Multi-chemical analysis combined with chemometrics to characterize PDO and PGI Italian apples

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Abstract

BACKGROUND: The use of PDO (protected designation of origin) and PGI (protected geographical indication) labels allows to protect and promote agricultural products characterized by unique features related to the place of origin and traditional know-how. However, the presence of non-authentic products in the market represents a fraud that can be tackled applying analytical techniques combined with chemometric analysis. In this study, we applied multi-element and multi-isotope analysis to characterize PDO and PGI apples cultivated in northern Italy, comparing them with Italian apples without labels of geographical indications.

RESULTS: The multi-element and multi-isotope approach allowed to characterize the apples cultivated in northern Italy. Despite a significant effect of the sampling sites on the apple composition, the comparison of the multi-chemical fingerprint of the apples significantly varied among cultivation areas. Results of this characterization were used to classify samples according to their cultivation area applying a linear discriminant analysis (LDA). Outputs of the LDA showed that correct sample classification can be successfully achieved (balanced accuracy > 96%). Moreover, using a selection of variables, it was possible to correctly classify samples also at regional level.

CONCLUSION: The presented evidences indicate that the multi-element and multi-isotope fingerprint can be successfully applied to traceability studies. The combination of this characterization with chemometric tools allows the classification of Italian apples based on their origin both on a national and regional scale. This approach represents an interesting tool to enhance and protect PDO and PGI Italian products.

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Keywords: multi-element analysis; isotope ratio; linear discriminant analysis; geographical origin; mass spectrometry; PDO and PGI labels

INTRODUCTION

Geographical origin of food products has a potential impact on consumers’ purchasing habits and represents an important information and marketing tool. 1–4 To contrast fraudulent activities related to misdeclaration of food origin it is important to be able to trace back food provenance. 5 However, traceability is largely based on paper-records, insufficient to meet specific requirements and it is even more difficult in international trading due to differences in regulatory systems. 6 Against this frame, the need for analytical tools able to recognize and attest food provenance has become a priority and their use might be determinant also in the management of several food crisis (e.g. food disease outbreaks). 5,7,8 Moreover, analytical indicators can be successfully applied to promote high-quality food products characterized by an undeniable link with their terroir, e.g. products with PDO (protected designation of origin) and PGI (protected geographical indication) labels. 9 For the tutelage of these products, analytical techniques can become essential to support legal cases and verify the authenticity of PDO and PGI products. 10–12 Different

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approaches have been tested to trace agricultural products, including the analysis of their multi-element and multi-isotope composition combined to several chemometric tools, as reported in several reviews.\textsuperscript{13–16}

The multi-element composition of horticultural products is linked to the soil features and environmental conditions of the growing areas and to crop management. Mineral elements are absorbed through the soil solution and then allocated to the different plant organs. While the uptake of essential nutrients is to some extent controlled by plant needs, the uptake of non-essential nutrients is more related to their concentration and bioavailability in the soil.\textsuperscript{17} This, in turn, depends on chemical-physical processes such as soil pH, moisture, mineral weathering, organic matter decomposition or on external factors such as anthropogenic pollution, atmospheric depositions, agricultural practices and the use of fertilizers or soil improvers.\textsuperscript{13,18,19} Therefore, the multi-element composition of horticultural products has been largely applied to verify food provenance.\textsuperscript{20–22}

Many elements are naturally present in soil and plants in two or more isotopic forms. The ratio between isotopes of specific elements can be related to food origin and hence applied as geographical tracers.\textsuperscript{23} Among these, the isotope composition of hydrogen (\(\text{H}/\text{H}\)) and oxygen (\(\text{O}/\text{O}\)) varies according to seasonality, latitude, altitude and continentality.\textsuperscript{24,25} This is due to the high mass difference characterizing isotopes of light elements compared to their atomic mass (e.g. \(\text{H} \) versus \(\text{H}\)) and hence during biochemical, chemical and physical processes a depletion or enrichment of one isotope against the others occurs (fractionation).\textsuperscript{24} Recent development of the analytical techniques have also determined a larger application of the isotope ratio of heavy elements as geographical tracers, particularly the strontium isotope (\(87\text{Sr}/86\text{Sr}\)) ratio.\textsuperscript{9} Indeed, the \(87\text{Sr}/86\text{Sr}\) ratio of horticultural products like apples is directly related to that of the strontium (Sr)-bioavailable fraction in the soil, which mainly depends and varies according to the geo-lithological features of the growing area.\textsuperscript{26–28} Its high potential as soil-derived traceability marker to distinguish different types of horticultural products according to their origin has been demonstrated in several studies.\textsuperscript{29–32}

