# Authenticity of PGI "Clementine of Calabria" by Multielement Fingerprint

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Ⓢ *Supporting Information*

**ABSTRACT:** Clementine is a citrus fruit that has found a peculiar habitat in specific areas of Calabria, a region located in southern Italy. Due to its peculiar characteristics it was recently awarded with protected geographical indications (PGI) from the European Union. In this work, stepwise linear discriminant analysis (S-LDA), soft independent modeling of class analogy (SIMCA), and partial least-squares discriminant analysis (PLS-DA) were used to build chemometric models able to protect PGI Clementine from others of different origin. Accordingly, the concentration of 24−26 elements was determined in peel and juice samples, respectively, obtained from Calabrian PGI clementine and from fruits cultivated in Algeria, Tunisia, and Spain. A cross-validation procedure has shown very satisfactory values of prediction ability for both S-LDA (96.6% for juice samples and 100% for peel samples) and SIMCA (100% for both peel and juice samples). PLS-DA models also yielded satisfactory results.

**KEYWORDS:** *clementine, trace elements, authenticity, statistical analysis, classification, protected geographical indications*

## ◼ INTRODUCTION

Clementine (*Citrus clementina* Hort. ex Tan.) is one of the most important cultivated variety of citrus mandarins in the Mediterranean basin. It is the result of a cross between mandarin and bitter orange achieved in Algeria in the early 20th century. Due to their peculiarly sweet juicy pulp and the absence of seeds, they are recognized worldwide. Clementines are cultivated in many countries located on different continents, and Italy is among the major European producers. Cultivations are located in the southern part of the country where the best weather conditions for their growth exist. Calabria is a region of southern Italy where the cultivation of clementines is widespread. The peculiar pedoclimatic conditions of Calabrian cultivation areas of clementines have contributed to develop a product that, due to its special qualities, was awarded with protected geographical indications (PGI) certification by the European Union as "Clementine di Calabria".[1] The European PGI brand was introduced with Council Regulation (EEC) 2081/92.[2] It is assigned to foodstuffs with a strong regional identity closely linked to a geographical area in at least one of the stages of production, preparation, or processing. This legislation was introduced with the aim of protecting products against fraud and imitation and to protect consumers by providing them with information on quality, organoleptic, and nutritional characteristics of foods.

Several papers concern the beneficial health effects of citrus fruits and citrus-derived products. Some of these properties have been found to include anticancer, antiviral, and anti-inflammatory activities, which are related to the presence of antioxidants including vitamin C, carotenoids, and phenolic compounds.[3−7]

Citrus juice has been the subject of many studies, most of which have concerned development and optimization of analytical methods for the identification of orange juice adulteration.[8−11] Other studies were carried out to determine the geographical origin of lemon juices,[12] orange juices,[13] and the discrimination of orange juices and frozen concentrate orange juices (FCOJ) using trace element contents.[14,15] A carotenoids profile determined by HPLC coupled with photodiode array detector was used to differentiate pure Valencia juices from five countries.[16,17] Recently, Rummel et al. successfully applied the combination of stable isotope abundance ratios of H, C, N, and S on $^{87}Sr/^{86}Sr$ for geographical origin assignment of orange juices.[18]

Nowadays, few studies concerning the discrimination of clementines are present in the literature. The profiles of the flavanone-7-*O*-glycosides and fully methoxylated flavones (FMFs) content of three clementine cultivars were subjected to discriminant analysis.[19] The content of antioxidant constituents narirutin, hesperidin, and total vitamin C was used for classifying different varieties of mandarin and orange.[20] Denaturing gradient gel electophoresis (DGGE) of 16S rDNA fragments, generated by polymerase chain reaction (PCR), was used to characterize the bacterial flora of clementines, proving the relationship between the bacterial communities of fruits and their geographical origins.[21]

It is noteworthy from the literature that, with respect to the use of phytochemicals, which is more affected by random environmental changes, the multielement content of macro- and microelements in foods clearly reflects the soil type and the environmental growing conditions.[22] Among these, a prom-inent role is played by rare earth metals due to their less anthropogenic presence in soil.[23] Because of that, evaluation of trace element content has been proposed to ensure a more

**Figure 1.** Calabria map. In black is spotlighted the cultivation areas specified in the PGI certification of "Clementine of Calabria".

reliable individuation of the geographical origin of food samples.

"Clementine of Calabria" is a fruit known throughout the world with a great impact on the regional and national gross internal product. Economically driven frauds are, therefore, surveyed because fruits produced in other countries, such as Spain, Tunisia, and Algeria, are often introduced in the market with the Calabria PGI brand name.[24] The Consortium for the protection of the PGI "Clementine of Calabria"[25] was therefore founded in 1998 to promote and protect the autochthon products according to the standard UNI 22005:2008. The latter defines the principles and specifies the requirements for the implementation of a system of traceability in the agrifood companies, but they hardly offer tools for identification of origin of food products based on reliable scientific methodologies.

The development of analytical methods for geographical origin identification of food products represents an important goal to ensure organoleptic and nutritional characteristics to consumers and to prevent unfair competition that can eventually damage the whole agricultural sector. Several scientific contributions are available in the literature, and a great number of different analytical techniques and parameters have been evaluated for geographical origin authentication purpose.[26−28] Multielement analysis has been applied to a range of foodstuffs to develop methods for the identification of their geographical origins.[22,29] The contribution of the mass

spectrometry group of the University of Calabria in the identification of useful markers for the traceability of foodstuffs is documented.[30−32] It has been oriented in the implementation of multielement profiling in the geographical characterization of olive oil, tomato, and tomato paste and the detection of buffalo milk adulteration.[33−35] Recently this approach was also successfully applied to Tropea red onion, a product with PGI certification.[36]

The capability of multielement profiling to act as a marker for the classification of the PGI "Clementine of Calabria" is now evaluated, and it represents, to the best of our knowledge, the first study aiming at providing markers to protect clementines identified with the European Union brand name. Forty-six elements were screened by ICP-MS in 54 juice and peel clementine samples grown according to the production regulations of the Consortium for the protection of the PGI "Clementine of Calabria" and in 34 juice and peel clementine samples produced in cultivation areas not indicated in the production regulations.

