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Detection of biosynthetic citric acid and sugar addition in Italian tomato passata using liquid chromatography–combustion–isotope ratio mass spectrometry

Silvia Pianezze [a,1], Domenico Masuero [b,1], Matteo Perini [a,*], Stefania Carpino [c], Nicola Barilaro [c], Vincenzo Di Martino [c], Luana Bontempo [b]

[a] Technology Transfer Center, Fondazione Edmund Mach, San Michele all'Adige, Italy
[b] Research and Innovation Center, Fondazione Edmund Mach, San Michele all'Adige, Italy
[c] Department of Central Inspectorate for Fraud Repression and Quality Protection of the Agri-Food Products and Foodstuffs (ICQRF), MASAF, Italy

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ABSTRACT

The citric acid is naturally present in tomatoes and can be added to tomato passatas (E330) with no restriction about its amount and origin. The additive E330 is mainly biosynthetically produced starting from the *Aspergillus niger* fed on cheap materials. For the first time, an isotopic method for the detection of biosynthetic citric acid addition to Italian tomato passata was provided. Italian tomato passata samples without added citric acid (287 samples) were analysed through LC-co-IRMS to establish reference values for tomatoes citric acid carbon isotopic ratio ($\delta^{13}C_{CA}$). The isotopic values of glucose ($\delta^{13}C_{G}$) and fructose ($\delta^{13}C_{F}$) have been considered to increase the method effectiveness and to be compared to the reference values provided by the AIJN (European Fruit Juice Association). The method showed good repeatability, with intra-day and inter-day standard deviations below 1.1 ‰ for citric acid, glucose, and fructose. The ratios between the isotopic values $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_{G}$) and $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_{F}$) were more effective in the detection of biosynthetic citric acid addition than the $\delta^{13}C_{CA}$ alone.

1. Introduction

Italy is the third largest producer of tomatoes in the world, reaching 5.4 million tons of tomatoes produced in 2023, corresponding to 12 % and 52 % of the global and European production, respectively. In 2022, the revenue amounted to 4.4 billion €, half of which derived from exportation. As for Italian tomato passata, in the last campaign season the difference between the exported and the imported products reached the record value of 2.5 billion € (September 2022–August 2023) (ISMEA Report January 2024). These data reflect the importance of tomato passata in the Italian economy.

In the last few years more and more food products like the tomato passata are being advertised as "100 % natural", "all natural", "naturally derived" or "containing no additives". Since consumers are concerned about health and environmental issues, they are willing to pay a premium for products labelled this way (Simão et al., 2022). Consequently, "natural" claims led to an increase in the litigations about this topic, with varying levels of success, primarily due to the lack of clear laws or guidelines defining what "natural" truly means (Debevoise and Plimpton, 2024).

Some definitions of "natural" products have been provided by different institutions, such as the Food and Drug Administration (FDA) (U.S. Food and Drug Administration, 2024) and the International Organisation for Standardization (ISO/TS 19657:2017). Some can also be found in regulations like the REACH (Research and Markets, 2024), assessing that a substance which occurs in nature is "a naturally occurring substance as such, unprocessed or processed only by manual, mechanical or gravitational means, by dissolution in water, by flotation, by extraction with water, by steam distillation or by heating solely to remove water, or which is extracted from air by any means" (Regulation (EC) No 1907/2006). Regulation (EC) No 1924/2006 on nutrition and health claims made on food statistics that food labelling criteria shall "aim to avoid a situation where nutrition or health claims mask the overall nutritional status of a food product." The same regulation also

* Corresponding author.
E-mail addresses: silvia.pianezze@fmach.it (S. Pianezze), domenico.masuero@fmach.it (D. Masuero), matteo.perini@fmach.it (M. Perini), s.carpino@masaf.gov.it (S. Carpino), n.barilaro@masaf.gov.it (N. Barilaro), v.dimartino@masaf.gov.it (V. Di Martino), luana.bontempo@fmach.it (L. Bontempo).
[1] The authors contributed equally to this work.

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provides a general definition of “natural” for food claims. According to Annex I, a product can be labelled as natural when it fulfils the conditions for the use of a nutritional claim. Nevertheless, these definitions are not always effective when it comes to their practical application, especially when talking about natural additives.