A multi-chemical approach is rather promising to detect the origin of agricultural products, especially when data are analysed through chemometric methods.\textsuperscript{8,20,22,31} The possibility of applying chemometric methods largely facilitates data interpretation because results are processed through a multivariate approach, a fact that maximizes the extractable information. For this purpose, supervised methods, which require a priori knowledge of the prediction ability is evaluated testing unknown samples, are often applied. Among these, linear discriminant analysis (LDA) is largely employed for food traceability and authentication allowing the development of classification models among classes.\textsuperscript{34,35}

The agri-food sector is a leading one in the Italian economy and Italy represents the country with the highest number of products certified with the PDO and PGI labels by the European Union (https://ec.europa.eu/agriculture/quality/door/list.html). The class with the highest number of certifications corresponds to ‘Fruit, vegetables and cereals fresh or processed’ and, within this class, the most important products in terms of both production volume and annual revenue are the apples (\textit{Malus × domestica} Borkh.) labelled as ‘Mela Alto Adige PGI’ and ‘Mela Val di Non PDO’.\textsuperscript{36,37} The Italian apple cultivation covers an area of about 70 000 ha, but most production derives from northern regions where climatic conditions, good exposures to sunlight and soil features favoured the apple cultivation in mountain valleys between 200 and 1100 m above sea level (a.s.l.).\textsuperscript{38}

The aim of the present study was to apply a multi-chemical approach combined with chemometrics (LDA) to classify Italian apples, according to their provenance. Our multi-chemical dataset was obtained combining the results of the apple multi-element analysis with the isotope ratios of hydrogen, oxygen, and strontium (\(\delta\text{D} / \text{H}, \delta^{18}\text{O} / \text{O}, \delta^{87}\text{Sr} / \delta^{86}\text{Sr}\)). Additionally, we verified the possibility to develop a second model, based on a restricted selection of variables, able to distinguish among multiple cultivation districts within a single cultivation area.

\section*{MATERIALS AND METHODS}

\subsection*{Sampling}

Site description and sampling procedure are reported in detail in a previous paper.\textsuperscript{35} Briefly, apples (cv. Golden Delicious, rootstock M9) were collected from orchards within the protected areas for the cultivation of ‘Mela Val di Non PDO’ (six orchards), ‘Mela Alto Adige PGI’ (17 orchards from three main cultivation districts, namely Bressanone, Val d’Adige, Val Venosta), ‘Mela di Valtellina PGI’ (three orchards), in addition to other cultivation areas located in northern Italy where apples with no geographical indication (GI) are produced (13 orchards) (Fig. 1). From now on, we will refer to apples from Val di Non as Val di Non apples PDO, those from South Tyrol as South Tyrolean apples PGI, those from Valtellina as Valtellina apples PGI. Hereafter the apples without any GI will be refereed as non-GI apples.

The sampling followed the same protocol in all the orchards and was performed 2 weeks before harvest (August–September 2017). In each orchard, ten apple trees were randomly selected and three fruits were collected from each one at 1–2 m from the ground. Each apple was peeled and, after removing the core, the equatorial disk (1 cm thickness) isolated. The equatorial disks of apples collected from the same tree were grouped together to create a bulk sample. Apple samples were frozen at −80 °C, freeze-dried and finely powdered. All samples were stored at −20 °C until further use. For the purpose of the current study, three bulk samples per sampling site were analysed (n = 117).

