

1 BULK AND COMPOUND-SPECIFIC STABLE ISOTOPE RATIO ANALYSIS FOR
2 AUTHENTICITY TESTING OF ORGANICALLY GROWN TOMATOES

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35 **Abstract**

36 Until now, there has been a lack of analytical methods that can reliably verify the authenticity of
37 organically grown plants and derived organic food products. In this study, stable isotope ratio analysis
38 of hydrogen (H, $\delta^2\text{H}$), carbon (C, $\delta^{13}\text{C}$), nitrogen (N, $\delta^{15}\text{N}$), oxygen (O, $\delta^{18}\text{O}$) and sulfur (S, $\delta^{34}\text{S}$) was
39 conducted along the tomato passata production process using organic and conventionally grown
40 tomatoes from two Italian regions over two years. A gas chromatography–combustion–isotope ratio
41 mass spectrometry (GC-C-IRMS) based method was developed and applied for analysis of C and N
42 isotope ratios in amino acids derived from tomatoes. Of the bulk isotope ratios, $\delta^{15}\text{N}$ was the most
43 significant parameter for discriminating organic from conventional products. The classification
44 power was improved significantly by compound-specific isotope analysis regardless of the production
45 years and regions. We conclude that isotope analysis of amino acids is a novel analytical tool for
46 complementing existing certification and control procedures in the organic tomato sector.

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49 **Keywords:** Amino acids; Compound-specific; Organic; Stable isotope ratios; Tomatoes

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1. Introduction

Food products, bearing the organic label, should be produced without synthetic fertilizers, hormones, genetic engineering, sewage sludge, or irradiation (Council Regulation (EC) No. 834/2007). Organic farmers, processors and traders, must comply with these regulations, if they want to use the EU organic logo and sell their products as organic. In 2008 the European Council of Agricultural Ministers agreed on a new Council Regulation (EC No 967/2008) setting out the principles, aims and rules for organic production as well as defining how organic products can be labelled was instituted. Despite the strict EU regulations for food and food products to be classified as organic, there has been a lack of analytical methods that can reliably verify the authenticity of organic produce (Kahl et al., 2014; Hong et al., 2017; Kelly & Mihailova, 2017; Laursen Schjoerring, Kelly, & Husted, 2014). Tomatoes are one of the most frequently consumed horticultural products worldwide with 182 million tons of tomatoes grown globally in 2017 (FAOSTAT data, accessed December 2019). Italy is the second main producer of tomatoes in Europe with 6 million tons produced in 2017 (FAOSTAT data, accessed December 2019). Italian production of organic tomatoes has almost doubled in two years, with a production of 162,619 tons of organic tomatoes in 2017 (http://www.tomatonews.com/en/italy-the-production-of-organic-tomatoes-has-almost-doubled-in-two-years_2_219.html). The choice of fertilizer and other agricultural management practices are critical in growing tomatoes and will differ depending on whether the tomatoes are grown organically or conventionally. The prohibition of synthetic fertilizers in organic production does potentially impact the availability of nitrogen including the ratio of $^{15}\text{N}/^{14}\text{N}$, expressed as $\delta^{15}\text{N}$ (Masclaux-Daubresse et al., 2010; Styring, Fraser, Bogaard, & Evershed, 2014a). Synthetic fertilizers have $\delta^{15}\text{N}$ values from -6 to +6‰, which is lower than those of organic fertilizers, ranging between +0.6 and +36.7‰ (manure between +10 and +25‰) (Bateman & Kelly, 2007). Plants grown conventionally with synthetic fertilizers therefore often have lower $\delta^{15}\text{N}$ values than those grown organically (Laursen, Schjoerring, Kelly, & Husted, 2014; Nakano & Uehara, 2007). The roots of non- N_2 -fixing plants take up nitrogen from soil as either ammonium (NH_4^+) or nitrate (NO_3^-) and the $\delta^{15}\text{N}$ value of the fertilizer, synthetic or organic, will be reflected in the plant (Bateman & Kelly, 2007). Assimilation of NO_3^- and NH_4^+ in plants occurs differently. Indeed, NH_4^+ , once taken up by the roots, is metabolized by glutamine synthetase and enters directly in the amino acids metabolism of the plant. On the other hand, NO_3^- is sequentially reduced by nitrate and nitrite reductase enzymes to nitrite and then ammonia (Lea & Azevedo, 2017). Through the assimilation of ammonia, glutamine synthetase produces the first amino acid, glutamine, and plays a central role in the amino acids metabolism. Due to the transfer of the glutamine amide to keto acids and transamination, plants produce glutamate and then other amino acids (Wada, 1995).

89 However, the impact of nitrogen assimilation on stable isotope signatures of plant-derived amino
90 acids is still poorly understood.

91 Several studies have shown that bulk tissue analysis of stable isotope ratios of N, sometimes in
92 combination with those of C, O, H and S, together with statistical methods, has the potential to
93 differentiate vegetables and fruits grown under different farming systems (Bateman & Kelly, 2007;
94 Camin et al., 2011; Cristea, Feher, Magdas, Voica, & Puscas, 2017; Flores, López, Fenoll, Hellín, &
95 Kelly, 2013; Georgi, Voerkelius, Rossmann, Graßmann, & Schnitzler, 2005; Mahne Opatić, Opatić,
96 Nečemer, Kocman, & Lojen, 2017; Rapisarda et al., 2010; Schmidt et al., 2005; Wada, 1995).
97 However, it has been shown that compound-specific isotope ratio analysis is more powerful (Laursen
98 et al., 2013; Mihailova, Pedentchouk, & Kelly, 2014; Novak, Adler, Husted, Fromberg, & Laursen,
99 2019; Paolini, Ziller, Laursen, Husted, & Camin, 2015; van Leeuwen, Prenzler, Ryan, & Camin,
100 2014). The application of bulk tissue stable isotope ratio analysis of N is challenged by the fact that
101 N₂ fixing-plants, used as cover crops or green manures in organic crop rotations, have $\delta^{15}\text{N}$ values
102 overlapping with those of conventionally grown plants due to the atmospheric origin of N in both
103 cases (Laursen et al., 2013; Paolini et al., 2015). This can be by-passed by stable isotope ratio analysis
104 of N and O from nitrate or of O from sulfate, which has proven to be promising for authentication of
105 organic vegetables (Novak, Adler, Husted, Fromberg, & Laursen, 2019; Laursen et al., 2013;
106 Mihailova et al., 2014). Another approach is GC-C-IRMS based isotope ratio analysis of amino acids,
107 which has already proven valuable for discriminating organic and conventional cereals (Paolini et al.,
108 2015).