Principal component analysis (PCA) was used to perform a preliminary inspection of the distribution of information in data. Three supervised pattern recognition chemometric procedures, linear discriminant analysis (LDA), soft independent model class analogy (SIMCA), and partial least-squares discriminant analysis (PLS-DA), were applied for building models able to discriminate between PGI and non-PGI

samples. Cross-validation was used to check the reliability of the chemometric models in terms of classification ability.

## ■ MATERIALS AND METHODS

**Chemicals and Instrumentation.** The mineralization was carried out using acids of Suprapur grade ($HNO_3$ (65%), $H_2O_2$ (30%), HCl (30%), HF (40%), $HClO_4$ (70%), and $H_3BO_3$) (Merck, Darmstadt, Germany). All other reagents used for analysis were of analytical reagent grade (Merck). Two multielement solutions of Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, V, U, and Zn (100 mg/L, Merck) and Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sm, Sc, Tb, Th, Tm, Y, and Yb (10 mg/L, PerkinElmer) were used for the preparation of aqueous calibration standard solutions after appropriate dilution. All glassware, polyethylene flasks, squeezer, and tubes involved in sample preparation and measurement process were cleaned with nitric acid (2%, v/v) by soaking overnight and rinsed with ultrapure water prior to use. Aqueous solutions were prepared using ultrapure water, with a resistivity of 18.2 M$\Omega$ cm, obtained from a Milli-Q plus system (Millipore, Bedford, MA, USA).

The sample preparation was carried out using the following system for the microwave digestion: Anton Paar Multiwave 3000 with programmable power control (maximum power 1400 W) and rotor XF100 (operating pressure up to 120 bar maximum; operating temperature, 260 °C maximum; construction material, PTFE-TFM for the liner and seal). The element determination was carried out utilizing an Elan DRC-e ICP-MS instrument (Perkin-Elmer SCIEX, Canada). The sample delivery system consisted of a PerkinElmer autosampler model AS-93 Plus with peristaltic pump and a cross-flow nebulizer with a Scott type spray chamber. The ICP torch was a standard torch (Fassel type torch) with platinum injector.

A solution containing Rh, Mg, Pb, Ba, and Ce (10 $\mu$g/L, Merck) was used to optimize the instrument in terms of sensitivity, resolution, and mass calibration.

**Sampling.** Clementine samples of variety 'Comune' with PGI brand "Clementine of Calabria" came from four different Calabrian cultivation zones located in the municipalities of Corigliano Calabro, Lamezia Terme, Pizzo Calabro, and Rosarno (Figure 1).

These samples were provided by six farms and hand-harvested in October, November, and December 2007. For each harvesting month three significant samples, each consisting of 10 fruits and harvested from three plants previously selected in the field, were chosen for each farm, so a total of 54 samples were collected. Non-PGI "Clementine of Calabria" samples came from Spain, Tunisia, and Algeria (Table 1). All samples were immediately stored at −20 °C.

**Table 1. Numbers of PGI "Clementine of Calabria" and Non-PGI "Clementine of Calabria" Samples**

| cultivation zone | region/state | juice samples | peel samples |
|---|---|---|---|
| Corigliano Calabro | Calabria | 18 | 18 |
| Lamezia Terme | Calabria | 9 | 9 |
| Pizzo Calabro | Calabria | 9 | 9 |
| Rosarno | Calabria | 18 | 18 |
| Algiers | Algeria | 10 | 10 |
| Blida | Algeria | 4 | 4 |
| Valencia | Spain | 8 | 8 |
|  | Tunisia | 12 | 12 |

**Analytical Procedure.** Clementines were thoroughly washed with tap water and rinsed with ultrapure water. Clementine juice was obtained by hand-squeezing using a plastic squeezer to prevent metal contamination. Each clementine juice sample was obtained from three clementines randomly chosen among those constituting each sample, and clementines were squeezed separately being careful to obtain the juice from only the edible part of the fruit without including the albedo. For each cultivation zone and harvesting month three replicates were used in the quantitative analysis. An aliquot of

clementine juice (5.0 g) at its natural Brix value was weighed directly into the PTFE-TFM digestion tube of the microwave system. Digestion was performed by adding 2.5 mL of $HNO_3$ to each sample. The operating conditions used for the microwave digestion system are

**Table 2. Mineralization Power Programs Used for the Microwave Digestion of Clementine Juice and Clementine Peel Samples**

| step | power (W) | time (min) | |
|---|---|---|---|
| | | juice | peel |
| 1 | 800 | 10:00 | 15:00 |
| 2 | 0 | 10:00 | 10:00 |
| 3 | 900 | 10:00 | 15:00 |
| 4 | 0 | 30:00 | 35:00 |

shown in Table 2. After a mineralization process, extracts were quantitatively transferred to a graduated polypropylene test tube and diluted with ultrapure water to 50 mL. Blank samples were prepared by subjecting 5 mL of ultrapure water to the same digestion procedure used for juice samples and by adding the same mineralization reagents.

Peel samples were prepared from the same clementines used for the preparation of the juice samples. Each clementine peel (albedo and flavedo) was grated on a plastic kitchen grater to shred the peel without metal contamination. For each zone and harvesting month, three clementines were used in quantitative analysis as for juice samples. An aliquot of shredded clementine peel (300 mg) was weighed directly into the PTFE-TFM digestion tube of the microwave system. Digestion was performed by adding 2 mL of $HNO_3$ and 4 mL of ultrapure water to each sample. The digestion was carried out using the microwave power program shown in Table 2. Digested samples were quantitatively transferred to a graduated polypropylene test tube, and the volume was adjusted to 50 mL with ultrapure water. Blank samples were prepared in a similar way as juice samples by using the microwave conditions and mineralization reagent of peel digestion.