\subsection*{Hydrogen and oxygen isotope ratio analysis}

Powdered apple samples were preventively oven-dried at 65 °C for 48 h to remove water traces. Then, samples were weighted in silver cups and tightly packed. Each sample was analysed in single form. The hydrogen and oxygen isotope ratios were determined with a TC/EA (high temperature conversion elemental analyzer; Thermo Fisher Scientific, Waltham MA, USA) coupled to a CF-IRMS (continuous flow-isotope ratio mass spectrometer; Delta V Advantage; Thermo Fisher Scientific).

Hydrogen and oxygen isotope ratios are reported in delta notation (\(\%\)) in relation to the international V-SMOW (Vienna-Standard Mean Ocean Water) standard, according to the general equation:

\[ \delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \]  

where \(R_{\text{sample}}\) and \(R_{\text{standard}}\) indicate the heavy to light isotope ratio (\(\text{H}^2 / \text{H}^1\) and \(\text{O}^{18} / \text{O}^{16}\)) of samples and standards. The isotope values were calibrated against international reference materials, namely NBS 22 Oil and IAEA-CH-7 polyethylene foil for \(\delta\text{D}\) and IAEA-601 benzoic acid for \(\delta^{18}\text{O}\), included in each running sequence.
together with blanks. The overall measurement precision was < 3‰ and < 0.3‰, for δ²H and δ¹⁸O, respectively.

Multi-element analysis

Acid digestion of the samples was performed adding to dried apple samples (0.5 g) 5 mL of nitric acid (HNO₃, 65%), purified by quartz sub-boiling distillation system (DUOPUR, Milestone, Sorisole, IT), and 1 mL of hydrogen peroxide (H₂O₂, 30%, Suprapur) by means of a microwave digestion apparatus (UltraWAVE, Milestone). The heating profile applied for the acid digestion was reported in a previous study.³⁹ Digest solutions were filtered, and an aliquot of each sample was diluted with distilled water to reach a 2% acid content and stored in the refrigerator until multi-element analysis.

Quantitative analysis of the multi-element composition was performed through inductively coupled plasma mass spectrometry (iCAP Q ICP-MS) (Thermo Scientific, Bremen, Germany) equipped with an autosampler ASX-520 (Cetac Technologies Inc., Omaha, NE, USA). The following elements were quantified: beryllium (Be), sodium (Na), magnesium (Mg), aluminium (Al), potassium (K), calcium (Ca), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), rubidium (Rb), strontium (Sr), molybdenum (Mo), silver (Ag), cadmium (Cd), antimony (Sb), barium (Ba), thallium (TI), lead (Pb), and uranium (U). The calibration curve was prepared diluting a standard solution to reach the following ranges: 0.005–50 μg g⁻¹ for Na, Mg, K, Ca, Fe and 0.025–250 ng g⁻¹ for the remaining elements. Instrumental blanks were also included. Starting from mono-element standard solutions of lithium (Li), scandium (Sc), germanium (Ge), yttrium (Y), rhodium (Rh), and lanthanum (La), a solution was prepared and used as internal standard to monitor and correct the instrumental drift. Operating conditions were reported in a previous study.³⁹ Limit of detection (LOD) and limit of quantification (LOQ) were determined as, respectively, three and ten times the standard deviations of the signal of independent blanks; element concentrations lower than LOQ were excluded from further data analysis. A certified reference material (TMDA-64.3) was used as quality control to measure instrument accuracy, comparing the measured concentrations with the certified value. On average, the instrument accuracy ranged between 95 and 115%. Repeatability and within-laboratory reproducibility were evaluated by repeatedly measuring a single apple sample used as internal reference material (five independent replicates per day, in different days) and were on average < 5% and < 15%, respectively. The same material was used to evaluate the average recovery through the method of the standard additions, which ranged between 90% and 105%, except for a few elements for which it was between 80% and 90% (Ni, Zn and Pb). The applied procedures to determine the method and instrument performances were reported in a previous study.³⁹ Element concentrations for apple samples are reported as μg g⁻¹ or ng g⁻¹ on a dry weight basis.
Strontium isotope ratio analysis

