



Original research article

## Determination of glycerol carbon stable isotope ratio for the characterization of Italian balsamic vinegars

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### ABSTRACT

The gas chromatographic-combustion-isotopic ratio mass spectrometry (GC-C-IRMS) approach was applied to determine the compound-specific  $^{13}\text{C}/^{12}\text{C}$  isotopic ratio of glycerol in balsamic vinegars of Modena (Italy). In particular, Italian Protected Designation of Origin and Protected Geographical Indication balsamic vinegars, namely the traditionally made Aceto Balsamico Tradizionale di Modena (ABTM) and the industrial Aceto Balsamico di Modena (ABM) products, were analyzed and a first attempt at classification was carried out. The carbon isotopic ratio of the glycerol polyalcohol varies on the basis of origin, varietal or provenance; therefore the discriminating potentiality of this species might be useful to elucidate the balsamic vinegar production process. To do this, a preliminary study was conducted and several marketable products, ABTM and ABM type, were subjected to measurements in addition to samples coming from three ABTM cask series (*batteria*). Experimental results highlighted the peculiarities of the two different production processes, suggesting the use of the carbon isotopic ratio of glycerol as an additional tool for balsamic vinegar authentication.

### 1. Introduction

Food characterization, in terms of determination of the chemical constituents, represents one of the main topics in current scientific research (Georgiou and Danezis, 2017). European Commission regulations promote food quality and food safety, with increased surveillance of products with geographical designations and/or indications such as the Protected Designation of Origin, PDO or the Protected Geographical Indication, PGI (Regulation (EC) No 510/2006; EUR-Lex document 52011DC0436, July 14th, 2011 &#x2013;).

To this end, a large variety of analytical methodologies have been developed in order to detect possible commercial frauds and to improve food authenticity (Danezis et al., 2016). Starting from the pioneering works of Bender (1971) and Bricout (1973), measuring the stable isotope ratios of  $^2\text{H}$ ,  $^{13}\text{C}$  and  $^{18}\text{O}$  in biomolecules of food, one of the most promising techniques used for food authentication is the compound-specific isotope ratio analysis, useful in distinguishing botanical and geographical origins of food (Van Leeuwen et al., 2014). For example, the stable isotopes ratio analysis of acetic acid has been reported as an assurance technique for the authenticity of vinegar (Perini et al., 2014; Dordevic et al., 2012) and measurement of  $^{13}\text{C}/^{12}\text{C}$  ratio of glycerol has been widely applied in wine authentication and for classification

purposes (Calderone et al., 2004; Jung et al., 2006; Cabanero et al., 2010).

Glycerol is a polyalcohol naturally formed in grape-derived products as a consequence of sugar fermentation by yeast and also, in some cases, by the presence of molds (Calderone et al., 2004; Lorenzini et al., 2012). It represents the most abundant by-product obtained from the alcoholic fermentation of sugars to ethanol. Moreover, owing to its ability of changing the mouthfeel properties of wines and vinegars or increasing the sugar-free extract, glycerol is sometimes fraudulently added in order to mask poor quality food (Pretorius, 2000).

Although the biochemical pathway for glycerol production is nowadays well known (Wang et al., 2001), the prediction of its amount in the final product is rather difficult. In fact, many variables may influence the biochemical mechanisms and the related yields, such as type of yeast and environmental conditions, i.e. pH, temperature and the presence of nutrients (Scanlan et al., 1998).