The quantitative determination of elements was carried out with external standards. Ten-point calibration curves covering the range 0.1−2000 $\mu$g/L were used. Standard solutions were prepared by diluting the multielement solutions cited under Chemicals and Instrumentation. The concentration range for the elements Ce, Dy, Er, Eu, Gd, Ho, La, Lu, Nd, Pr, Sc, Sm, Tb, Th, Tm, U, Y, and Yb was 0.1−150 $\mu$g/L, whereas the concentration range for the elements Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, V, and Zn was 0.1−2000 $\mu$g/L.

**Statistical Analysis.** Principal component analysis (PCA) was performed by using the Statistica 7.1 statistical package. Classification was carried out by three multivariate chemometric techniques: linear discriminant analysis (LDA), soft independent modeling of class analogy (SIMCA), and partial least squares-discriminant analysis (PLS-DA). LDA were performed by using Statistica 7.1 (StatSoft 2005 edition), and SIMCA was executed by V-Parvus 2009,[37] whereas the PLS-DA algorithm was supported by the software package The Unscrambler 9.1 (Camo Process As., Oslo, Norway).

## ■ RESULTS AND DISCUSSION

**Analytical Performances.** To choose the best mineralization conditions, some preliminary tests were conducted. Amounts of 0.3, 0.5, and 1 g of peel sample and 2.0, 5.0, and 8.0 g of juice sample were subjected to mineralization using the microwave condition of Table 2. The mineralization mixture was constituted by 2.5 mL of $HNO_3$ and 3.5 mL of ultrapure water for each juice sample and 2 mL of $HNO_3$ plus 4 mL of ultrapure water for each peel sample. The addition of ultrapure water was needed to reach the minimum volume of sample that can be mineralized (6 mL). Pressure and temperature profiles of the mineralization and limpidity of digests were monitored

**Table 3. Summary of Calibration Parameters, Limits of Detection (LODs), and Limits of Quantitation (LOQs)**

| | isotope | calibration range ($\mu$g/L) | $R^2$ | juice LOD (ng/g) | juice LOQ (ng/g) | peel LOD (ng/g) | peel LOQ (ng/g) |
|---|---|---|---|---|---|---|---|
| Ag | 107 | 0.1−2000 | 0.9999 | 0.507 | 0.939 | 9.54 | 24.5 |
| Al | 27 | 1−2000 | 0.9998 | 2.54 | 4.27 | 29.1 | 47.4 |
| As | 75 | 1−2000 | 0.9996 | 2.92 | 4.15 | 101 | 279 |
| Ba | 138 | 0.1−2000 | 0.9999 | 0.311 | 0.470 | 4.28 | 5.74 |
| Be | 9 | 0.1−2000 | 0.9999 | 0.482 | 0.82 | 11.4 | 30.4 |
| Bi | 209 | 1−2000 | 0.9999 | 4.14 | 6.29 | 91.1 | 216 |
| Ca | 44 | 100−2000 | 0.9933 | 823 | 912 | 7167 | 8314 |
| Cd | 114 | 0.2−2000 | 0.9999 | 1.04 | 1.85 | 23.4 | 55.6 |
| Ce | 140 | 0.1−150 | 0.9999 | 0.010 | 0.017 | 0.058 | 0.097 |
| Co | 59 | 0.1−2000 | 0.9999 | 0.067 | 0.109 | 8.27 | 21.1 |
| Cr | 52 | 0.1−2000 | 0.9999 | 0.758 | 1.08 | 1.67 | 2.18 |
| Cr[a] | 52 | 1−2000 | 0.9998 | 3.72 | 8.4 | 10.4 | 18.1 |
| Cs | 133 | 1−2000 | 0.9999 | 0.732 | 1.77 | 7.26 | 20.3 |
| Cu | 63 | 1−2000 | 0.9999 | 3.30 | 4.84 | 91.2 | 227 |
| Dy | 164 | 0.1−150 | 0.9999 | 0.005 | 0.012 | 0.144 | 0.373 |
| Er | 166 | 0.1−150 | 0.9999 | 0.019 | 0.043 | 0.206 | 0.406 |
| Eu | 153 | 0.1−150 | 0.9999 | 0.005 | 0.011 | 0.027 | 0.039 |
| Fe[a] | 54 | 10−2000 | 0.9999 | 22.6 | 31.4 | 81.7 | 95.3 |
| Ga | 69 | 1−2000 | 0.9999 | 0.419 | 0.518 | 1.92 | 2.47 |
| Gd | 158 | 0.1−150 | 0.9999 | 0.005 | 0.009 | 0.012 | 0.016 |
| Ho | 165 | 0.1−150 | 0.9999 | 0.003 | 0.008 | 0.049 | 0.116 |
| In | 115 | 0.1−2000 | 0.9999 | 0.111 | 0.156 | 1.46 | 1.92 |
| K | 39 | 50−2000 | 0.9991 | 184 | 195 | 3073 | 3243 |
| La | 139 | 0.1−150 | 0.9999 | 0.005 | 0.007 | 0.110 | 0.134 |
| Li | 7 | 1−2000 | 0.9999 | 0.214 | 0.257 | 5.11 | 9.48 |
| Lu | 175 | 0.1−150 | 0.9999 | 0.002 | 0.003 | 0.145 | 0.364 |
| Mg | 24 | 1−2000 | 0.9993 | 6.33 | 7.66 | 249 | 389 |
| Mn | 55 | 1−2000 | 0.9999 | 3.19 | 5.21 | 22.4 | 30.7 |
| Na | 23 | 100−2000 | 0.9997 | 37.4 | 64.5 | 843 | 1225 |
| Nd | 142 | 0.1−150 | 0.9999 | 0.003 | 0.006 | 0.005 | 0.011 |
| Ni | 58 | 0.1−2000 | 0.9999 | 0.336 | 0.392 | 1.52 | 2.01 |
| Pb | 208 | 0.1−2000 | 0.9999 | 0.076 | 0.123 | 1.96 | 2.85 |
| Pr | 141 | 0.1−150 | 1 | 0.003 | 0.005 | 0.015 | 0.029 |
| Rb | 85 | 0.2−2000 | 0.9999 | 1.18 | 2.74 | 13.4 | 36.6 |
| Sc | 45 | 5−150 | 0.9998 | 31.4 | 59.1 | 660 | 1541 |
| Se | 82 | 1−2000 | 0.9995 | 0.858 | 1.42 | 24.8 | 58.3 |
| Sm | 152 | 0.1−150 | 0.9999 | 0.010 | 0.023 | 0.051 | 0.096 |
| Sr | 88 | 0.1−2000 | 0.9999 | 0.281 | 0.684 | 7.16 | 11.9 |
| Tb | 159 | 0.1−150 | 0.9999 | 0.005 | 0.009 | 0.124 | 0.227 |
| Th | 232 | 0.1−150 | 0.9999 | 0.120 | 0.188 | 9.30 | 24.2 |
| Tl | 205 | 0.1−2000 | 0.9999 | 0.498 | 0.587 | 13.9 | 32.6 |
| Tm | 169 | 0.1−150 | 1 | 0.006 | 0.014 | 0.0699 | 0.138 |
| U | 238 | 0.1−150 | 0.9999 | 0.169 | 0.274 | 4.13 | 10.9 |
| V | 51 | 10−2000 | 0.9999 | 52.1 | 114 | 1227 | 3347 |
| Y | 89 | 0.1−150 | 1 | 0.002 | 0.007 | 0.006 | 0.011 |
| Yb | 174 | 0.1−150 | 0.9999 | 0.004 | 0.007 | 0.175 | 0.384 |
| Zn | 64 | 1−2000 | 0.9999 | 8.08 | 16.7 | 109 | 151 |