Apple sample preparation and analysis to determine the Sr isotope ratio was comprehensively described in a previous paper.32 Briefly, all the digested samples went through a Sr/matrix separation to isolate the Sr fraction and remove the elements that potentially interfere with the Sr isotope ratio analysis, specifically Ca and Rb. Then, the $^{87}$Sr/$^{86}$Sr was measured with a double-focusing multicollector inductively coupled plasma mass spectrometer (Neptune Plus™, Thermo Scientific, Bremen, Germany) in dry-plasma conditions (CETAC Aridus apparatus as aerosol drying unit and Jet sample cone + Ni ‘H’ skimmer cone). Within each sequence, blank and standard (SRM 987) solutions were measured at the beginning, at the end and for every block of samples. Results of the SRM 987 analysis were consistent with both its certified and ‘generally accepted’ value,50 with a precision, expressed as relative standard deviation, better than 15 ppm. Details about instrument configuration, operating conditions, data corrections and about the performance of the analysis are reported in previous studies.32,39

Statistical analysis

After data logarithmic transformation, two different models were tested to highlight statistical differences among cultivation areas. The first one was a linear regression model in which only a fixed effect was considered, namely the cultivation area. The second model was a linear mixed model in which, beside the fixed effect (cultivation area), the sampling site was included as random effect in order to take into account the hierarchical structure of the data. Results of the two models were compared through the analysis of variance (ANOVA) test and the outputs of the model with the lowest AIC (Akaike information criterion) were chosen. Level of significance was fixed at $P$-value = 0.05. Tukey HSD (Honestly significant difference) post hoc test was applied for multiple comparisons among cultivation areas.

To improve the identification of sample origin based on the results of the multi-chemical approach, multivariate data analysis was performed based on a supervised classification method, namely the LDA. A first model was developed analysing the multi-chemical composition of 117 apple samples divided in four main groups according to their cultivation areas. Then, a second model was developed limiting the analysis to the multi-chemical composition of 51 apple samples. For this second model, the grouping factor was the cultivation district (three groups). Prior to LDA, data were centred and scaled. The discriminant models were validated by ‘leave-one-out’ cross-validation. Results of the confusion matrix were elaborated to get the sensitivity, specificity, precision, false discovery rate and balanced accuracy of the developed model applying the following equations:

$$\text{sensitivity} = \frac{TP}{TP + FN}$$

(2)

$$\text{specificity} = \frac{TN}{TN + FP}$$

(3)

$$\text{precision} = \frac{TP}{TP + FP}$$

(4)

false discovery rate = 1 − precision

(5)

balanced accuracy = $\frac{1}{2}$ (sensitivity + specificity) = $\frac{1}{2}$ ($\frac{TP}{TP+FN}$ + $\frac{TN}{TN+FP}$)

(6)

where TP stands for true positive, TN for true negative, FP for false positive, and FN for false negative.41

The statistical analysis was performed using the computing environment R (R Core Team, Vienna, Austria, 2016).

RESULTS AND DISCUSSION

Multi-element fingerprint

The multi-element composition of apple pulps, grouped according to their cultivation areas, is reported in Table 1. In addition to element concentration, also the ratio between the concentrations of certain elements which share common chemical features (K/Rb, Ca/Sr, Ca/Ba, Sr/Ba) has been included, since they can provide additional information about the cultivation areas.42,43 Some elements (Be, V, As, Ag, and Sb) were excluded from the table, since their concentration was below the LOQ.

Results highlighted that all the collected apples had a similar composition in terms of the main macro- and micro-nutrients, with K being on average the most abundant element among those quantified in apple pulps (6832 ± 1159 μg g$^{-1}$), followed by Mg (331 ± 58 μg g$^{-1}$) and Ca (195 ± 57 μg g$^{-1}$). This is in agreement with published literature.44–46 While the concentration of essential macro- and micro-nutrients in the apple mainly depends on both soil nutrient availability and on the typical allocation pattern of the absorbed nutrients, the presence of non-essential elements in the pulp is more heterogeneous and may be related to the bioavailability of these elements in the soil solution, chemical-physical processes, and other external factors.13,17,18