The citric acid is naturally present in the tomatoes, and its concentration depends on the variety, the maturation stage and the growing conditions, varying between 0.3 % and 0.9 % over the total fruit weight (Stevens et al., 1977). According to the European Union, citric acid can be legally added (E330) to the tomato passata with no restrictions about its amount and origin (Reg. EU 1129/2011). It is used in the food industry as a preservative, flavour enhancer and stabiliser (Książek, 2023). The vast majority (99 %) of the world's production of manufactured citric acid is carried out using *Aspergillus niger* fungus (Sweis & Cressey, 2018) and global market reports on citric acid production highlight that the request for the biosynthetic citric acid is driven by the food and beverage industry (The Business Research Company, 2024). Therefore, even though it would be tempting to think that the citric acid added to the tomato passata comes from some citrus fruit, it is much more likely to derive from *A. Niger* (Amato et al., 2020). The addition of a biosynthetic ingredient to a product claimed as “100 % natural” could therefore raise some doubts about its correct labelling.

The economic implications are also not to be underestimated: producers who use naturally occurring citric acid face higher production costs compared to those who employ cheaper biosynthetic citric acid. This creates an uneven playing field, stressing the need for analytical tools and regulatory frameworks to distinguish natural from biosynthetic components.

Besides the economical side of this topic, the possibility of health issues can also be considered. As previously mentioned, E330 is produced by the *A. Niger*, which is a well-known allergen (Martínez et al., 2021; Sander et al., 1998). Moreover, in a study published in 2011, Frisvard and coworkers found that some of these industrially used *A. Niger* strains can produce fumonisins and ochratoxins, at conditions mimicking industrial citric acid production conditions (Frisvad et al., 2011). Furthermore, since no scientific studies had been performed to evaluate the safety of the biosynthetic citric acid when ingested in high amounts and/or through chronic exposure, Sweis and coworker presented a 4-case study of patients with a history of significant and repetitive inflammatory reactions after the ingestion of food/beverage/vitamins containing E330 (Sweis & Cressey, 2018). It must be reported that the authors stated that additional research is mandatory to evaluate the potential of biosynthetic citric acid to cause inflammatory symptoms in the body, but they recommended that the correlation between the occurrence of the symptoms and the ingestion of biosynthetic citric acid be further addressed.

A method to detect the addition of the biosynthetic citric acid to tomato passata is therefore required. The same approach could be also considered for future applications on food, beverages and supplements.

The analysis of stable isotope ratios of bioelements, in particular of carbon ($\delta^{13}C$) and hydrogen ($\delta^{2}H$) has been effectively applied for the discrimination between natural molecules and their synthetic or biosynthetic homologues (Perini et al., 2024). Compounds of natural origin, obtained through various processes from C3 plants (e.g. monacolin K from rice) (Perini et al., 2017) or Crassulacean Acid Metabolism (CAM) plants (e.g. vanillin from *Vanilla* species) (Greule et al., 2010) show different ranges of variability than the biosynthetic analogues, usually obtained through the fermentation of low cost sugars (e.g. from cane, corn and beet processing). To the best of our knowledge, no reference values for the citric acid $\delta^{13}C$ of tomatoes are available in the literature. Nevertheless, the AIJN (European Fruit Juice Association) provides reference $\delta^{13}C$ for tomato sugars.

The aim of this project was therefore to state first reference values for citric acid $\delta^{13}C$ ($\delta^{13}C_{CA}$) analysing authentic Italian tomato passata and to develop a robust method to detect the addition of the biosynthetic analogue in these products estimating the inter-days and intra-day repeatability. Once the analytical method was optimised, the limit values for an authentic tomato passata were defined. Therefore, the method effectiveness was improved, considering also the isotopic values of the individual sugars glucose ($\delta^{13}C_G$) and fructose ($\delta^{13}C_F$) measured in the authentic tomato passata samples and by using them to calculate the ratios $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$). A database made of 287 samples of authentic Italian tomato passata samples was analysed through Liquid Chromatography–combustion–Isotope Ratio Mass Spectrometry (LC-co-IRMS) together with a smaller group of 59 commercial samples. The $\delta^{13}C_{CA}$, $\delta^{13}C_G$, and $\delta^{13}C_F$ as well as the ratios $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$) of the authentic samples have been used to test the commercial group.