[a]Analyzed in DRC mode.

during the tests to choose the amount of sample that allows the best sensitivity to be achieved without stressing the microwave oven system. The best results were obtained using 0.3 g of peel sample and 5.0 g of juice sample.

Initially, it was decided to monitor a great number of elements including the rare earth metals because these elements often play an important role in food authentication.[23,38] The 46 elements investigated were [7]Li, [9]Be, [23]Na, [24]Mg, [27]Al, [39]K, [44]Ca, [45]Sc, [51]V, [52]Cr, [53]Cr, [55]Mn, [54]Fe, [56]Fe, [58]Ni, [60]Ni, [59]Co, [63]Cu, [64]Zn, [66]Zn, [69]Ga, [75]As, [82]Se, [85]Rb, [88]Sr, [89]Y, [107]Ag, [114]Cd, [115]In, [133]Cs, [138]Ba, [139]La, [140]Ce, [141]Pr, [142]Nd, [152]Sm, [153]Eu, [158]Gd, [159]Tb, [164]Dy, [165]Ho, [166]Er, [169]Tm, [174]Yb, [175]Lu, [205]Tl, [208]Pb, [209]Bi, [232]Th, and [238]U. The determination of some of these elements by ICP-MS is known to suffer from polyatomic isobaric interferences. The dynamic reaction cell (DRC) is proved to be an effective method for relieving such isobaric interferences. It is pressurized with an appropriate gas, and elimination or reduction of polyatomic interferences takes place through the reaction of the interfering polyatomic species in the incoming ion beam with the reaction gas. The formation of new
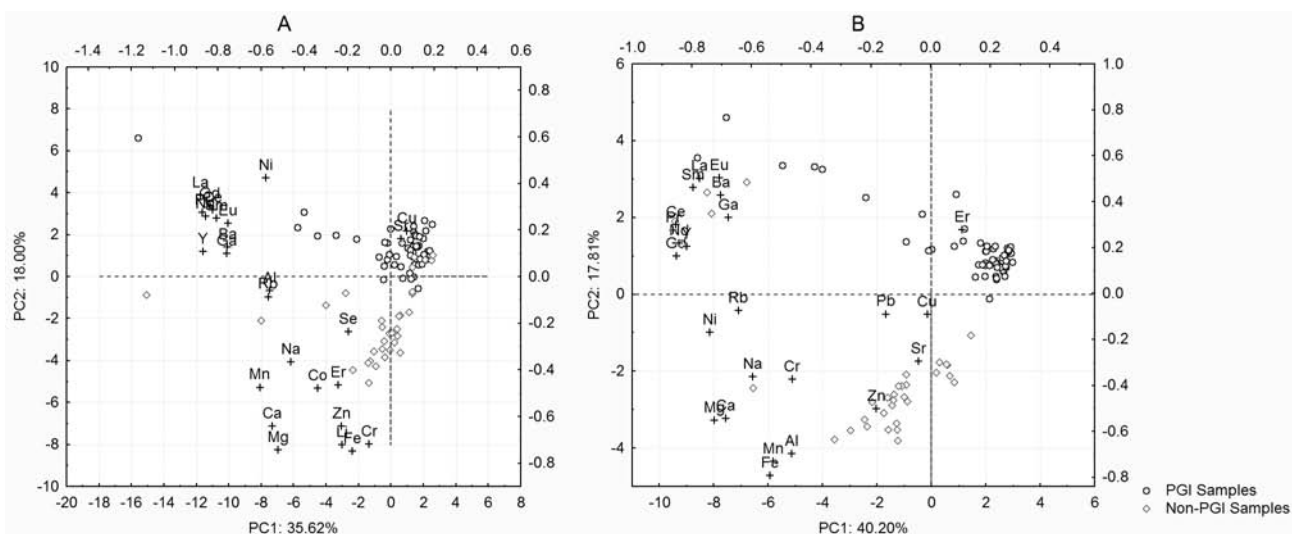
**Figure 2.** Biplot of PC1 versus PC2 scores and loadings for clementine juice samples (A) and for clementine peel samples (B).