Focusing on the concentration of single elements, some differences among cultivation areas were highlighted by statistical analysis. Testing a simple linear regression model with a more complex linear mixed model, in which also the cultivation site is included as random effect, it appears that the hierarchical structure of the data is noteworthy to define differences among cultivation areas (Supporting Information, Table S1). Indeed, considering the multi-element fingerprint of apples within single cultivation areas, some variability among cultivation sites was detected (the whole dataset is reported in Tables S2 and S3). For instance, considering the pool of Val di Non apples PDO, apples from orchard no. 1 were characterized by significantly higher concentrations of Cr, Sr and Ba (49.7, 636 and 888 ng g$^{-1}$, respectively) compared to those from other sites in the same area, which were on average more similar to each other (16.1, 248 and 305 ng g$^{-1}$, respectively). For other variables, the difference in concentration of some elements determined the subdivision of the cultivation sites in multiple groups. This is the case of Sr concentration in South Tyrolean apples PDO, for which three ranges of Sr concentration were identified: 1567 ± 269 μg g$^{-1}$, 1006 ± 78 μg g$^{-1}$ and 498 ± 92 μg g$^{-1}$. The difference in Sr concentration among sites does not correlate with the geographical position of the sites within the cultivation area of South Tyrol, but it seems to depend more on local features. Moreover, the described range of Sr concentrations overlaps with the Sr levels found in apples from other cultivation areas. Hence, in this case, Sr concentration cannot represent a marker to distinguish South Tyrolean PDO apples. Similar considerations apply to other variables. Therefore, not only the cultivation areas, but also the sampling site within each cultivation area influence the multi-element composition of samples,18 hence the possibility to discriminate samples according to their origin might be negatively affected. For the purpose of this contribution, only differences among cultivation areas, assessed using the linear mixed model with cultivation site as random effect, are further discussed.
As reported in Table 1, the concentration of several elements significantly varies in apples from different cultivation areas. Val di Non apples PDO were characterized by the lowest levels of Ca (145 ± 34 μg g⁻¹), Fe (3.8 ± 1.1 μg g⁻¹), Co (4.6 ± 1.8 ng g⁻¹), Rb (1.6 ± 0.6 μg g⁻¹), Sr (313 ± 182 ng g⁻¹), Mo (26.2 ± 9.3 ng g⁻¹), and Ba (402 ± 271 ng g⁻¹), and the highest Ca/Sr ratio (0.58 ± 0.26) compared to the apples from the other cultivation areas. In particular, Sr concentration and Ca/Sr ratio could represent two markers for Val di Non apples PDO, since their values differ significantly from the values measured in apples from the other cultivation areas (881 ± 130 ng g⁻¹ and 0.25 ± 0.05, on average, respectively).

South Tyrolean apples PGI were characterized by the highest concentration of Ca (212 ± 58 μg g⁻¹) and Rb (4.5 ± 3.0 μg g⁻¹), but these concentrations are significantly different only compared to the concentrations measured in Val di Non apples PDO, as Valtellina and non-GI apples had in-between values. Except for these two element concentrations, South Tyrolean apples PGI did not show peculiar trends. This might depend on the fact that South Tyrolean apples PGI were collected from three main cultivation districts, namely Bressanone, Val d’Adige, and Val Venosta, characterized by different geological features. Hence, we hypothesized that this difference might also be reflected on the multi-element composition of the apples of the single districts. Indeed, if we consider more in detail only South Tyrolean apples PGI, the concentration of few elements was useful to establish differences among the three main districts, as reported in Table 2.