2. Material and methods

2.1. Samples description and preparation

In this study, 287 authentic Italian samples (275 tomato passata and 12 concentrate samples) coming from different Italian regions were collected during 2022 and 2023 by the technicians of the Italian Ministry of Agriculture, Food Sovereignty and Forestry directly at the production plants. Moreover, 59 commercial tomato passata samples available on the market were also considered, reaching a total number of samples of 346. All samples except the concentrate ones were centrifuged to separate the juice from the residual (ALC multi speed refrigerated centrifuge PK131R, Thermo Scientific, Bremen, Germany). The liquid phase was therefore diluted 1:10 in milli-Q water and filtered through a 0.22 μm polyvinylidene fluoride filter (Merck, Massachusetts, USA). As for the concentrate samples, 40 mg/ml solutions were prepared in milli-Q water and filtered through a 0.22 μm polyvinylidene fluoride filter.

2.2. UHPLC-IRMS

The working principle of the LC-co-IRMS consists in the oxidation in acid conditions of all the carbon-based compounds the sample mixture is made of, previously separated from each other thanks to an appropriate analytical column. In this study, an Ultra-High-Performance Liquid Chromatography (UHPLC) (UHPLC UltiMate 3000, Dionex, California, USA) linked with an isotope ratio mass spectrometer (IRMS) (Delta V Plus, Thermo Scientific, Bremen, Germany) via the LC-Isolink interface (Thermo Scientific, Bremen, Germany) was used. A more detailed description of the system that has been used, is reported by Perini et al. (Perini et al., 2023).

The separation of citric acid, glucose and fructose was performed through a Rezex ROA-Organic Acid H+ 300 × 0.8 mm (Phenomenex, Torrance, CA, USA), equipped with a precolumn, both set at 70 °C. A 0.005 N H_2SO_4 (Sigma Aldrich products, St. Louis, MO, USA) milli-Q solution was used as the mobile phase. The oxidant (sodium persulphate, Honeywell Fluka, ≥99.0 %) and acid (orthophosphoric acid, Honeywell Fluka, ≥99.0 %) reagents were prepared in milli-Q water (Arium Ultra-pure Lab Water System, Sartorius Stedim, Göttingen, Germany). The fluxes were set at 500 μL/min for the LC system and at 50 μL/min for each of the two reagents (acid and oxidant).

The standard citric acid, D-(+)-glucose and D-(−)-fructose used to prepare the standard solutions (1 mg/ml) were purchased from Sigma Aldrich products, St. Louis, MO, USA.

2.3. Data expression and correction

According to the IUPAC protocol (Prohaska et al., 2022), the $^{13}C/^{12}C$ values are expressed in the delta scale (δ‰), against the international standards V-PDB (Vienna-Pee Dee Belemnite) according to the Eq. (1):

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

where *ref* is the international measurement standard, *sample* is the analysed sample, and iE/jE is the isotope ratio between heavier and lighter isotopes. The delta values were multiplied by 1000 and expressed in units "per mil" (‰).

Each sample was analysed in quadruplicate. The $\delta^{13}C$ values were corrected for the instrumental drift and calculated against working standard materials: citric acid (−11.9 ± 0.2 ‰), D-(+)-glucose (−20.0 ± 0.2 ‰) and D-(−)-fructose) (−26.5 ± 0.2 ‰). The standard solutions (1 mg/ml) were injected in quadruplicate at the beginning and at the end of each analytical sequence. The working standards were calibrated using an EA-IRMS (Flash 1112, Thermo Scientific, Bremen) against the international standards fuel oil NBS-22 with $\delta^{13}C = -30.03 \pm 0.05$ ‰, sucrose IAEA-CH-6 with $\delta^{13}C = -10.45 \pm 0.04$ ‰ (IAEA-International Atomic Energy Agency, Vienna, Austria) and L-glutamic acid USGS 40 with $\delta^{13}C = -26.39 \pm 0.04$ ‰ (U.S. Geological Survey, Reston, VA, USA). For calibration, each standard was analysed ten times. The deviation between the measured and certified values was determined, and the average of these deviations was calculated. This average deviation was then applied to correct the value of the working standard.

2.4. Statistics

The software Statistica 14.0.1.25 was used to calculate the mean values and the standard deviation, as well as to carry out the *T*-Test to check for differences between the values of tomatoes harvested in different years (2022 and 2023).

3. Results and discussion

3.1. Inter-days and intra-day repeatability

The repeatability of the method was evaluated by measuring the isotopic values of a standardized tomato passata sample under two conditions: intra-day, consisting of repeated measurements performed within the same day under identical conditions to assess short-term precision, and inter-day, consisting of repeated measurements performed on different days to evaluate long-term precision while accounting for day-to-day variations.