interferences is avoided by eliminating unwanted reaction byproducts through the appropriate use of the RF value in the reaction cell. For example, with regard to the elements of our interest, the scandium signal at $m/z$ 45 is affected by the possible presence of $^{13}C^{16}O_2^+$ and $^{29}Si^{16}O^+$, the chromium signal at $m/z$ 53 by $^{40}Ar^{13}C^+$ and $^{37}Cl^{16}O^+$, the iron signal at $m/z$ 56 by $^{40}Ca^{16}O^+$, the nickel signal at $m/z$ 58 by $^{42}Ca^{16}O^+$, the zinc signal at $m/z$ 64 by $^{48}Ti^{16}O^+$ and $^{32}S_2^+$, the selenium signal at $m/z$ 80 by $^{40}Ar^{40}Ar^+$ and, finally, the europium signal at $m/z$ 153 by $^{137}Ba^{16}O^+$. Consequently, Sc, Cr, Fe, Ni, Zn, Se, and Eu have been monitored in both modes (standard and DRC modes) using methane (99.996% purity) as reaction gas. The $CH_4$ flow rate and the RPq value were optimized using matrix blank solution prepared with HCl (2%), $HNO_3$ (1%), $CH_3OH$ (1%), Ca at 20 mg/L, and Si and Ba at 50 $\mu$g/L and a matrix blank solution spiked with Sc, Cr, Fe, Ni, Zn, and Se at 1 $\mu$g/L and Eu at 0.1 $\mu$g/L. The best background equivalent concentrations (BEC) were obtained at a flow rate of 0.7 mL/min for Cr, Fe, Se, and Zn and at a flow rate of 1.15 mL/min for Sc, Ni, and Eu. The best S/N ratio was obtained with a RPq value of 0.6 for all elements except Eu (0.5), Cr (0.7), and Fe (0.7). The operating conditions and parameters of ICP-MS analyses are identical to those used for analysis in an onion traceability study.[36]

Limits of detection (LOD) and quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry, as

$$S_{LOD} = S_{RB} + 3\sigma_{RB}$$

$$S_{LOQ} = S_{RB} + 10\sigma_{RB}$$

where $S_{LOD}$ and $S_{LOQ}$ are the signals at the LOD and LOQ, respectively, $S_{RB}$ is the signal of the mineralization blank, and $\sigma$RB is the standard deviation for the mineralization blank. The concentrations were calculated from the standard curve. The obtained values of LOD and LOQ in mineralized samples are presented in Table 3.

The chemometric treatments have been applied to the data set containing the concentration of those elements that have shown concentration values above the LOD values. Although the concentration values for K were above the LOD, it was not

considered in the statistical analysis because its use as fertilizer is permitted by the production regulations. The concentrations of $^{23}Na$, $^{24}Mg$, $^{27}Al$, $^{44}Ca$, $^{52}Cr$, $^{54}Fe$, $^{55}Mn$, $^{58}Ni$, $^{63}Cu$, $^{64}Zn$, $^{69}Ga$, $^{85}Rb$, $^{88}Sr$, $^{89}Y$, $^{138}Ba$, $^{139}La$, $^{140}Ce$, $^{141}Pr$, $^{142}Nd$, $^{152}Sm$, $^{153}Eu$, $^{158}Gd$, $^{166}Er$, and $^{208}Pb$ in peel samples and the concentrations of $^{7}Li$, $^{23}Na$, $^{24}Mg$, $^{27}Al$, $^{44}Ca$, $^{52}Cr$, $^{54}Fe$, $^{55}Mn$, $^{58}Ni$, $^{59}Co$, $^{63}Cu$, $^{64}Zn$, $^{69}Ga$, $^{82}Se$, $^{85}Rb$, $^{88}Sr$, $^{89}Y$, $^{138}Ba$, $^{139}La$, $^{140}Ce$, $^{141}Pr$, $^{142}Nd$, $^{152}Sm$, $^{153}Eu$, $^{158}Gd$, and $^{166}Er$ in juice samples were submitted to statistical analysis. Among the elements monitored in standard and DRC modes, significant differences have been observed in the assay of $^{56}Fe$ and $^{52}Cr$. Therefore, the statistical analysis has been carried out by means of the data acquired in standard mode except for the chromium in juice samples and iron in both peel and juice samples, for which DRC values were used. The mean concentration and the standard deviation of the elements considered in juice and peel samples analyzed by ICP-MS can be seen in the Supporting Information (Tables S1 and S2, respectively).

**Chemometric Analysis.** The data matrices containing the concentration values ($\mu$g/kg) of the selected elements for peel and juice samples were submitted to an unsupervised technique such as PCA and three supervised approaches (LDA, SIMCA, PLS-DA). PCA is a very important tool, especially in preliminary steps of a multivariate analysis, to perform an exploratory analysis for obtaining an overview of data and finding patterns in complex experimental data. The supervised pattern recognition techniques were chosen to get classification rules for distinguishing between clementine samples grown according the PGI production regulations and non-PGI samples. The reliability of these classification rules was validated through a cross-validation procedure. The samples set was randomly divided into a training set and a validation set, the latter containing $1/k$ of the samples ($k$ is called the cancellation group). Such a division allows for a sufficient number of samples in the training set and a representative number of members in the validation set considered as unknown. The process was reiterated $k$ times with different random constitutions of both sets to ensure that all of the samples were included in the validation set at least once. Classification goodness for LDA and SIMCA was estimated in terms of prediction ability, which is equal to the percentage of the validation set members correctly classified. PLS-DA