### Table 1. Concentration of the quantified elements and ratios between concentration in apple samples (cv. Golden Delicious) from different cultivation areas

| Element | Val di Non apples PDO | South Tyrolean apples PGI | Valtellina apples PGI | Non-GI apples |
|---------|-----------------------|---------------------------|-----------------------|--------------|
| Na (μg g⁻¹) | 10.1 ± 5.6 b | 12.2 ± 3.9 b | 11.4 ± 2.1 b | 53.7 ± 36.7 a |
| Mg (μg g⁻¹) | 3371 ± 35 | 334 ± 72 | 281 ± 20 | 337 ± 48 |
| Al (μg g⁻¹) | 0.49 ± 0.10 bc | 0.80 ± 0.48 b | 0.36 ± 0.07 c | 1.28 ± 0.51 a |
| K (μg g⁻¹) | 7444 ± 1272 | 6622 ± 955 | 5937 ± 435 | 7030 ± 1292 |
| Ca (μg g⁻¹) | 145 ± 34 b | 212 ± 58 a | 204 ± 54 ab | 194 ± 53 ab |
| Cr (ng g⁻¹) | 21.7 ± 16.6 ab | 25.2 ± 21.3 ab | 103.7 ± 1.7 b | 34.6 ± 36.1 a |
| Mn (ng g⁻¹) | 1518 ± 298 | 1746 ± 413 | 2289 ± 646 | 1617 ± 480 |
| Fe (μg g⁻¹) | 3.8 ± 1.1 b | 5.5 ± 1.3 a | 5.8 ± 1.1 a | 6.6 ± 2.2 a |
| Co (ng g⁻¹) | 4.6 ± 1.8 b | 8.4 ± 5.0 ab | 8.5 ± 4.9 ab | 10.2 ± 5.4 a |
| Ni (ng g⁻¹) | 78 ± 34 | 97 ± 70 | 47 ± 16 | 105 ± 78 |
| Cu (μg g⁻¹) | 2.3 ± 0.5 | 2.4 ± 0.5 | 1.8 ± 0.4 | 2.5 ± 0.8 |
| Zn (μg g⁻¹) | 1.1 ± 0.2 | 1.4 ± 0.3 | 1.2 ± 0.3 | 1.3 ± 0.3 |
| Rb (μg g⁻¹) | 1.6 ± 0.6 b | 4.5 ± 3.4 a | 2.9 ± 2.8 ab | 4.0 ± 2.7 a |
| Sr (ng g⁻¹) | 313 ± 182 b | 740 ± 340 a | 908 ± 207 a | 996 ± 457 a |
| Mo (ng g⁻¹) | 26.2 ± 9.3 c | 75.7 ± 35.7 ab | 50.7 ± 27.4 bc | 127.6 ± 85.0 a |
| Cd (ng g⁻¹) | 0.24 ± 0.17 | 0.37 ± 0.19 | 0.44 ± 0.20 | 0.34 ± 0.21 |
| Ba (ng g⁻¹) | 402 ± 271 b | 720 ± 370 ab | 1197 ± 154 a | 605 ± 546 ab |
| Ti (ng g⁻¹) | 0.9 ± 0.4 | 1.7 ± 1.4 | 1.0 ± 1.1 | 1.4 ± 0.9 |
| Pb (ng g⁻¹) | 4.8 ± 1.8 | 5.7 ± 2.9 | 4.3 ± 0.6 | 10.2 ± 12.7 |
| U (ng g⁻¹) | 0.28 ± 0.08 b | 0.77 ± 0.61 a | 0.20 ± 0.05 b | 0.65 ± 0.60 ab |
| K/Rb | 5556 ± 2871 a | 2111 ± 1332 b | 4275 ± 2855 ab | 2691 ± 1709 b |
| Ca/Sr | 577 ± 264 a | 316 ± 93 b | 228 ± 54 bc | 219 ± 76 c |
| Ca/Ba | 471 ± 212 ab | 353 ± 149 ab | 177 ± 63 b | 632 ± 560 a |
| Sr/Ba | 0.84 ± 0.23 b | 1.26 ± 0.71 b | 0.78 ± 0.26 b | 2.81 ± 2.35 a |

Data are reported as mean ± standard deviation and refer to the dry sample. In rows, different letters indicate significant differences for the element concentration among the cultivation areas.

As isotopic ratios were determined on a sample basis, the values shown in the tables are averages of the individual measurements. The standard deviation of the mean values is reported in parentheses. The δ13C values range from −29.3 ± 0.3 atom% to −16.1 ± 0.3 atom% and the δ18O values range from −17.1 ± 1.1 atom% to −2.2 ± 1.0 atom% for all the sampling sites.