109 In the present study, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values were measured along the production chain
110 of tomato passata (soil – tomato – passata), from tomatoes grown organically and conventionally in
111 two regions of Italy. Furthermore, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were determined in amino acids extracted from a
112 set of organic and conventional tomatoes from Italy and Denmark. The overall aim of the study was
113 to identify the most suitable isotope parameters for authenticity testing of organically grown
114 tomatoes.

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116 **2. Materials and Methods**

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118 **2.1 Standards and reagents**

119 The standards L-Alanine ($\geq 98\%$); Glycine ($\geq 99\%$); L-Valine ($\geq 98\%$); L-Isoleucine ($\geq 98\%$); L-
120 Leucine ($\geq 98\%$); L-nor Leucine ($\geq 98\%$); L-Proline ($\geq 99\%$); L-Aspartic acid ($\geq 98\%$); L-Glutamic
121 acid ($\geq 99\%$); L-Glutamine ($\geq 99\%$); L-Asparagine ($\geq 98\%$); L-Phenylalanine ($\geq 98\%$); L-Threonine
122 ($\geq 98\%$) and the analytical grade Amberlite® IR120 cation-exchange resin (hydrogen form) were
123 purchased from Sigma-Aldrich (Milan, Italy). Triethylamine ($\geq 99.5\%$) and acetic anhydride ($\geq 99\%$)

124 reagents were purchased from Sigma-Aldrich (Milan, Italy). Analytical grade acetone, isopropanol,
125 diethyl ether, dichloromethane and ethyl acetate were purchased from VWR (Milan, Italy) and Sigma-
126 Aldrich (Milan, Italy). Petroleum ether (40-60%) was purchased from VWR Chemicals (Milan, Italy).

128 **2.2 Tomato samples and growth conditions**

129 Samples of tomato (*Solanum lycopersicum* L.) were obtained directly from farms (on-farm trials)
130 during 2012 and 2013, in two different Italian regions, viz. Emilia Romagna (in Central Italy) and
131 Basilicata (in Southern Italy). In both regions, the tomatoes were grown under two different systems:
132 organic (Org) or conventional (Conv). Tomatoes grown within the organic regimen were managed in
133 compliance with the EU regulations for organic farming (European Community Council Regulation
134 EC 834/2007) and had been organic for more than 10 years prior to 2012. The systems were all based
135 on stockless crop production, but were managed differently from each other, due to differences in
136 climate, soil types and the structure of the farm. In the organic production, organic fertilizers and
137 mechanical weed management were applied, and in the conventional production a combination of
138 organic and mineral fertilizers was used as well as chemical weed protection agents. Irrigation was
139 implemented on both the organic and conventional fields. At each location and with both systems, 2
140 varieties were grown; Roma (long tomato) and San Marzano (round tomato). The tomatoes were
141 planted at the end of April/beginning of May each year and were harvested at maturity from mid-
142 August to early September depending on the region. The total number of tomato samples was 48 (2
143 locations, 2 years, 2 systems, 2 varieties and 3 replicate sampling times).

144 Organically grown tomato plants were grown in large plots of a minimum of 1000 m². Tomatoes were
145 sampled on three separate occasions whereby all tomatoes that were ripe on the day of collection
146 were harvested in a large batch and then a small sub-sample of 2 kg was selected for analysis. Due to
147 extreme drought in 2012 a plot in Emilia Romagna was lost and could not be sampled. Harvesting
148 quantities were 20–30 tonnes/ha in the organic system compared to 50–80 tonnes/ha in the
149 conventional one in Basilicata. In Emilia Romagna, 50–60 tonnes/ha was harvested in the organic
150 system and 70–90 tonnes/ha in the conventional system. A soil sample from each region and growth
151 system was also collected (8 in total). The soil in Basilicata was loamy, whereas the soil in Emilia
152 Romagna consisted of clay. Details regarding tomato sample information like varieties, farming
153 system details, climatic data and fertilizers application and use are shown in Tables 1, 2, 3 and 4 of
154 the Supplementary Material.

