{00}$ values of -26.30 ± 0.4 (mean $\pm$ sd) and -26.29 ± 0.4, -25.84 ± 0.2, and -26.08 ± 0.4 before and after the topping up procedure for glucose and fructose, respectively, demonstrated a nearly constant trend across the series of casks. Consequently, the impact of this operational procedure on the differentiation of $\delta^{13}C$ values of vinegars between barrels can be considered negligible. However, further data collection is required to confirm and generalize this observation.

To gain a deeper understanding of the effects of the alcoholic fermentation of sugars and the bio-oxidation of ethanol to acetic acid, the $\delta^{13}C$ of acetic acid was also assessed. Fig. 7 shows the $\delta^{13}C$ values for acetic acid and sugars determined in the ABTM and ABM products.

The isotope ratios of sugars are expressed as the weighted average of glucose and fructose. The $\delta^{13}C$ for sugars was comprised between $-28.5°/_{00}$ and $-24°/_{00}$, while the $\delta^{13}C$ values of acetic acid were within the range of -28 to $-22°/_{00}$ for the vinegars sampled from the batteries. Generally, the trend of $\delta^{13}C$ values of acetic acid showed a gradual increase as the product ages, likely due to isotopic fractionation, which causes the heavier isotope to concentrate in the liquid phase. Moreover, the trend and the values of the $\delta^{13}C$ of acetic acid were not significantly different in *bacteria* **F** before and after the topping up procedure. However, a comparison of the isotopic ratio of sugars with that of acetic acid did not reveal a consistent trend across all batteries.

In the younger ABTM products, the ratio values between $\delta^{13}C$ of acetic acid and $\delta^{13}C$ of glucose-fructose were greater than unity, a phenomenon attributed to the statistical non-equivalence of carbon atoms in the two monosaccharides. However, this complexity likely arose from the overlapping processes of acetic acid evaporation, topping up procedure between barrels, and potential sugar degradation, as well as the limited data availability, making it difficult to extrapolate a generalized and clear trend.

The $\delta^{13}C$ values determined for sugars and acetic acid for ABM samples, although within the uvic range, were completely independent. This independence stemmed from the acid fraction derived from wine vinegar being added to a concentrated must and not obtained from

fermentation, as in the case of ABTM.

3.3. Multivariate PCA analysis

Principal Component Analysis (PCA) was employed as an effective tool to comprehensively evaluate and rationalize the obtained results. The selected model includes two principal components, which account for 79.24 % of the total variance. Figs. 8 and 9 reports the scores and loadings plots respectively.

From the scores plot, two key patterns emerged. First, the sample of ABM clustered predominantly in the higher left quadrant, with distinct values for both PC1 and PC2 compared to ABTM and vinegar samples, which were more broadly distributed across the remaining three quadrants. This distinction in spatial distribution on the PCA scores plot indicated substantial differences in the underlying properties of the ABM and ABTM samples. Additionally, a temporal trend was evident in the vinegars-ABTM samples coming from each *bacteria*, which shift across the plot as aging progresses. Younger ABTM samples were positioned at more negative PC1 and PC2 values, specifically concentrated in the lower left quadrant. As the aging time increases, the samples gradually move to higher values for both PC1 and PC2.

Analysis of the loadings plot, Fig. 9, further elucidates the relationships between the variables and sample distribution. Water $\delta^{18}O$ was located in the opposite quadrant to that of ABM samples, indicating that these samples exhibited the lowest values for this parameter. Therefore, water $\delta^{18}O$ is the main parameter that allows to distinguish ABM and ABTM samples. Conversely, ABM samples showed higher values for $\delta^{13}C$ of fructose, glucose, and total sugars.

The total acidity parameter was positioned in the lower left quadrant, and it was characterized by a very low PC2 value and a nearly-zero PC1 value. The total acidity was lower in the ABM samples compared to the ABTM ones. However, some ABTM samples with longer aging times, such as A8, B5, E3, E4, E5, F5, F15_5, showed high PC2 values in the higher right quadrant, since they have reached acidity levels that are comparable to, or even lower than, those of the ABM samples. As aging time increased, the glucose and fructose content, along with density and refractive index, tended to progressively increase, clustering at higher PC1 and PC2 values in the higher right quadrant. Simultaneously, the water content decreased (lower left quadrant).