### Isotope fingerprint

Results of the isotope ratio analysis are illustrated in Fig. 2. In this case tool, substantial variability was found from site to site within each cultivation area. The results of the linear mixed model, which was built considering the sampling site as random effect, were chosen as they were more representative of the data hierarchical structure (Table S1). Average data for all the sampling sites are reported in Table S4.
Moreover, irrigation differences among cultivation areas were highlighted only for the larger extension of the sampling area. Nonetheless, statistically significant differences for the element concentration among the districts. As shown in Fig. 2, cultivation areas are split in two main groups.

Both $\delta^2\text{H}$ and $\delta^{18}\text{O}$ showed slightly higher values in non-GI apples ($-47 \pm 10$ and $29 \pm 1.5$, respectively), compared to apples cultivated within the cultivation areas of PDO and PGI apples ($-57 \pm 3$ and $28 \pm 0.3$, on average, respectively). This trend may be due to the different morphology of the cultivation areas, as shown in Fig. 1. PDO and PGI apples are indeed cultivated in mountain areas, while non-GI apples were collected from sites located in the flat area of the Po Valley. Non-GI apples were also characterized by higher variability, probably as a consequence of the larger extension of the sampling area. Nonetheless, statistical differences among cultivation areas were highlighted only for the $\delta^2\text{H}$. Specifically, a significant difference was found between the $\delta^2\text{H}$ of non-GI apples and South Tyrolean apples PGI ($-60.1 \pm 8.1$), while in-between values were measured in apples from the other cultivation areas. Despite the visible trend, $\delta^{18}\text{O}$ did not represent a significant marker of geographical origin compared to $\delta^2\text{H}$, probably due to the lower natural range of this delta ratio in plants compared to the $\delta^2\text{H}$ and the lack of such a strong geographical effect on O isotope fractionation in apples from northern Italy.

While $\delta^2\text{H}$ and $\delta^{18}\text{O}$ are related to geographical features, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio is linked to the geology of the cultivation areas. As shown in Fig. 2, cultivation areas are split in two main groups.

Val di Non apples PDO and non-GI apples were characterized by low values of $^{87}\text{Sr}/^{86}\text{Sr}$ ratio ($0.7082 \pm 0.0008$ and $0.7089 \pm 0.0003$, respectively), significantly different from those of South Tyrolean and Valtellina apples PGI, which were on the contrary characterized by higher ratios and also higher variability ($0.7119 \pm 0.0035$ and $0.7119 \pm 0.0020$, respectively). More details about the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of apples collected from the different cultivation areas were reported in a previous study.1 2

Considering only South Tyrolean apples PGI, the $\delta^{18}\text{O}$ confirmed not to be a representative marker of apple origin, while significant differences were found for both the $\delta^2\text{H}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ ratio (Table 2). Apples cultivated in Val Venosta had rather characteristic values for both the parameters ($-66.7 \pm 8.2$ and $0.7158 \pm 0.0030$, respectively). These values significantly differed from those of apples cultivated in Val d’Adige and Bressanone, which were on average statistically similar, with more positive values for the $\delta^2\text{H}$ and lower values for the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio. The more negative values obtained for $\delta^2\text{H}$ in apples cultivated in Val Venosta might be attributed to the higher altitude of the sampling sites, compared to Bressanone and Val d’Adige, affecting the isotope composition of local precipitations and hence of soil water.4 7,4 8 Moreover, irrigation water used in Val Venosta largely derives from glacier and snow-melt, hence we hypothesize that it might be characterized by more

![Figure 2](https://example.com/image2.png)

**Figure 2.** Box and whisker plots of $\delta^2\text{H}$, $\delta^{18}\text{O}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ for apple samples (cv. Golden Delicious) grouped according to the cultivation area. The line in the boxes indicates the median, the * symbol the mean, the ° symbol the outliers. Different letters in each panel indicate significant differences among cultivation areas.