**Table 4. Summing of the Forward Stepwise LDA: Selected Elements for Juice Samples and the 11 Most Important Elements for Peel Samples**

| | juice samples | | | | | peel samples | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Wilks' $\lambda$ | Parziale Wilks' $\lambda$ | F remove | p level | | Wilks' $\lambda$ | Parziale Wilks' $\lambda$ | F remove | p level |
| Cr | 0.190443 | 0.757903 | 24.27667 | 0.000005 | Fe | 0.060440 | 0.722102 | 25.78462 | 0.000003 |
| Ni | 0.188653 | 0.765096 | 23.33397 | 0.000007 | Ga | 0.054743 | 0.797263 | 17.03753 | 0.000104 |
| Y | 0.183784 | 0.785367 | 20.77007 | 0.000019 | Cr | 0.052886 | 0.825243 | 14.18822 | 0.000351 |
| Mg | 0.180554 | 0.799415 | 19.06946 | 0.000039 | Ba | 0.052332 | 0.833981 | 13.33755 | 0.000511 |
| Gd | 0.176614 | 0.817249 | 16.99496 | 0.000095 | Ca | 0.050504 | 0.864177 | 10.53040 | 0.001834 |
| Sr | 0.172065 | 0.838855 | 14.59965 | 0.000270 | Sm | 0.046042 | 0.947913 | 3.68158 | 0.059278 |
| Cu | 0.167593 | 0.861239 | 12.24492 | 0.000784 | Ce | 0.046018 | 0.948424 | 3.64352 | 0.060572 |
| Na | 0.166547 | 0.866648 | 11.69425 | 0.001011 | Zn | 0.045950 | 0.949817 | 3.53991 | 0.064254 |
| Co | 0.153686 | 0.939174 | 4.92218 | 0.029499 | Mn | 0.045762 | 0.953727 | 3.25069 | 0.075890 |
| Al | 0.151891 | 0.950269 | 3.97734 | 0.049703 | Cu | 0.045580 | 0.957539 | 2.97102 | 0.089380 |
| Zn | 0.148973 | 0.968887 | 2.44056 | 0.122389 | La | 0.045011 | 0.969642 | 2.09770 | 0.152185 |

prediction ability was evaluated using an external test set constituted of samples submitted to the model as unknown.

**PCA.** PCA is a basic chemometric tool widely used in data analysis. It allows the dimensionality of a data set to be reduced and most of the information present in the original data to be retained. PCA transforms the original variables, using an orthogonal linear transformation, to a new set of uncorrelated variables known as principal components (PCs). Representation of the PCs scores and loadings in a bidimensional plot can be used as an overview of the data, pointing out patterns hidden in the data set and finding possible correlations between variables. The scores and loadings values of the first two PCs for juice and peel samples are plotted in Figure 2, panels A and B, respectively.

Juice samples of the two production areas have similar score values on the first principal component, whereas a separation of objects on the second PC is clear. The elements at the top of the plot (i.e., Eu, Gd, Ce, Pr, Nd, Sm, La, and Ni) are present at higher concentration in PGI samples, whereas clementine samples from abroad have higher concentrations in elements with the highest negative loading values on the PC2 (i.e., Mg, Ca, Zn, Li, Fe, Cr, and Na). Strontium and copper, which have loading values near zero for both considered PCs, have similar concentrations in all clementine samples regardless of the production zone. The four samples from the Algerian province of Blida are characterized by higher score values on the PC1 and slightly negative on the PC2.

For peel samples, the biplot of the first two PCs, which explain, respectively, 40.20 and 17.81% of the total variance, shows the presence of two different clusters corresponding to clementine samples with PGI brand and clementine samples from abroad (Figure 2B). Also in this case, separation of the groups corresponding to the two production areas occurs principally for the second principal component, and the positions of variables in the loadings plot are similar to those observed for juice samples. Moreover, the cluster corresponding to the samples from the Algerian province of Blida is again characterized by higher negative score values on the PC1 and slightly positive on the PC2.

**LDA.** LDA is a chemometric technique that defines a set of delimiters (depending on the number of considered classes) so that the multivariate space of the objects is divided in as many subspaces as the number of classes. Discriminant functions are obtained as a linear combination of descriptors that maximize the ratio of between-class variance and minimize the ratio of within-class variance. This chemometric tool computes a

number of orthogonal linear discriminant functions equal to the number of classes minus one. LDA belongs to the "hard" classification techniques. This means that even if the samples that will be predicted by LDA did not belong to any of the classes of the model, each object will be assigned anyway to one of them. Moreover, each object can fall into one and only one class because the multidimensional space is divided into many subspaces as classes. To obtain models that have good stability, the number of samples must be at least 3 times the number of variables, so the application of a variables reduction technique is needed. S-LDA permits the variables with a major discriminant capacity to be selected, discarding redundant information. In this study, S-LDA was used to classify clementines according to the categories PGI (samples grown in accordance with the production regulations) and non-PGI (samples cultivated in zones different from those cited in the production regulations) used as input a priori. The forward stepwise analysis performed on juice samples (F to enter = 2.00 and F to remove = 1.00) retained 11 elements (Table 4), allowing the number of variables to be halved. Among the most discriminating elements, the presence of alkali metals and alkaline earth metals (Mg, Na, and Sr) is in agreement with other works carried out by our research group on the geographical traceability of oil,[34] tomatoes,[35] and Tropea red onions.[36] On the other hand, these results are not in agreement with those obtained in the differentiation between frozen orange juice from Brazil and Florida[14,15] or Spain and Morocco.[13] To verify the goodness of method in terms of prediction ability, cross-validation with cancellation group 10 was performed. The proposed model showed a total prediction ability of 96.6%, and misclassification involved one sample belonging to the PGI category and two samples belonging to the non-PGI category.

For peel samples, LDA was carried out on the concentration values of the 24 elements reported under Analytical Performances. As for juice samples, forward S-LDA has been performed (F to enter = 0.4 and F to remove = 0.00) and four variables have been eliminated (Al, Eu, Ni, and Pb, Table 4). The cross-validation procedure showed a prediction ability of 100% for each category.