To test the intra-day repeatability, 10 runs in a row were performed, giving standard deviation of 1.1 ‰, 0.9 ‰ and 0.9 ‰ for citric acid, glucose and fructose, respectively. To test the inter-days repeatability, the same sample was injected in every sample batch during an 8-week period of time ($n_{TOT} = 19$ measurements), giving standard deviation of 1.1 ‰, 0.8 ‰ and 0.9 ‰ for citric acid, glucose and fructose, respectively.

3.2. Reference ranges for the isotopic ratios of Italian tomato passata without added citric acid

The $\delta^{13}C_{CA}$, $\delta^{13}C_{G}$ and $\delta^{13}C_{F}$ results obtained for the authentic Italian samples, together with their standard deviation and maximum/minimum limit are reported in Table 1. The maximum/minimum limit considering a confidence interval of 95 % has been calculated as the mean value plus/minus two times the standard deviation.

Table 1
Mean carbon isotopic ratio, standard deviation, maximum and minimum limits for citric acid ($\delta^{13}C_{CA}$), glucose ($\delta^{13}C_{G}$) and fructose ($\delta^{13}C_{F}$) of Italian tomato passata samples without added citric acid.

	$\delta^{13}C_{CA}$ (‰, vs V-PDB)	$\delta^{13}C_{G}$ (‰, vs V-PDB)	$\delta^{13}C_{F}$ (‰, vs V-PDB)
mean	−26.5	−27.9	−27.8
st. dev.	1.3	1.2	1.1
max. Limit	−23.9	−25.6	−25.6
min. Limit	−29.1	−30.3	−30.1

3.2.1. Carbon isotopic ratio reference values for the citric acid naturally present in Italian tomato passata

The $\delta^{13}C_{CA}$ of Italian tomato passatas without added citric acid ranged from −31.3 ‰ to −23.0 ‰ (the mean $\delta^{13}C_{CA} \pm 2\sigma$ are reported in Fig. 1). The complete dataset is reported in Table 2S. Since the samples were produced starting from tomatoes harvested in two different years (2022 and 2023), a *T*-Test was performed to check for the absence of statistical differences. This evidence was expected, since seasonality does not noticeably influence the $\delta^{13}C$ of the products (Riddle et al., 2022).

Although no reference values are reported in the literature for tomatoes $\delta^{13}C_{CA}$, a comparison with the isotopic values already reported for other fruits can be considered. Bononi et al., in a study carried out on 20 authentic Italian lemon samples, reported values of −25.0 ± 1.1 ‰ for the $\delta^{13}C_{CA}$ (Bononi et al., 2016). Another work on orange and tangerine samples of various geographical origins reported mean $\delta^{13}C_{CA}$ of −23.6 ± 1.0 ‰ and − 25.5 ± 1.1 ‰, respectively (Jamin et al., 1998). Moreover, Jamin and coworkers reported $\delta^{13}C_{CA}$ of −23.8 ± 0.7 ‰ for oranges, −24.8 ± 1.1 ‰ for lemon, −25.3 ± 0.3 ‰ for grapefruit, −24.3 ± 1.0 ‰ for raspberry, −24.4 ± 0.8 ‰ for strawberry and − 24.7 ± 0.6 ‰ for black currant samples (Jamin et al., 2005). Despite the difference in the matrix considered, the reported values are comparable with the results obtained in the present study and fall within the range from −30 ‰ to −23 ‰ reported for the $\delta^{13}C$ of the C3 plants (Camin et al., 2004, Camin et al., 2008; Knobbe et al., 2006; Molkentin, 2009).

The citric acid biosynthesis is based on the submerged fermentation mediated mainly by the fungus *A. Niger* (Behera et al., 2021). To allow its growth, the fungus is normally fed on cheap starting materials, generally cane sugar solutions as carbon source (Almakki et al., 2019). Unlike tomatoes (C3 plants), cane belongs to the C4 plant group, that uses a different photosynthetic cycle for CO_2 fixation, resulting in different $\delta^{13}C$ which normally range between −14 and − 12 ‰ (O'Leary, 1988). In this way, the biosynthetic citric acid produced by the *A. Niger* will have different $\delta^{13}C$ values from those of the citric acid naturally present in the tomato passata. As demonstrated in this study (Table 1), $\delta^{13}C$ values above −23.9 % indicate the presence of biosynthetic citric acid added to the tomato passata.