156 Fresh tomatoes were stored at 10 °C for a maximum of 5 days and were then transported to a
157 processing plant in refrigerated vehicles. The processing from tomatoes to passata was only physical,
158 through a ‘cold-break treatment’ (Hayes, Smith, & Morris, 1998). Briefly, tomatoes were cut into

159 pieces and were then boiled with salt at a temperature less than 60 °C. The tomato flesh was separated
160 mechanically from peels and seeds using a pulper with a hole size of around 1.0-1.3 mm, before being
161 vacuum-concentrated to around 8 °Brix. Passata was then bottled and pasteurized. This type of
162 treatment preserves the bright red color, fragrance and full taste of fresh harvested tomatoes. In
163 Basilicata, 4 passata were produced for both organic and conventional systems, whereas 2 passata per
164 agricultural system were sampled in Emilia Romagna (12 samples in total).
165 In order to test the applicability of the developed analytical method for organic authenticity testing
166 across countries and production methods, a test sample set consisting of 13 different greenhouse
167 grown tomatoes was collected from Danish growers. Of these samples, 8 were organic and 5 were
168 conventional and included a wide range of tomato varieties (classic, plum, snack, cocktail, cherry or
169 San Marzano) and different genotypes, growth medias and fertilization strategies. Organic tomato
170 samples were grown either in soil or in peat mixture. In contrast, conventional samples were grown
171 in artificial growth media (rock wool) or fibers. Organic samples were fertilized with animal manure,
172 garden waste or vegetable-based liquid fertilizer mixture, whereas conventional samples were
173 fertilized with liquid inorganic fertilizers.
174 All tomato and passata samples were subject to H, O, N, C and S stable isotope ratios analysis on
175 bulk tissue. A number of samples of Italian tomatoes (N=16), randomly selected from the complete
176 set of samples in order to equally cover the agricultural systems, regions and years, were also used
177 for C and N stable isotope ratio analysis of amino acids. In addition, the Danish tomatoes test set was
178 analyzed for C and N stable isotope ratios of amino acids. All the soils from field-grown tomatoes
179 were analyzed for bulk N and C stable isotope ratios.

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181 **2.3 Sample preparation and bulk analysis**

182 Approximately 2 kg of mature tomato fruits were taken for each sample. Samples were rinsed in
183 ultrapure water (Milli-Q Element, Millipore), frozen and freeze-dried (SvanVac Coolsafe 95-15 PRO)
184 at 1.0 mbar until complete dryness. Samples were crushed in a plastic bag followed by milling into a
185 fine powder using a 200 µm mesh size titanium sieve and a titanium rotor in a centrifugal mill (ZM
186 200 Retsch GmbH, Haan, Germany) and stored in plastic bags at room temperature until analysis.
187 The soil was air-dried (at room temperature) and sieved (<2 mm), while passata samples were freeze-
188 dried and then ground into fine powder.

189 Tomato and passata samples were measured for stable isotope ratios of C, N, S, H and O. The bulk
190 sample (0.5 mg) was weighed into tin capsules, and was combusted in an elemental analyser (VARIO
191 CUBE, Elementar Analysensysteme GmbH, Germany) coupled to an isotope ratio mass spectrometer
192 (IRMS, Isoprime, Isoprime Ltd., UK) to measure the $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ and $^{34}\text{S}/^{32}\text{S}$ isotopic ratios in
193 one run. In the case of soils, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were determined using an elemental analyser (Flash EA

1112, Thermo Scientific, Bremen, Germany), interfaced through a ConFlo IV dilutor device (Thermo
 Finningan, Bremen, Germany) with a DELTA V Thermo Scientific isotope ratio mass spectrometer.
 A pyrolyser (Finnigan TC/EA, high temperature conversion elemental analyser, Thermo Scientific)
 coupled to an IRMS (Finnigan DELTA XP, Thermo Scientific) was used to measure the $^2\text{H}/^1\text{H}$ and
 $^{18}\text{O}/^{16}\text{O}$ isotopic ratios in one run for the bulk tomato and passata sample (0.5 mg) in silver capsules.
 Samples were measured in duplicate. The isotope ratios were expressed in $\delta\text{‰}$ against Vienna
 Standard Mean Ocean Water for $\delta^{18}\text{O}$ and $\delta^2\text{H}$, Vienna-Pee Dee Belemnite for $\delta^{13}\text{C}$, air for $\delta^{15}\text{N}$ and
 Vienna Canyon Diablo Troilite for $\delta^{34}\text{S}$ according to the following equation:

$$\delta i E = \frac{i R_{SA} - i R_{REF}}{i R_{REF}} \quad (1)$$

where R_{SA} is the isotope ratio measured for the sample and R_{REF} is the international standard isotope
 ratio. The isotopic values for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ were calculated against working in-house standards,
 which were themselves calibrated against international reference materials: potassium nitrate IAEA-
 NO3 (IAEA-International Atomic Energy Agency, Vienna, Austria) for $\delta^{15}\text{N}$, L-glutamic acid USGS
 40 (U.S. Geological Survey, Reston, VA, USA) for $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, fuel oil NBS-22, IAEA-CH-
 6 for $^{13}\text{C}/^{12}\text{C}$, barium sulphates IAEASO-5, NBS 127 (IAEA) and USGS 42 and USGS 43 for $\delta^{34}\text{S}$.
 Through the development of a linear equation, the values were calculated against two working
 standards. The isotopic values for $\delta^2\text{H}$ and $\delta^{18}\text{O}$ were calculated against KHS (Kudu Horn Standard,
 $\delta^2\text{H} = -35.3 \pm 1.1\text{‰}$ and $\delta^{18}\text{O} = +20.3 \pm 0.3\text{‰}$) and CBS (Caribou Hoof Standard $\delta^2\text{H} = -157 \pm 0.9\text{‰}$
 and $\delta^{18}\text{O} = +3.8 \pm 0.3\text{‰}$) through the development of a linear equation and implementing the
 comparative equilibration procedure (Dunn & Carter, 2018). These keratinous standards were used
 because of the absence of any international and matrix matched organic reference material. For the
 $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^{18}\text{O}$ and $\delta^2\text{H}$ the uncertainty of measurement (2 Std Dev) was 0.1‰, 0.2‰, 0.3‰,
 0.3‰ and 1‰, respectively.