To further elucidate the aging effect observed in the PCA analysis, a plot of PC1 *vs.* sample, available as Supplementary Material (Fig. S1),
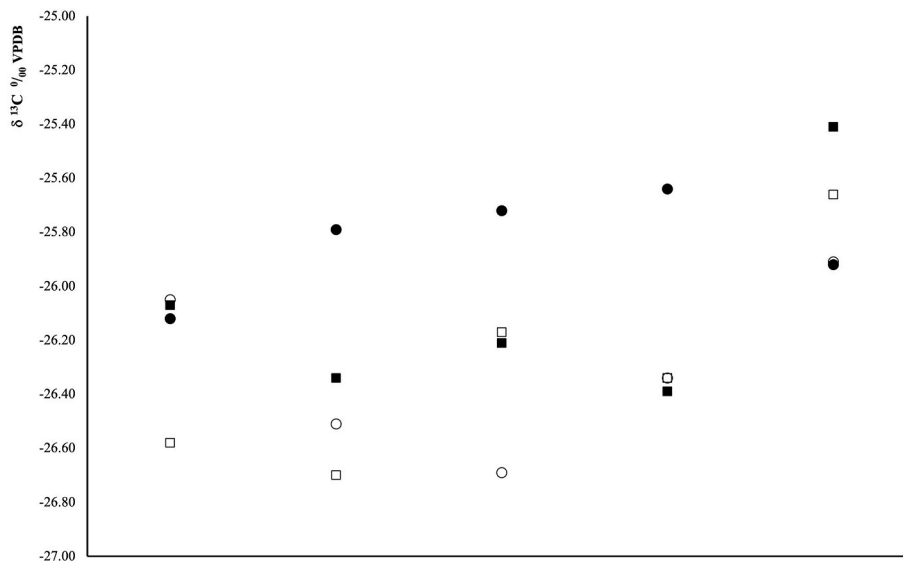

Fig. 6. Trend of the $\delta^{13}C^0/_{00}$, data reported in the VPDB scale, of glucose and fructose determined in the **ABTM** vinegars sampled from *bacteria* **F** before and after the topping up procedure (**F15**, 15 months). Symbols are as follow: (○) $\delta^{13}C$ glucose and (●) $\delta^{13}C$ fructose before topping up; (□) $\delta^{13}C$ glucose and (■) $\delta^{13}C$ fructose after 15 months. **ABTM** ageing increases from left to right.
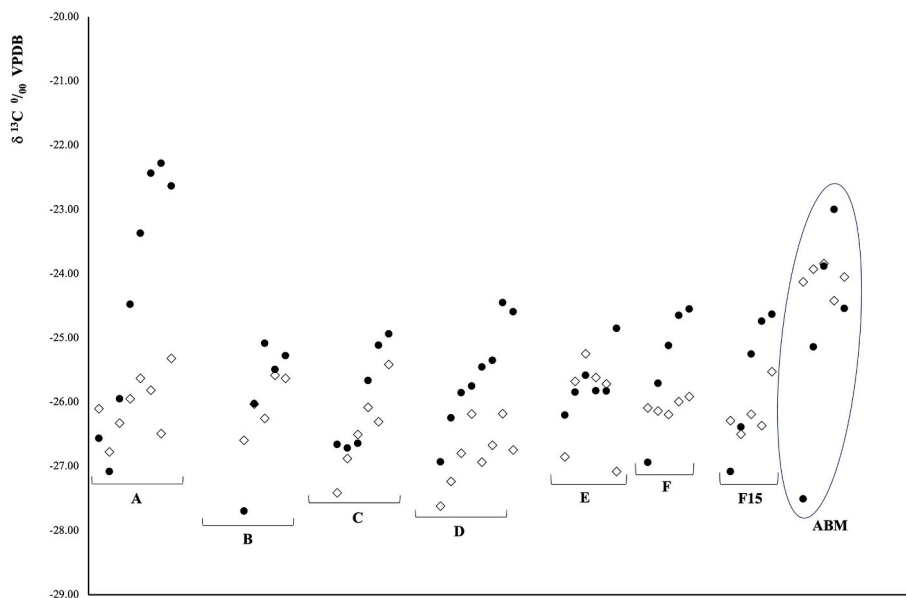
Fig. 7. Trend of the $\delta^{13}C^0/_{00}$, data reported in the VPDB scale, of monosaccharides and acetic acid determined in the vinegar samples obtained from *batterie* **A** to **F** (**F15** data are obtained from vinegars of *batteria* **F** after topping up procedure) and **ABM** samples. Symbols are as follow: (◇) $\delta^{13}C$ monosaccharides and (●) $\delta^{13}C$ acetic acid. **ABTM** ageing increases from left to right.
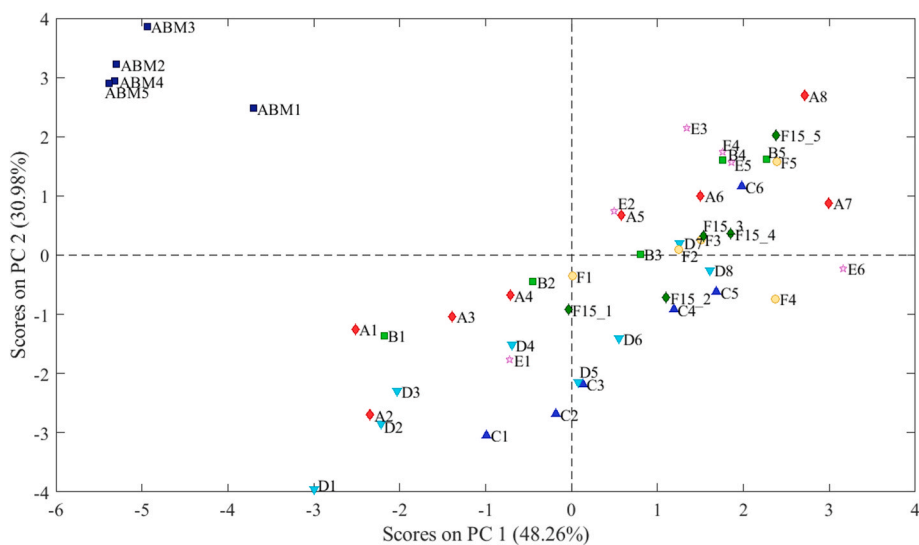