### Table 2. Concentration of selected mineral elements, ratios between element concentration and isotope ratios of South Tyrolean apples PGI (cv. Golden Delicious) divided for the three main cultivation districts

| Element | Bressanone | Val d’Adige | Val Venosta |
|---------|------------|-------------|-------------|
| Mn (ng g$^{-1}$) | 2138 ± 335 a | 1510 ± 391 b | 1758 ± 270 ab |
| Fe (μg g$^{-1}$) | 5.7 ± 0.9 ab | 4.7 ± 1.0 b | 6.3 ± 1.4 a |
| Rb (μg g$^{-1}$) | 2.0 ± 0.7 b | 5.9 ± 3.4 a | 4.6 ± 2.3 a |
| Mo (ng g$^{-1}$) | 47.1 ± 17.9 b | 97.2 ± 37.0 a | 69.7 ± 27.4 a |
| U (ng g$^{-1}$) | 0.29 ± 0.11 b | 0.91 ± 0.31 a | 0.91 ± 87.2 a |
| K/Rb | 3734 ± 1314 a | 1573 ± 1081 b | 1656 ± 537 b |
| $\delta^2\text{H}$ | $-55.5 ± 5.7$ b | $-56.8 ± 5.0$ b | $-66.7 ± 8.2$ a |
| $^{87}\text{Sr}/^{86}\text{Sr}$ | 0.7099 ± 0.0012 b | 0.7096 ± 0.0008 b | 0.7158 ± 0.0030 a |

Only variables for which significant differences among districts were detected are included. Data are reported as mean ± standard deviation and concentrations are referred to the dry sample. In rows, different letters indicate significant differences for the element concentration among the districts.
negative values compared to the irrigation water used in the two other districts, even though no irrigation water sample was collected for isotope analysis in the different districts during the samplings of this study. For what it concerns the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio, a polymetamorphic basement characterized by relatively high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios is present in Val Venosta, determining the Sr isotope ratio of the apples locally cultivated.32

Both the results of the multi-element and multi-isotope analysis provided useful information about the origin of apples, and several variables can be used as markers of the cultivation areas. Nonetheless, none of the considered elements or isotope ratios was able to differentiate simultaneously apples from the different cultivation areas using a univariate approach.

Linear discriminant analysis
To obtain a comprehensive evaluation of the multi-element and multi-isotope composition of PDO and PGI apples cultivated in northern Italy, in comparison with non-GI apples, LDA was applied as classification method of the apple samples. LDA results are summarized in Fig. 3 and Table 3.

The best model was obtained excluding from the dataset Cr and Mg concentration. The three linear discriminant (LD) functions explained respectively 54%, 31% and 15% of the total variance. The variables with the highest contribution to classification in LD1, identified by means of their discriminant coefficients, were given by three concentration ratios: Ca/Ba ($-3.07$), Sr/Ba ($+2.60$), and Ca/Sr ($+2.16$). The concentration of Ca, Mn, and Ba contributed mostly to LD2 ($-1.03$, $+1.01$ and $+1.00$, respectively). The three main contributors to LD3 were Sr concentration ($+1.49$), Ca concentration ($-1.25$) and Sr/Ba concentration ratio ($-1.02$).

The separation between apples from different cultivation areas can be appreciated in Fig. 3, where the two-dimensional (2D) plots including LD1 versus LD2 and LD2 versus LD3 are reported. As
CONCLUSIONS

This study demonstrated the capability of a multi-chemical approach combined with chemometric tools to provide accurate information about the apple origin. Despite a non-negligible effect of the sampling site in determining the element concentration and isotope composition of apple samples, this regional variability did not affect the recognition of apple origin when multivariate data analysis was applied. While a univariate statistical analysis did not result in a clear separation between GI and non-GI apples, a multivariate approach based on the LDA was successful. The developed classification model allowed reaching almost a complete recognition of all the samples according to their origin, with a balanced accuracy > 96%, on average. Such a study might represent an important tool to enhance and tutelage apples. Additionally, we proved that a restricted selection of variables successfully characterizes apples from the three main districts of the South Tyrol cultivation area.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.
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SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

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