**SIMCA.** SIMCA is a class modeling technique that builds a class model based on the significant PCs of the category. In SIMCA, each category is independently modeled using PCA and can be described by a different number of principal components. In this technique the models (one for each class) can overlap and/or leave some regions of the multivariate space unassigned. Unlike LDA, SIMCA is a soft modeling techniques;

**Table 5. Prediction Matrices for SIMCA of the Cross-Validation Procedure for Juice and Peel Clementine Samples (Rows Represent the True Class, Columns Report the Assigned Class)**

| | juice samples | | | | | peel samples | | | |
|---|---|---|---|---|---|---|---|---|---|
| | PGI | non-PGI | SENS (%) | SPEC (%) | | PGI | non-PGI | SENS (%) | SPEC (%) |
| PGI | 54 | 0 | 83.3 | 91.2 | PGI | 54 | 0 | 85.2 | 100 |
| non-PGI | 0 | 34 | 79.4 | 100 | non-PGI | 0 | 34 | 94.1 | 100 |
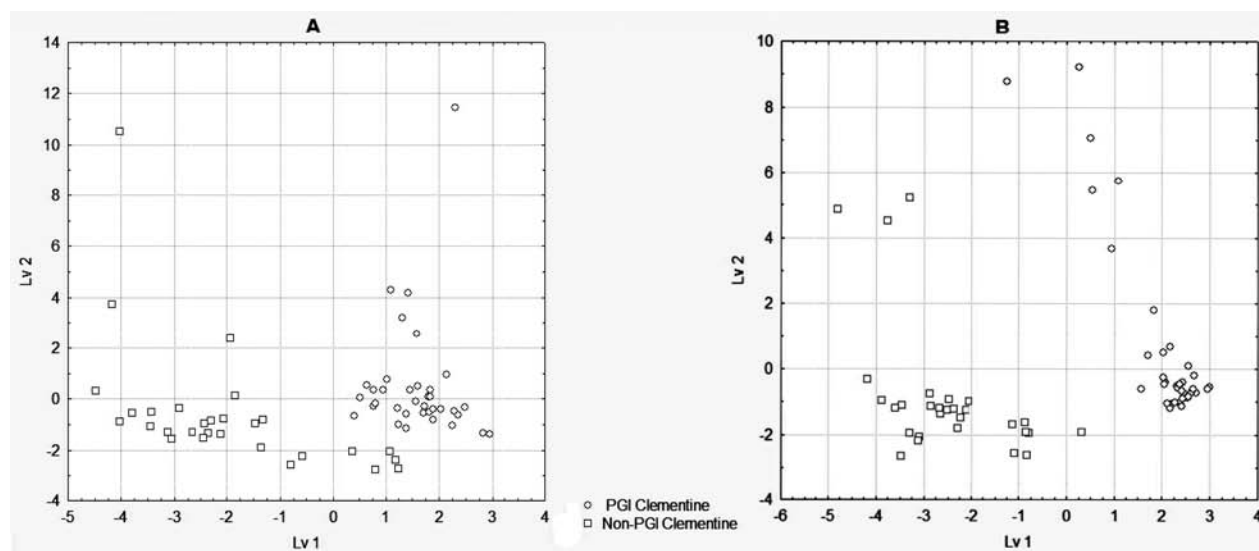


**Figure 3.** PLS-DA plot LV1 versus LV2 for clementine juice samples (A) and for clementine peel samples (B).

thereby, an object can belong to one category, to more than one category simultaneously, or to none of the categories. An important consequence of this feature is that SIMCA is able to detect the number of false positives/negatives for each class. Validation of the model can be evaluated considering not only the prediction ability but also the sensitivity (SENS) and specificity (SPEC).[39,40] The SENS of a class is referred to the percentage of objects belonging to the class correctly accepted by the class model. The SPEC of a class corresponds to the percentage of objects not belonging to the class correctly rejected by the class model.

SIMCA was applied to the same data matrices used for LDA, and validation was carried out by 10-fold cross-validation procedure for both juice and peel samples. With regard to juice samples, the model obtained was based on nine PCs for the first class (PGI samples) and six PCs for the second class (non-PGI samples), which explain 89.2 and 91.2% of total variance, respectively. The SIMCA model has provided very good prediction ability, classifying correctly all samples submitted (Table 5).

Moreover, the SIMCA model is satisfactory in terms of mean sensitivity (81.8%) and mean specificity (96.6%). Better results are achieved by the SIMCA model built using a peel sample data matrix. For these data, the two SIMCA classes are modeled considering eight PCs for the first class (explained variance of 89.7%) and seven PCs for the second class (explained variance of 90.2%). As for juice samples, the SIMCA model is capable of correctly classifying all samples submitted during the cross-validation procedure and, in this case, shows better values of mean sensitivity (88.6%) and mean specificity (100%) (Table 5).

**PLS-DA.** DA carried out by PLS regression is a widely used tool in pattern recognition. The PLS-DA algorithm allows the

establishment of a regression model between the data matrix **X**, where each sample is described by a number of variables, and a dummy variable represented by a vector **Y** for each class. This vector has binary response with a value of 1 for members of that class and 0 for nonmembers. During the calibration process, the PLS-DA model computes the membership value of each sample and an object is assigned to one class if its value is above a specific prediction threshold. Indeed, because it is hard for the predicted value to be exactly 1 or 0, values $\geq 0.5$ are interpreted as indicating a membership of the considered class, whereas results $\leq 0.5$ indicate nonmembership.[41]

Conversely to PCA, the PLS-DA model is able to classify unknown samples. During PLS-DA the PCs are rotated to generate latent variables (LVs), which represent those directions that maximize the variance between different classes rather than the total variance as in PCA.