217

218 **2.4 Preparation and GC-C-IRMS analysis of amino acids from tomatoes**

Extraction, hydrolysis, purification and derivatization of amino acids from tomatoes was carried out
 as described for wheat by Paolini et al. (2015). Protein was hydrolyzed with HCl which was
 subsequently derivatized to obtain the N-acetyl-isopropyl esters of the amino acids. GC-C-IRMS
 analysis determined the isotopic ratios and retention times for derivatized individual amino acids
 alanine (Ala), glutamate (Glx), glycine (Gly), isoleucine (Ile), leucine (Leu), phenylalanine (Phe),
 proline (Pro), threonine (Thr), and valine (Val). The amino acid glutamine was converted to glutamic
 acid, due to acid hydrolysis. Accordingly, the isotopic values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ for Glx reflect the
 isotopic values for carbon and nitrogen for both glutamate + glutamine (Paolini et al., 2015; Styring,

Fraser, Bogaard, & Evershed, 2014b; Styring et al., 2012). The analysis was carried out according to the method set up by Paolini et al. (2015), with a Trace GC Ultra (GC Isolink + ConFlo IV, Thermo Scientific) furnished with an autosampler (Triplus, Thermo Scientific) and for $\delta^{15}\text{N}$ analysis a HP-INNOWAX column (60 m x 0.32 mm i.d. x 0.25 μm film thickness, Agilent). Instrumental performance was monitored using a standard mixture of derivatized L-amino acids with the addition of an internal standard, L-norleucine (Nle), and their isotopic values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were measured during each analytical run as recommended by Yarnes and Herszage (Paolini et al., 2015; Yarnes & Herszage, 2017). Because of its absence in tomatoes, Nle was chosen as the internal standard for the samples and standard mix and its isotopic value was measured in each sample run. The L-amino acid EA-IRMS isotopic values determined for Nle were -27.6‰ for $\delta^{13}\text{C}$ and +14.0‰ for $\delta^{15}\text{N}$. When the difference between the values determined by EA-IRMS and GC-C-IRMS for the Nle for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were no more than 1.5‰ and 1.0‰, respectively, the analytical run was accepted.

239

240 2.5 Data analysis and corrections

The derivatized amino acid $\delta^{13}\text{C}$ values are a composite of the initial starting amino acid carbon and of the reagents implemented in the derivatization reaction. Due to the addition of these extra carbons, an empirical correction was applied to determine the $\delta^{13}\text{C}$ value of the amino acid (Docherty, Jones, & Evershed, 2001; Paolini et al., 2015).

The $\delta^{13}\text{C}$ values measured by EA-IRMS for the non-derivatized amino acid standards and by GC-C-IRMS for the derivatized amino acid standards were utilized to calculate the correction factors:

$$247 \quad n_{cd}\delta^{13}\text{C}_{cd} = n_c\delta^{13}\text{C}_c + n_d\delta^{13}\text{C}_d \quad (2)$$

where n is the number of moles of carbon, and the subscripts c, d, and cd represent the compounds of interest, the derivative group, and the derivatized compound, respectively. Measurement errors as well as the total analytical error, which arises from the different derivatization steps, were considered when calculating the $\delta^{13}\text{C}$ measurement of uncertainty:

$$252 \quad \sigma_c^2 = \sigma_s^2 \left(\frac{n_s}{n_c} \right)^2 + \sigma_{sd}^2 \left(\frac{n_s + n_d}{n_c} \right)^2 + \sigma_{cd}^2 \left(\frac{n_c + n_d}{n_c} \right)^2 \quad (3)$$

where n is the number of moles of carbon, and the subscripts c, s, d, cd and sd represent the non-derivatized compound, the non-derivatized standard, the derivatizing agent, the derivatized compound and the derivatized standard, respectively.

Accuracy and precision of GC-C-IRMS analysis is the same reported in Paolini et al. (2015). In particular, precision was on average $\pm 0.4\text{‰}$ for $\delta^{15}\text{N}$ and $\pm 0.8\text{‰}$ for $\delta^{13}\text{C}$ (1 Std Dev) and uncertainty was on average $\pm 0.3\text{‰}$ for $\delta^{15}\text{N}$ and $\pm 1.0\text{‰}$ for $\delta^{13}\text{C}$ (1 Std Dev).

259

260 2.6 Statistical analysis

The data were analyzed with Statistica (version 9, StatSoft Inc., Tulsa, OK, USA). To examine trends for different ratios along the production chain, a paired t-test was applied. Analysis of Variance (ANOVA) and Tukey test (HSD or for unequal number of samples) were applied to test differences related to agricultural system, production year, region and type of sample (soil, tomato, passata). Kolmogorov-Smirnov and Shapiro-Wilk tests were used to check if data were normally distributed. Glutamine and glutamic acid isotopic values are central to all amino acid isotopic values as all amino acids are derived from these. Single amino acid isotopic values were therefore normalized against corresponding Glx values to control differences associated with peripheral sources of nitrogen, such as locality (Paolini et al., 2015; Styring et al., 2014b). Principal Component Analysis (PCA) was performed on bulk data for $\delta^2\text{H}$, $\delta^{18}\text{O}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ in tomatoes and on data for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the amino acids from tomatoes. PCA allows multidimensional data to be projected onto low dimensional subspaces, highlighting the direction of maximum variability in the original dataset. PCA results are presented in graphical form as score and loading plots. In the score plot, each point represents the position of each sample in the isotopic space. In contrast, the loading plot shows the contributions of each isotope ratio to the principal components. All PCA analyses were performed on mean centered and scaled datasets. Finally, a full cross-validation was applied to test the model.