Nevertheless, it must be mentioned that also sugar beet and beet molasses represent a source for citric acid production through *A. niger* fermentation (Latif et al., 2024). In this case, citric acid $\delta^{13}C$ would not be enough for the detection of biosynthetic citric acid additions and would have to be supported by other isotopic data, such as for example the deuterium isotopic ratio.

3.2.2. Carbon isotopic ratio reference values for the glucose and fructose of Italian tomato passata

The $\delta^{13}C$ of the Italian authentic tomatoes considered in the present study ranged from −31.3 ‰ to −25.3 ‰ for glucose and from −31.0 ‰ to −25.1 ‰ for fructose (the mean $\delta^{13}C_{G} \pm 2\sigma$ and $\delta^{13}C_{F} \pm 2\sigma$ are reported in Fig. 1). The complete dataset is reported in Table 2S. According to the AIJN, the reference values for tomato sugars range between −28 ‰ and − 25 ‰ (AIJN European Fruit Juice Association, 2024). The upper limit is respected for both glucose and fructose of all the authentic samples included in this study (Table 1). On the other hand, around 46 % of the authentic samples fell below the lower limit value. This may be likely due to the intrinsic nature of Italian tomatoes, whose $\delta^{13}C$ variability for the sugars might be influenced by the geographical location where the tomatoes were cultivated and harvested.

Nevertheless, it is worth mentioning that the addition of exogenous sugars of C4 origin is among the most frequent adulterations to tomato passata. Therefore, the limit that is the most of our interest to be

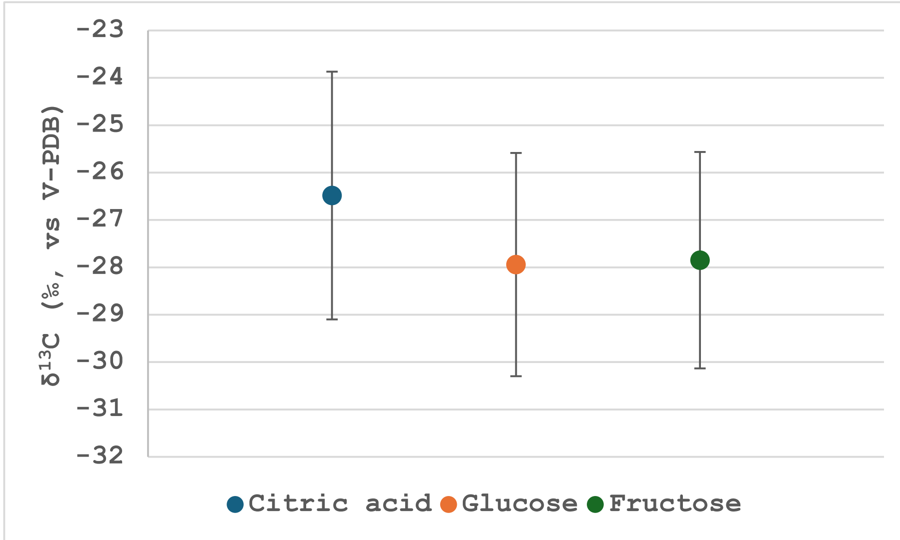

Fig. 1. Mean carbon isotopic ratios (δ^{13}C‰, vs V-PDB) of citric acid, glucose and fructose for authentic Italian tomato passata samples. The upper and lower bars correspond to the 2σ of each dataset.

Table 2
Mean ratios $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$), standard deviation, maximum and minimum limits of Italian tomato passata samples without added citric acid.

	$R_{CA/G}$	$R_{CA/F}$	$R_{G/F}$
mean	0.95	0.95	1.00
St. Dev.	0.04	0.04	0.02
max. Limit	1.04	1.04	1.05
min. Limit	0.86	0.87	0.96

respected, is the upper one.

To the best of our knowledge, no reference values for the δ^{13}C of tomato glucose and fructose are available in the literature. Nevertheless, considering that sugars are among the most abundant tomato constituents (Ali et al., 2020), the δ^{13}C of both glucose and fructose could be compared to the δ^{13}C of the bulk samples. The values are in agreement with previous studies on bulk tomatoes from Italy having δ^{13}C ranging from −29.9 to −26.0 ‰ according to Bontempo et al. (Bontempo et al., 2020) and mean $\delta^{13}C = -27.6 \pm 1.1$ ‰ according to Mahne Opatić et al. (Mahne Opatić et al., 2018). Moreover, the results were also comparable to previous studies regarding tomato coming from Slovenia (mean $\delta^{13}C = -27.4 \pm 0.6$ ‰), Spain (mean $\delta^{13}C = -26.6 \pm 0.7$ ‰) and Morocco (mean $\delta^{13}C = -27.3 \pm 0.9$ ‰) (Mahne Opatić et al., 2018).