Concerning the  $^{13}\text{C}/^{12}\text{C}$  ratio of glycerol, Weber et al. (1997) demonstrated that the isotope ratio is directly dependent on the composition of the glucose substrate and on the metabolic pathway that leads to its production. Therefore, all the variables that influence the  $^{13}\text{C}/^{12}\text{C}$  ratio of sugars, namely the C3, C4 or CAM carbon fixation pathway (O'Leary, 1988), the yeast strain, the process temperature, etc., as well

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as the climate and/or geography of the production area, also influence the  $^{13}\text{C}/^{12}\text{C}$  ratio of glycerol. Fronza et al. (1998) reported that climatic conditions, due to seasonal or geographical variation, influence the glycerol  $^{13}\text{C}/^{12}\text{C}$  in wines. In particular, wet and cold years lead to a depletion of the  $^{13}\text{C}$  of glycerol while dry and hot years lead to an enrichment of the same isotope. Moreover, the same authors found a direct correlation between  $^{13}\text{C}/^{12}\text{C}$  of glycerol and  $^{13}\text{C}/^{12}\text{C}$  of ethanol, from the same wine, specifically a depletion of  $^{13}\text{C}/^{12}\text{C}$  in glycerol by 2.5–3.5‰ compared to ethanol, while other authors report a depletion of 3.6–4.1‰ (Cabanero et al., 2010 and references herein reported). These findings allow the definition of a  $^{13}\text{C}/^{12}\text{C}$  scale for glycerol to be used for grape-derived products starting from the measured  $^{13}\text{C}/^{12}\text{C}$  of ethanol and *vice versa*.

In addition, since glycerol can be obtained also from animal fats, the  $^{13}\text{C}/^{12}\text{C}$  ratio will account for the diet of the animals too. As a consequence, the possibility to discriminate between natural or synthetic glycerol, obtained from the chemical hydrolysis of vegetal/animal fats, from industrial synthesis or from the fermentation of sugars of different plants, represents a powerful tool to determine food quality and authenticity (Fronza et al., 1998).

At present and to our knowledge, measurements of carbon isotopes of glycerol in balsamic vinegars, of both traditional and industrial origin, are not reported in literature. Balsamic vinegars, such as Aceto Balsamico Tradizionale di Modena, ABTM and Aceto Balsamico di Modena, ABM, represent interesting food matrices to investigate owing to their peculiar food chain processes. In particular, the European Commission, in 2000, awarded ABTM with the PDO recognition and in 2009 registered the PGI protection for ABM (Regulation (EC) No 813/2000; Regulation (EC) No 583/2009).

Several analytical methods have been employed for the chemical characterization of these products and in particular for ABTM (Consonni et al., 2008a). Furthermore, the type and amount of characteristic metabolic by-products of *Acetobacter* fermentation, present in vinegar, have been used as origin and authenticity proof for vinegars (Belitz and Grosch, 1992; Consonni et al., 2008b; Papotti et al., 2015) and as quality markers (Giudici et al., 2009).

The ABTM production chain, as reported in the production rule (ABTM PDO production rule) starts from the alcoholic fermentation of condensed cooked musts, obtained from selected grapes, coming from the Modena province and successive acetic bio-oxidation of the produced ethanol. The procedure for making ABTM is briefly described to better understand the peculiarities of the product chain process. Cooked must is the raw starting material for making ABTM. It is obtained from a must of selected grapes that is condensed by simmering gently over an open fire in uncovered pans. The aging process is carried out in a set of barrels composed of a variable number of wooden casks, generally from 5 to 10, of different volumes and made of different woods. During the aging process the liquid in each cask is kept constant by transferring a certain amount of vinegar from one cask to another in a decreasing progression. This procedure is called “topping up”. The first operation consists of taking from the oldest cask an aliquot of aged balsamic vinegar, which is marketed as ABTM inside the typical 100 mL bottle designed by Giugiaro and authorized by the ABTM Consortium. From the next oldest cask, vinegar is added to the oldest one in order to replace the volume that is lost. This procedure goes on by topping up one cask from the neighboring one until the youngest cask is reached. This one is then fed with the new cooked must (Cocchi et al., 2002). The vinegar is aged inside the barrels until it reaches the organoleptic features of the PDO rule: “affinato”, 12 years of ageing, and “extravecchio”, 25 years of ageing (Cocchi et al., 2007).