278

279 **3. Results and Discussion**

280

281 **3.1 Trends of bulk tissue isotope values along the tomato production chain**

To determine the behavior of the five isotope ratios during passata production for the two different production systems, eight production chains (from soil to tomatoes to passata), randomly selected, were considered (4 from Emilia Romagna, 4 from Basilicata, 4 from organic systems and 4 from conventional systems) (Figure 1). A paired t-test was applied to check statistically significant differences among the matrices collected along the production chain (soil versus tomato and versus passata, tomatoes versus passata) for the five isotope ratios. The differences between the tomatoes and passata were not statistically different according to the paired t-test ($p>0.05$), even when considering separately the organic and conventional production chains. The only exception was $\delta^{13}\text{C}$ between tomatoes and passata when considering only organic samples or all the samples together ($p<0.01$). It is worth noting that $\delta^{13}\text{C}$ of passata presented higher values ($p<0.001$) compared to tomatoes, also at Tukey test (Table 1). This can be explained by the loss of peel and seeds during the preparation of passata. In a previous study, apple peels were reported to have more depleted values compared to pulp (Mimmo et al., 2015).

295 Except for $\delta^{13}\text{C}$, comparable results were found in a previous study (Bontempo et al., 2011; Mimmo
296 et al., 2015), where statistically significant differences were not observed in the processing from
297 tomatoes to juice and finally to passata for the five isotope ratios. From these results, it was evident
298 that the production of passata did not alter the isotopic content systematically in either of the two
299 agricultural systems. This was also the case for oxygen and hydrogen isotope ratios, despite potential
300 evaporation during the production. This could be explained by the fact that during the ‘cold-break’
301 process the heating of the chopped tomatoes was carried out at low temperatures (lower than 60°C)
302 (Hayes, Smith, & Morris, 1998). Significant differences were found for $\delta^{13}\text{C}$ values between tomatoes
303 and soils ($p<0.001$, Table 1)). In particular, the $\delta^{13}\text{C}$ values of the soils were higher with respect to
304 the corresponding tomatoes, with a mean difference of 2‰. Similar results were previously observed
305 in another study on apples, pears and grapes (Dehelean, Magdas, & Cristea, 2013). The C of plant
306 materials is derived from atmospheric CO_2 and not from the soil, which may explain the lack of
307 relationship. Carbon isotope ratios of soil are instead expected to be related to the input of organic
308 matter from previous growth years. For $\delta^{15}\text{N}$, no statistically significant differences were observed
309 between soils and tomatoes. This close relationship was expected as N in plants is derived from plant
310 available N in the soil during the growth season.

311

312 3.2 Bulk data in the different matrices

313 In Table 1 the means and standard deviations of $\delta^{13}\text{C}$, $\delta^2\text{H}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^{18}\text{O}$ values in soils, tomatoes
314 and passata are presented according to the origin and the agricultural system. The impact of product
315 type (soil, tomatoes and passata), production year (2012 versus 2013), tomato variety (long versus
316 round), geographic origin and conventional versus organic products was statistically evaluated. No
317 statistically significant differences ($p>0.05$) were found according to the variety or the production
318 year for all the isotope ratios. ANOVA test found statistically significant differences ($p<0.001$)
319 according to the agricultural system and the geographic origin.

320 Considering the geographical origin, significant differences were found for $\delta^{18}\text{O}$ and $\delta^{34}\text{S}$ in tomatoes
321 ($p<0.001$) and $\delta^{34}\text{S}$ of passata ($p<0.001$) (Table 1). In particular, higher $\delta^{18}\text{O}$ values were found in
322 Basilicata tomatoes than Emilia Romagna samples (mean values and standard deviations
323 $+26.7\text{‰}\pm 0.9\text{‰}$ versus $+24.2\text{‰}\pm 1.2$), as expected due to the different latitudes and climatic conditions
324 of the two regions. Furthermore, lower $\delta^{34}\text{S}$ values were found in Basilicata tomatoes and passata
325 (mean values and standard deviations $-3.0\text{‰}\pm 1.5\text{‰}$ and $-4.4\text{‰}\pm 0.2\text{‰}$) than for the Emilia Romagna
326 samples ($-0.3\text{‰}\pm 2.9\text{‰}$ and $-0.3\text{‰}\pm 1.9\text{‰}$) which were likely related to the different geological

characteristics of the soils, differences in the microbial activity as well as the different proximities to the sea (Krouse, & Grinenko, 1991).

3.3 Discrimination between organic and conventional tomatoes using bulk isotope data

Statistically significant differences ($p < 0.001$) were found for $\delta^{15}\text{N}$ in tomatoes (Table 1). As expected, tomatoes grown conventionally had lower values of $\delta^{15}\text{N}$ compared to organic samples (mean values $4.5 \pm 2.1\text{‰}$ versus $9.2 \pm 3.3\text{‰}$), irrespective of geographic origin. The $\delta^{15}\text{N}_{\text{bulk}}$ values of the tomatoes ranged between $+4.0\text{‰}$ to $+14.6\text{‰}$ for the organic system and $+1.6\text{‰}$ to $+8.2\text{‰}$ for the conventional system, respectively, and these values depict the nature of the fertilization or farming systems implemented. Fertilizers synthetically obtained via the Haber-Bosch process, have nitrogen derived from the air and therefore have $\delta^{15}\text{N}$ values between -6‰ and $+6\text{‰}$. On the other hand, organic fertilizers derived from animal waste or vegetable matter, have isotopic values of $\delta^{15}\text{N}$ ranging from $+0.6\text{‰}$ to $+36.7\text{‰}$ (Bateman & Kelly, 2007). In this study, farmyard manure was e.g. used for the organic systems which, according to Bateman and Kelly (2007), has $\delta^{15}\text{N}$ values ranging from $+3.5\text{‰}$ to $+16\text{‰}$, whereas KNO_3 used in the conventional systems have much lower values of $\delta^{15}\text{N}$ (from -1‰ to -1.5‰ according to Bateman and Kelly) (Tables 2 and 3 Supplementary Material).