3.2.3. Ratios between the isotopic values to increase method effectiveness

Previous studies demonstrated that the combination of different isotopic parameters is more effective than considering them individually in detecting possible additions of exogenous compounds to a product (Bononi et al., 2016; Guyon et al., 2011). Therefore, it was of our interest to check if the inclusion of the ratios $R_{CA/G}$ and $R_{CA/F}$ in the detection of biosynthetic citric acid addition to tomato passata samples was more efficient than considering the $\delta^{13}C_{CA}$ only.

First, using the data reported in Section 3.2 for tomato passata without added citric acid, the ratio between the δ^{13}C of both citric acid and sugars were calculated and expressed as $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$) (Table 2). The maximum/minimum limit of the Rs has been calculated as the mean value plus/minus two times the standard deviation.

Second, to test the effectiveness of this approach to improve the possibility of identifying biosynthetic citric acid additions, a tomato passata sample (having a δ^{13}C value of −27.7 ‰, which will formally correspond to the 0 % addition) has been chosen to simulate a spiked product. Ten aliquots of the mentioned tomato passata sample were added with growing concentrations (10 % to 100 %) of biosynthetic citric acid with a δ^{13}C value of −18.7 ‰. The $\delta^{13}C_{CA}$ was therefore measured in each aliquot and the $R_{CA/G}$ and $R_{CA/F}$ were calculated (Table 3).

As reported in Table 3, the $\delta^{13}C_{CA}$ was efficient in the detection of biosynthetic citric acid when added in a concentration higher than 40 % in the selected tomato passata sample without added citric acid. Since the limit for authentic samples was set at $\delta^{13}C_{CA} = -23.9$ ‰, tomato passata samples having less negative $\delta^{13}C_{CA}$ values than this threshold would have been declared as added with biosynthetic citric acid (in grey, Table 3). On the other hand, additions of biosynthetic citric acid higher than 10 % can be detected when considering $R_{CA/G}$ and higher than 20 % when considering $R_{CA/F}$. Therefore, a much higher effectiveness in the detection of added biosynthetic citric acid can be improved when using these ratios to characterise a sample. It must be noted that for samples having a less negative $\delta^{13}C_{CA}$ starting value (corresponding to the $\delta^{13}C_{CA}$ at 0 % addition, which is −27.7 ‰ in the present study, Table 3) the addition of biosynthetic citric acid might be detected at lower concentrations of biosynthetic citric acid added. Nevertheless, in this situation, the $R_{CA/G}$ and $R_{CA/F}$ values would still be detecting the addition at lower concentration of biosynthetic citric acid added than the $\delta^{13}C_{CA}$ itself.

Table 3
Citric acid carbon isotopic ratio ($\delta^{13}C_{CA}$), ratios $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$) and $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) of an Italian tomato passata sample added with growing concentrations (%) of biosynthetic citric acid; grey cells indicate samples detected as added according to the limits stated for each parameter.

Citric acid addition (%)	$\delta^{13}C_{CA}$ (‰, vs V-PDB)	$R_{CA/G}$	$R_{CA/F}$
0	−27.7	0.92	0.93
10	−25.7	0.85	0.87
20	−24.3	0.79	0.80
30	−24.6	0.79	0.80
40	−23.5	0.77	0.81
50	−22.4	0.74	0.74
60	−22.4	0.73	0.70
70	−20.3	0.66	0.67
80	−21.2	0.68	0.68
90	−19.5	0.64	0.64
100	−18.7	0.64	0.64

The mean $R_{CA/G}$ and $R_{CA/F}$ for the Italian tomato passatas without added citric acid were calculated as 0.95 ± 0.04 and 0.95 ± 0.04, respectively (Table 2). Values falling above the maximum limit for these ratios will indicate the addition of biosynthetic citric acid, while values below the minimum limit of $R_{CA/G}$ and $R_{CA/F}$ will indicate the addition of, respectively, glucose and fructose of C4 origin. The ratio between glucose and fructose isotopic values was considered as well. The mean $R_{G/F}$ of the Italian samples is 1.00 ± 0.02 (Table 2). Values falling above the maximum limit will indicate the addition of C4-glucose, while values falling below the minimum limit will indicate the addition of C4-fructose.