For Aceto Balsamico di Modena, ABM, the starting raw material generally consists of condensed grape musts obtained by multiple effects concentrators operating under vacuum conditions and relatively low temperatures (40–60 °C). In some cases, producers can also use blends obtained by mixing condensed and cooked musts to obtain balsamic vinegars with a caramel-like taste. The condensed must or the

blend is then added with a certain amount of wine vinegar in order to achieve a total acidity in the final product of 6%, expressed as  $v/v$  of acetic acid, as stated by the PGI rule. The liquid must be then aged at least three months inside barrels before commercialization (Regulation (EC) No 583/2009). Unlike ATBM, the ABM production rule does not impose any geographical origin on its raw materials.

On the basis of the production process, it is clear that ABTM is characterized by the presence of a greater amount of glycerol with respect to ABM, since the polyalcohol, produced during the alcoholic fermentation, owing to its low vapor pressure, can accumulate in the product during the long aging period. A lower amount of glycerol is expected in ABM since, generally, no fermentation of the musts occurs and the polyalcohol should mainly come from the added wine vinegar fraction.

The present work represents a preliminary study to test the potentialities of the  $^{13}\text{C}/^{12}\text{C}$  carbon isotope ratio of glycerol as a tool for food authentication. This includes the development of an analytical procedure to measure the glycerol  $^{13}\text{C}/^{12}\text{C}$  in balsamic vinegars, using GC-C-IRMS, and the characterization of balsamic vinegars in terms of glycerol content and  $^{13}\text{C}/^{12}\text{C}$  values. Several marketable ABTM, “extravecchio” type, in addition to samples coming from three different ABTM cask series, namely BatA, BatB and BatC, were characterized. Moreover, ABM samples from different production years, 2008 to 2011, representative of the different producers of the Modena province, were also analyzed using the same variables.

## 2. Materials and methods

### 2.1. Chemicals

Two different brands of glycerol were supplied by Carlo Erba (Milan, Italy) and J.T.Baker-Avantor (Milan, Italy), respectively. KOH (38%), used for the basic hydrolysis of different types of fats, and 37% HCl, were purchased from Sigma-Aldrich (Milan, Italy). Ethanol (98%) was supplied by Fluka (Thermo Fisher Scientific, Milan, Italy). Solutions, sample dilution as well as HPLC mobile phase were always prepared by using high purity deionized water, type 1, obtained from a Milli Q 185Plus apparatus (Millipore, Bedford, MA). Physical and chemical parameters for Type 1 water comply with ASTM and ISO 3696 grade purity specification (ASTM D1193, 1999).

### 2.2. Samples

To investigate the glycerol  $^{13}\text{C}/^{12}\text{C}$  differences among the ABTM samples, aged at least 25 years (“extravecchio” type), and the ABM samples, the analytical procedure was performed on 20 samples of marketable ABTM and 92 samples of ABM of different production years: 2008 (15), 2009 (26), 2010 (25) and 2011 (26). Furthermore, to evaluate changes of the glycerol  $^{13}\text{C}/^{12}\text{C}$  during the ABTM chain process, 25 samples coming from three different cask series, BatA(11), BatB (6), BatC(8), were also examined.

Moreover, in order to evaluate possible fraudulent addition of the polyalcohol, glycerol samples of different origins were analyzed. In particular, as far as samples of natural origin are concerned, 5 samples of glycerol prepared by sugars fermentation of different cereals (malt barley, malt cider, 2 malt maize and malt rice), 2 samples obtained by hydrolysis of vegetal fats (olive oil and sunflower oil), and 2 samples coming from the hydrolysis of animal fats (butter and lard), were investigated. While, as glycerol of unknown origin, 2 samples of different commercial brands (A and B), were taken into consideration. Glycerol samples coming from fermentation processes were obtained from batch cultures of *Saccharomyces cerevisiae* growing on maize, barley and apple juice (cider) and of *Saccharomyces bayanus* growing on maize and rice. Glycerol samples coming from vegetal fats (olive oil and sunflower oil) and animal fats (butter and lard) were obtained by basic hydrolysis with concentrated KOH, successive filtration, neutralization with HCl

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