The $\delta^{13}\text{C}$ mean values for the organic and conventional systems were not significantly different in this study (Table 1). The $\delta^{13}\text{C}_{\text{bulk}}$ values of the tomatoes (-29.9‰ to -26.0‰) were representative of C3 plants (-34‰ to -24‰) (Krueger & Reesman, 1982), as they are mainly influenced by the plant's photosynthetic pathway. However, nutrient availability, soil types, CO_2 concentrations, and water availability can also affect the plant $\delta^{13}\text{C}$ values (Longobardi et al., 2017). Moreover, these values relate to the uptake of CO_2 from the atmosphere and therefore to the factors that have an impact on stomatal aperture and photosynthesis, such as temperature, salinity and light intensity (O'Leary, 1981). The use of different farming systems, organic and conventional, could lead to different $\delta^{13}\text{C}$ values, as usually under conventional conditions the availability of nitrogen is high and leads to lower $\delta^{13}\text{C}$ values, whereas lower nitrogen availability leads to higher $\delta^{13}\text{C}$ values, which could help to differentiate between the systems (Paolini et al., 2015).

The $\delta^2\text{H}_{\text{bulk}}$ values of the tomatoes (-53‰ to -24‰) and the $\delta^{18}\text{O}_{\text{bulk}}$ values (22.5‰ to 28.2‰) are determined by a number of factors such as temperature, precipitation and humidity, longitude, latitude, and altitude. There were no significant differences between the conventional and the organic system for $\delta^2\text{H}_{\text{bulk}}$ (mean values and standard deviations $-37\text{‰} \pm 8\text{‰}$ for both) and $\delta^{18}\text{O}_{\text{bulk}}$ (mean values and standard deviations $25.0\text{‰} \pm 1.8\text{‰}$ and $25.9\text{‰} \pm 1.3\text{‰}$), which means that the farming system (organic and conventional) in this specific case did not influence these isotopic values. The

361 $\delta^{34}\text{S}_{\text{bulk}}$ values of the tomatoes were between -6.6‰ and +2.1‰ and did not allow discrimination
362 between conventional and organic tomatoes (-1.7‰ $\pm$ 2.3‰ for conventional samples and -
363 1.7‰ $\pm$ 3.0‰ for organic ones).

364 Principal component analysis was conducted using the bulk data for $\delta^2\text{H}$, $\delta^{18}\text{O}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$
365 in tomatoes (Figure 1 Supplementary Material). The majority of the data variability was explained by
366 the first three PCs with 40% variance explained for the first PC, 27% for the second PC and 19% for
367 the third PC. The first PC had three major contributors, $\delta^{13}\text{C}$, $\delta^{34}\text{S}$ and $\delta^2\text{H}$, whereas $\delta^{15}\text{N}$ mainly
368 contributed to the second PC. No clear grouping according to the farming system was seen (Figure 1
369 Supplementary Material) when plotting the first two PCs.

370 Collectively, results from the multi-element bulk tissue isotope ratio analysis did not provide
371 sufficient classification power to discriminate organically and conventionally grown tomatoes. Of the
372 isotope ratios analyzed, $\delta^{15}\text{N}$ was the most promising marker as it could reveal the use of organic
373 manure. This is contrary to the results of Paolini et al (2015) who found that $\delta^{15}\text{N}_{\text{bulk}}$ could
374 differentiate organically and conventionally grown cereal grains. However, $\delta^{15}\text{N}$ has showed clear
375 limitations for organic authenticity testing when organic manures are used in conventional production
376 or when legume based green manures are used in organic production (Laursen et al., 2013; Paolini et
377 al., 2015).

378

379 **3.4 Discrimination of organic and conventional tomatoes using amino acid isotope analysis**

380 Amino acid isotope ratio analysis has previously eliminated the limitations of bulk tissue isotope ratio
381 analysis in cereal grains (Paolini et al., 2015) and was therefore further explored for authentication of
382 organic tomatoes in the present study. Amino acids (aa), Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and
383 Phe, in tomato samples were analyzed for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ on a subset of 16 samples randomly selected
384 in order to equally cover the agricultural systems, regions and harvest years. In Table 2, the means of
385 the $\delta^{13}\text{C}_{\text{aa}}$ and $\delta^{15}\text{N}_{\text{aa}}$ values for the different farming systems, regions and years are reported. A Tukey
386 test showed significant differences ($p<0.001$) between the organic and conventional farming systems
387 for $\delta^{15}\text{N}_{\text{Ileu}}$, $\delta^{15}\text{N}_{\text{Glx}}$ and $\delta^{15}\text{N}_{\text{Phe}}$. For $\delta^{13}\text{C}$ there was only a significant difference ($p<0.001$) between
388 systems for Glx. There were no significant effects for $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ for all amino acids between the
389 different years, 2012 and 2013, and the regions.