3.3. Evaluation of commercial samples

It is important to note that the addition of citric acid (E330) to tomato passata is permitted under EU legislation (Regulation (EU) No 1129/2011) when properly declared on the label. Therefore, the detection of biosynthetic citric acid should not be interpreted as an adulteration per se but may indicate either a lawful addition or, if undeclared, a potential labelling non-compliance.

The reference values defined for tomato passata without added citric acid set for both $\delta^{13}C_{CA}$, $\delta^{13}C_G$ and $\delta^{13}C_F$ (Table 1) and $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$) (Table 2) have been applied to the 59 commercial samples to check for any addition. The results are reported in Table 2S of the Supplementary Materials.

Based on the $\delta^{13}C_{CA}$, 37 % of the commercial samples considered in this study had values falling above the maximum stated limit ($\delta^{13}C_{CA\ min}$ = −23.9 ‰) (Light grey, Table 2S) and therefore showed values consistent with the presence of biosynthetic citric acid (Table 4), while 3 % had values of $\delta^{13}C_{CA}$ falling below the minimum stated limit ($\delta^{13}C_{CA\ max}$ = −29.1 ‰) (Dark grey, Table 2S).

As for the sugars, according to the maximum and minimum limits for $\delta^{13}C_G$ and $\delta^{13}C_F$ defined in the present study, 10 % and 5 % of the commercial samples could be considered as added with exogenous C4-glucose and C4-fructose, respectively (Table 4). On the other hand, none of the commercial samples showed values falling below the minimum limit stated in this work. As already mentioned, unlike citric acid, for which no reference values were available, the bulk sugar $\delta^{13}C$ is provided by the AIJN (−28 ‰ < $\delta^{13}C_{CA}$ < −25 ‰). Therefore, when evaluating the $\delta^{13}C$ of both glucose and fructose in the commercial products, it is also worth considering the AIJN limits and comparing them to the results obtained in the present study. Values falling above/below these maximum/minimum limits are labelled (as "*" and "#", respectively) in Table 2S. Among commercial samples, up to 5 % were considered as added with exogenous C4-sugar (either glucose or fructose) based on the AIJN maximum limit. On the other hand, 39 % and 32 % of the commercial samples had respectively $\delta^{13}C_G$ and $\delta^{13}C_F$ values falling below the minimum limit stated by the AIJN for the bulk sugars. A considerable proportion of authentic samples fell below the AIJN minimum limit, suggesting that this threshold may not be fully suitable for Italian tomatoes. This should be taken into account when evaluating commercial products. Once again, it must be highlighted that the limit to be respected when authenticating a fruit sample based on the $\delta^{13}C$ of the sugars is the upper one.

To better interpret the information given by the R values, it must be considered that samples having values falling below the $R_{CA/G}$ and $R_{CA/F}$ minimum limit are considered as added with biosynthetic citric acid. On the other hand, samples having values falling above the $R_{G/F}$ and $R_{CA/F}$ maximum limit are considered as added with C4-derived fructose. Finally, samples having values falling above the $R_{CA/G}$ maximum limit and below the $R_{G/F}$ minimum limit are considered as containing added C4-derived glucose.

The application of $R_{CA/G}$ and $R_{CA/F}$ confirmed their usefulness, as they identified a higher proportion of samples containing added biosynthetic citric acid compared to $\delta^{13}C_{CA}$ alone (Table 5). Values of $R_{CA/G}$ and $R_{CA/F}$ falling below the minimum limit stated in this study indicated, as just mentioned, the addition of biosynthetic citric acid to the tomato passata (Dark grey, Table 2S). Regarding the detection of added biosynthetic citric acid, 41 % of the sample were identified as added with biosynthetic citric acid based on both $R_{CA/G}$ and $R_{CA/F}$ (Table 5), which represents a higher percentage compared to the 37 % resulting from the analysis of the $\delta^{13}C_{CA}$ alone (Table 4).

As for the sugars, the best results were obtained considering the combination of both $\delta^{13}C_G$, $\delta^{13}C_F$ (Table 4) and the R values (Table 5). Samples added with C4-glucose were more efficiently detected thanks to the $\delta^{13}C_G$ (10 %, Table 4), than the $R_{CA/G}$ maximum limit (3 % samples above this limit, Table 5) or the $R_{G/F}$ minimum limit (3 % samples below this limit, Table 5). On the other hand, samples added with C4-fructose were more efficiently detected thanks to the $R_{CA/F}$ (7 % samples above this limit, Table 5) than the $R_{G/F}$ maximum limit (2 % samples above this limit, Table 5) or the $\delta^{13}C_F$ (5 % samples above this limit, Table 4).