Fig. 8. Scores plot of PC1 *vs.* PC2.

provides additional clarity. Fig. S1 shows a clear trend, with samples distributed from lower to higher PC1 values, corresponding to younger vinegars to older samples (ABTM), respectively. This trend was driven by the higher water content (negative PC1 values) of the younger vinegars and by the greater density, refractive index, glucose and fructose content, and water $\delta^{18}O$ (positive PC1 values) of the older ABTM.

Finally, samples F and F15 were positioned at similar PC1 and PC2 values, with the same aging step. This indicates that, despite being sampled 15 months apart, the products had maintained nearly constant chemical and physical characteristics.

4. Conclusions

This study investigated the effects of the aging process on the isotopic ratios of specific compounds in ABTM, particularly focusing on $\delta^{18}O$ of water and $\delta^{13}C$ of glucose, fructose, and acetic acid. The findings revealed a progressive increase in $\delta^{18}O$ values as aging advanced,

effectively distinguishing traditional balsamic vinegar from substitute products, such as ABM. Isotopic dilution methods further highlighted the correlation between aging and oxygen isotope ratios, with ABTM samples consistently showing higher $\delta^{18}O$ values than ABM, providing a clear marker for its authenticity.

In terms of $\delta^{13}C$ values, the study observed that glucose, fructose, and acetic acid were influenced by various factors including the aging process, production conditions, and the origin of the raw materials. While the ratio between $\delta^{13}C$ values for glucose and fructose remained stable across certain *batterie*, such as *batteria* **B**, no universal trend could be established across all samples due to the complexity of the matrix and production variability. Notably, the depletion of ^{13}C in ethanol and subsequent enrichment in acetic acid during aging contributed to shifts in $\delta^{13}C$ values, especially in the older casks.

Overall, the study provides valuable insights into how isotopic analysis can be leveraged to assess the authenticity and geographical origin of balsamic vinegar, thereby supporting the certification
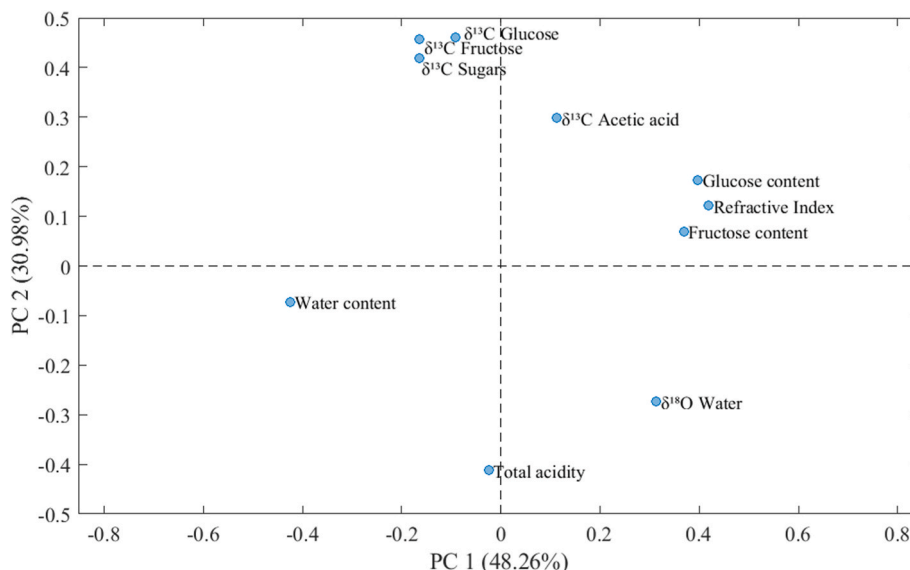
Fig. 9. Loadings plot of PC1 *vs.* PC2.

requirements of the PDO designation. This research represents a pioneering effort in applying stable isotope analysis for the detailed examination of ABTM food chain, offering a robust framework for future studies aimed at refining quality control measures and authentication techniques within the production process.

CRediT authorship contribution statement

Lisa Lancellotti: Methodology, Study design, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **Veronica D'Eusanio:** Formal analysis, Writing – original draft, Writing – review & editing. **Lorenzo Morelli:** Validation, Investigation. **Eleonora Truzzi:** Validation, Formal analysis. **Andrea Marchetti:** Conceptualization, Methodology, Study design, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100953.

Data availability

Data are reported in the manuscript.

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