390 The $\delta^{15}\text{N}$ amino acid values were normalized against the $\delta^{15}\text{N}_{\text{Glx}}$ (Figure 2 Supplementary Material)
391 (Paolini et al., 2015; Styring et al., 2014b). The amino acids are all derived from glutamine and
392 glutamic acid, therefore the single amino acid isotopic values were normalized against the
393 corresponding glutamine + glutamate values (Glx) to cancel out the differences due to peripheral
394 sources of nitrogen other than the agricultural system (*i.e.* the release of ammonia and/or nitrate during
395 organic matter decomposition or the conversion of atmospheric nitrogen into these compounds by

396 natural processes, such as lightning, and biological nitrogen fixation by bacteria). The $\delta^{15}\text{N}$ values,
 397 for both farming systems, of Pro ($6.0\text{‰} \pm 2.1\text{‰}$) and Phe ($3.0\text{‰} \pm 1.6\text{‰}$) were higher when compared
 398 to the $\delta^{15}\text{N}$ values for Glx. Phe is enriched in $\delta^{15}\text{N}$ compared to Glx due to a kinetic isotope effect in
 399 the synthesis of phenylpropanoids (for structural and protection purposes in plants) where it is
 400 deaminated as an intermediate (Styring et al., 2014b; Werner & Schmidt, 2002a). Pro is also enriched
 401 in $\delta^{15}\text{N}$ compared to Glx and this is due to a kinetic isotope effect associated with its catabolism via
 402 enzymatic reactions (Hermes, Weiss, & Cleland, 1985; Styring et al., 2014b). The $\delta^{15}\text{N}$ values for
 403 Ala ($-2.9\text{‰} \pm 1.3\text{‰}$), Ileu ($-4.6\text{‰} \pm 1.7\text{‰}$) and Leu ($-7.1\text{‰} \pm 1.6\text{‰}$) were lower when compared to
 404 the $\delta^{15}\text{N}$ values for Glx. Similarly, the majority of the values of Val ($-1.6\text{‰} \pm 1.7\text{‰}$) Gly ($-1.3\text{‰} \pm$
 405 1.9‰) and Thr ($-3.6\text{‰} \pm 2.1\text{‰}$) were lower when compared to the $\delta^{15}\text{N}$ values for Glx. Val, Ileu and
 406 Leu are branch-chain amino acids (BCAA) and are precursors to synthesizing proteins (Styring et al.,
 407 2014b; Werner & Schmidt, 2002b). Val is synthesized directly from pyruvate, which is derived from
 408 photosynthesis (Bryan, 1980) and Leu is synthesized from the Val metabolic pathway via a succession
 409 of reactions branching off from it (Kochevenko & Fernie, 2011; Pátek, 2007) and these successive
 410 reactions could be the cause of the greater discrimination for $\delta^{15}\text{N}$ in Leu than for Val (Bol, Ostle, &
 411 Petzke, 2002; Hofmann, Jung, -J. Segschneider, Gehre, & Schüürmann, 1995). Ileu is synthesized
 412 from Thr, which is deaminated, and during this metabolic process, there is a slight depletion in $\delta^{15}\text{N}$
 413 for Ileu compared to Thr. Gly is involved in the photorespiratory nitrogen cycle and is derived from
 414 the Glu, Ala or Asp amino group (Styring et al., 2014b; Werner & Schmidt, 2002b). Gly $\delta^{15}\text{N}$ is
 415 enriched compared with Ala and is slightly depleted to Glx. Ala is also slightly depleted compared to
 416 Glx.

417 Also for $\delta^{13}\text{C}$, to remove the external factors that could be affecting the isotopic value such as the
 418 atmosphere, the amino acid $\delta^{13}\text{C}$ values were normalized to the Glx $\delta^{13}\text{C}$ value (Figure 2
 419 Supplementary Material). The $\delta^{13}\text{C}$ values for both farming systems and regions of Pro ($1.7\text{‰} \pm$
 420 2.1‰), Ileu ($2.1\text{‰} \pm 1.1\text{‰}$) and Thr ($4.5\text{‰} \pm 2.1\text{‰}$) are higher when compared to the $\delta^{13}\text{C}$ values for
 421 Glx. The $\delta^{13}\text{C}$ values for Ala ($0.1\text{‰} \pm 1.0\text{‰}$) are almost always very similar as for Glx. The $\delta^{13}\text{C}$
 422 values for Val ($-6.2\text{‰} \pm 1.0\text{‰}$), Leu ($-9.0\text{‰} \pm 0.8\text{‰}$), Gly ($-11.2\text{‰} \pm 1.9\text{‰}$) and Phe ($-1.6\text{‰} \pm 1.0\text{‰}$)
 423 were lower when compared to the $\delta^{13}\text{C}$ values for Glx.

424 The fixation of CO_2 in plants by the enzyme RuBisCO has a kinetic isotope effect. RuBisCO
 425 generates glucose and aids in the photorespiration of the plant; these affect the amino acids involved
 426 in these systems, such as glycine in photorespiration (Paolini et al., 2015; Styring et al., 2014b;
 427 Werner & Schmidt, 2002b). Val is derived from photosynthesis as is Leu, by subsequent branching
 428 reactions from the Val metabolic pathway. Gly is derived from photosynthesis and this can explain
 429 the lower $\delta^{13}\text{C}$ values for these 3 amino acids: Val (-35.3‰ to -32.7‰), Leu (-38.0‰ to -34.8‰) and
 430 Gly (-44.2‰ to -36.7‰) (Table 2).

431 A separation between the farming systems, organic and conventional, were observed when $\delta^{13}\text{C}_{\text{Glx}}$
 432 values were plotted against $\delta^{15}\text{N}_{\text{Glx}}$ values (Figure 2). A clear improvement of the discrimination
 433 capacity was evident when compared with the plot of $\delta^{13}\text{C}_{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{bulk}}$ determined for the same
 434 16 samples (Figure 3 Supplementary Material). This demonstrated that compound-specific isotope
 435 analysis provided added classification power for organic authentication compared to traditional bulk
 436 tissue isotope analysis.