4. Conclusions

In the present paper, the need for a method to detect additions of biosynthetic citric acid to Italian tomato passatas was addressed. To perform the analysis, a new approach through LC-co-IRMS has been adopted, and the $\delta^{13}C$ of citric acid, glucose and fructose have been measured. The mean $\delta^{13}C_{CA}$, $\delta^{13}C_G$ and $\delta^{13}C_F$ for Italian tomato passata samples without added citric acid resulted as −26.5 ± 1.3 ‰, −27.9 ± 1.2 ‰ and − 27.8 ± 1.1 ‰, respectively.

The mean ratios $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$) on the samples without added citric acid have also been considered, resulting as 0.95 ± 0.04, 0.95 ± 0.04 and 1.00 ± 0.02, respectively.

Once assessed the carbon isotopic variability of the citric acid for the Italian tomato passata, the ranges were applied to 59 commercial samples available on the market. Among these, the $\delta^{13}C_{CA}$ of 37 % of the samples fell above the maximum limit stated in this work, indicating the addition of biosynthetic citric acid to the tomato passata. Nevertheless, when $R_{CA/G}$ and $R_{CA/F}$ were considered, the percentage of detected addition increased to 41 %. The effectiveness in detecting the addition of biosynthetic citric acid to samples was therefore higher when considering the ratios $R_{CA/G}$ and $R_{CA/F}$ rather than the $\delta^{13}C_{CA}$ alone.

Moreover, the $\delta^{13}C_G$ and $\delta^{13}C_F$ have been compared to the reference

Table 4
Percentage of commercial samples detected as having added citric acid according to the maximum/minimum $\delta^{13}C_{CA}$, $\delta^{13}C_G$ and $\delta^{13}C_F$ limits either stated in this study or provided by the AIJN (where available).

	$\delta^{13}C_{CA}$ (‰, vs V-PDB)	$\delta^{13}C_G$ (‰, vs V-PDB)	$\delta^{13}C_F$ (‰, vs V-PDB)
Above this study maximum limit	37 %	10 %	5 %
Below this study minimum limit	3 %	0 %	2 %
Above AIJN-derived maximum limit	–	5 %	3 %
Below AIJN-derived minimum limit	–	39 %	32 %

Table 5
Percentage of commercial samples detected as having added citric acid according to the maximum/minimum $R_{CA/G}$ ($\delta^{13}C_{CA}/\delta^{13}C_G$), $R_{CA/F}$ ($\delta^{13}C_{CA}/\delta^{13}C_F$) and $R_{G/F}$ ($\delta^{13}C_G/\delta^{13}C_F$) limits stated in this study. The biosynthetic citric acid (*), C4-glucose (#) and C4-fructose (§) additions are highlighted by symbols.

	$R_{CA/G}$	$R_{CA/F}$	$R_{G/F}$
Above this study maximum limit	3 % #	7 % §	2 % §
Below this study minimum limit	41 % *	41 % *	3 % #

values provided by the AIJN for tomato bulk sugar. A higher number of commercial samples was labelled as added with C4 exogenous sugar when considering the maximum limit of $\delta^{13}C_G$ and $\delta^{13}C_F$ stated in the present study (10 % and 5 %, respectively), rather than the AIJN maximum limit (3 % for both $\delta^{13}C_G$ and $\delta^{13}C_F$).

The method also demonstrated good repeatability, with intra-day and inter-day standard deviations below 1.1 ‰ for citric acid, glucose and fructose.

Since the $\delta^{13}C_{CA}$ in this work has been analysed on Italian samples only, foreign samples should be included in further studies to make the maximum/minimum limits of this work even more general.

CRediT authorship contribution statement

Silvia Pianezze: Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis, Data curation. **Domenico Masuero:** Validation, Formal analysis. **Matteo Perini:** Writing – review & editing, Software. **Stefania Carpino:** Writing – review & editing, Project administration, Conceptualization. **Nicola Barilaro:** Supervision. **Vincenzo Di Martino:** Supervision. **Luana Bontempo:** Writing – review & editing, Project administration, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.146924.

Data availability

Data will be made available on request.

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