437 Principal component analysis was conducted using the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the amino acids that
 438 showed statistically significant differences at ANOVA test for $p < 0.001$ ($\delta^{15}\text{N}$ values of Ileu, Glx and
 439 Phe and $\delta^{13}\text{C}_{\text{Glx}}$) (Figure 4 Supplementary Material). The majority of the data variability was
 440 explained with the first two PCs with 86% variance explained for the first PC and 10% for the second
 441 PC. The first PC had a number of amino acid contributors: all the $\delta^{15}\text{N}$ of Ileu, Glx and Phe that were
 442 highly correlated. The major contributor of PC2 was Glx for $\delta^{13}\text{C}$. Analyzing the PCA for farming
 443 system for amino acid $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (Figure 4 Supplementary Material) a much better separation
 444 between the organic and conventional systems was obtained compared to the bulk tomato data. The
 445 majority of the organically grown samples had higher values of $\delta^{15}\text{N}_{\text{aa}}$ and $\delta^{13}\text{C}_{\text{Glx}}$ compared to the
 446 conventionally grown ones. This corresponds with the findings of Paolini and colleagues (Paolini et
 447 al., 2015) for wheat samples.

448 To further test the efficacy of the isotopic profile of amino acids for the differentiation of organic and
 449 conventional tomatoes, the method was applied to 13 tomato samples collected from Danish growers
 450 (Table 3). Samples were initially analyzed for stable isotope ratios of H, C, N, O and S, on bulk tissue.
 451 The ranges of values for the five stable isotope ratios were overlapped between the conventional and
 452 organic products, thus not allowing for discrimination of these samples. In particular, three organic
 453 samples showed low values of $\delta^{15}\text{N}$ probably due to the use of organic liquid fertilizers derived from
 454 nitrogen fixing plants. Furthermore, it should be noted that the test samples had very low values of
 455 $\delta^{13}\text{C}$ (both bulk and amino acids). Schmidt and colleagues (2005) suggested that in German tomatoes
 456 $\delta^{13}\text{C}_{\text{bulk}}$ lower than -28‰ indicated greenhouse-cultivated tomatoes with methane based heating. Data
 457 of $\delta^{13}\text{C}_{\text{Glx}}$ was plotted against $\delta^{15}\text{N}_{\text{Glx}}$ values to visualize separation between the farming systems;
 458 organic and conventional (Figure 5 Supplementary Material). Due to the lower values of $\delta^{13}\text{C}$ from
 459 the greenhouse grown tomatoes, it was decided to normalize $\delta^{13}\text{C}_{\text{Glx}}$ values against $\delta^{13}\text{C}_{\text{bulk}}$ and plot
 460 the normalized data against $\delta^{15}\text{N}_{\text{Glx}}$ (Figure 3). The conventional and organic samples of the test set
 461 grouped in two different parts of the plot similarly to the organic and conventional samples of the
 462 subset of 16 Italian field-grown tomatoes, with the exception of three organic test samples that fell in
 463 the conventional group. These samples were found to have comparably lower values of $\delta^{15}\text{N}_{\text{bulk}}$ and
 464 $\delta^{15}\text{N}_{\text{aa}}$ (Table 3). An explanation of this could be that these three samples were the only ones grown
 465 on peat and that the fertilization strategy was partly based on legumes. The stable isotopic

composition of peat-forming plants, including $\delta^{15}\text{N}$, is well preserved in a peat formed in cold regions (Skrypek et al., 2008) and we propose that the peat used as media in this study originated from nitrogen fixing plants.

4. Conclusions

In this study, the isotopic signatures ($\delta^2\text{H}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{18}\text{O}$ and $\delta^{34}\text{S}$) of soils, tomatoes and tomato passata samples collected from two Italian regions, produced organically or conventionally in two years, were investigated along all steps of the production chain of passata. The main factors influencing the bulk isotopic signatures of tomatoes and passata were the geographical origin and the agricultural production system. Differences between conventional and organic agricultural practices were primarily detected for $\delta^{15}\text{N}$, as expected, even if this parameter alone is not able to completely distinguish organic and conventionally grown products.

By analyzing stable isotope ratios in amino acids from field-grown tomatoes (Ala, Val, Ileu, Leu, Gly, Pro, Thr, Glx and Phe), it was possible to improve the discrimination power according to the production system. In particular, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of Glx provided added value for organic authentication compared to isotopic bulk data. However, when applying the compound-specific analysis to a test set of greenhouse-grown tomatoes, the discrimination of organic and conventional tomatoes was less successful. In particular, $\delta^{13}\text{C}_{\text{Glx}}$ was biased by greenhouse growth conditions likely due to a higher CO_2 concentration and air humidity compared to field growth conditions. This clearly demonstrates that the growth conditions (greenhouse or field) should be taken into consideration when authenticating organic produce. Overall, it can be concluded that compound-specific isotopic analysis was superior for authentication of organic, field-grown tomatoes and efficiently eliminated the limitations of bulk isotope analysis. However, for greenhouse-grown tomatoes further studies should be conducted to investigate the applicability of amino acid isotope analysis for authentication of organically grown produce. Furthermore, a wider sampling plan and complementary analytical techniques including pesticide residue analysis and isotope ratio analysis of oxy-anions such as nitrate and sulfate, are also advisable in order to improve the discrimination power and the applicability of the developed method for authentication of organically grown produce.

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Figure captions

Figure 1: Trend of stable isotope ratios of H, C, N, O and S determined along the production chain of tomato passata in soils, tomatoes and passata collected in 2012 and 2013 in two Italian regions (Basilicata and Emilia Romagna).

Figure 2. Tomato $\delta^{15}\text{N}_{\text{Glx}}$ samples plotted against $\delta^{13}\text{C}_{\text{Glx}}$ samples. Org (organic), Conv (conventional).

Figure 3: $\delta^{15}\text{N}_{\text{Glx}}$ and $\delta^{13}\text{C}_{\text{Glx}}$ values normalized according to $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ plotted against each other for tomato samples of model and test samples. Org (organic), Conv (conventional).