PLS computes a number of latent variables with decreasing explained variance. After a number of latent variables, the variation explained by the others LVs can be mostly attributed to noise. Therefore, to avoid overfitting, it is extremely important to choose the correct number of LVs in the calibration step of model development. An external test set is often used to evaluate the true prediction performance of the optimized regression model even though, in the case of PLS, it does not seem strictly necessary.[42] In this work, the whole data set for peel and juice samples was split into two groups: a calibration set and a test set. To have a balanced calibration set, comparable numbers of samples belonging to each class were included.[43] The remaining samples constituted the test set (18 PGI samples and 5 non-PGI samples). The model was validated by full cross-validation (leave one out) on the calibration set samples. The procedure gives a first estimation of the prediction performance. The optimum number of LVs
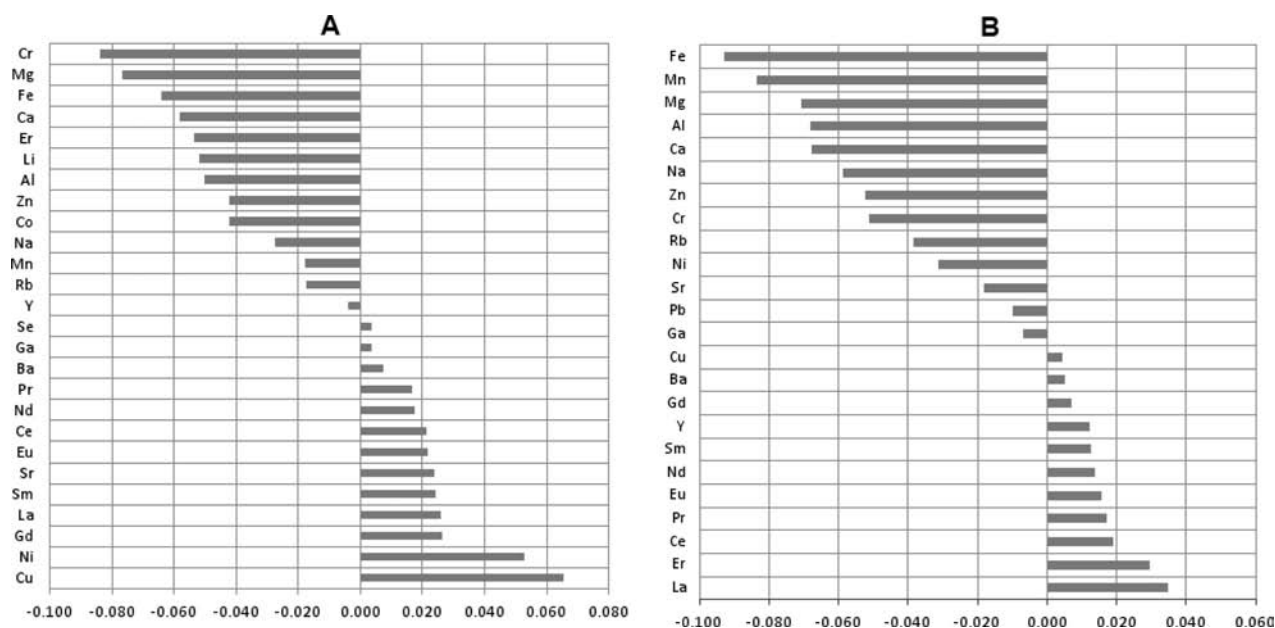
**Figure 4.** Weighted regression coefficients for the variables in the PLS-DA models: clementine juice samples (A) and clementine peel samples (B).

was selected by evaluating the parameter root mean square error of prediction (RMSEP) as a function of the number of LVs. The accuracy of the PLS model developed was evaluated as prediction ability on the basis of the correct classification of test set samples, which were submitted as unknowns to the regression model. The original data were column centered and standardized by 1/standard deviation.

The weighted regression coefficients of the PLS model were used to identify the most important variables. Their value gives an indication of experimental variables, which have a significant impact on the response variables. The use of a weighted coefficient is preferred because it allows the real importance of variables to be identified as their sizes do not depend on the range of variation.[44,45]

Variables that have weighted regression coefficients with high values play an important role in the regression model and, in particular, positive values indicate a great deal in the relationship with the response for the PGI category, whereas negative values mean a great deal to the non-PGI category.[46]

For juice samples two LVs were chosen by which the RMSEP function has reached the first minimum. The 2D plot of the scores of the first three LVs is shown in Figure 3A. The model explained 73.8% of total variance, and RMSEP and RMSEC values were 0.28 and 0.25, respectively. The closeness between these values can be interpreted as a lack of overfitting and good ability of the model to describe other data well.[42,47] All samples of the test set were submitted to the model, and only two of them (one PGI sample and one non-PGI sample) were wrongly classified. The analysis of the PLS regression coefficients (Figure 4A) shows that copper, nickel, and lanthanides are the most representative elements for the PGI category, whereas magnesium and calcium are representative for the non-PGI category.

For peel samples 2two LVs were chosen by which the RMSEP function has reached the first minimum. The 2D plot of the scores of the first two LVs is shown in Figure 3B.

The model explained 92% of total variance, and RMSEP and RMSEC values were 0.16 and 0.14, respectively. Also in this case, the PLS model shows good prediction ability because only

one sample belonging to the non-PGI category was erroneously predicted. By looking at the regression coefficients (Figure 4B), it is possible to observe that most of the elements (8 of 11) characterizing PGI peel samples belong to lanthanides. This result confirms the important role that these elements can play in applications of food authentication.[23] On the other hand, iron and manganese are the most representative elements for the non-PGI category. Moreover, as observed for juice data, also for peel samples magnesium, calcium, and sodium have an important role in the distinction of geographical origin.

In conclusion, in this work three pattern recognition chemometric models were evaluated to ascertain the geographical origin of "Clementine of Calabria" and to develop a reliable analytical tool for traceability purposes. Multielement fingerprint of both peel and juice samples, suggested as discriminative marker, was determined by a simple and rapid method based on a mineralization process assisted by microwaves and a subsequent ICP-MS analysis of the digested samples. The results of chemometric analysis expressed in terms of prediction ability show that all of the statistical techniques involved (S-LDA, SIMCA, and PLS-DA) can be successfully employed for traceability purposes.

In particular, all of the considered chemometric approaches show better prediction abilities by considering the multielement distribution of peel samples. S-LDA and SIMCA for juice samples are capable of predicting 96.6 and 100% of sample origin, respectively, whereas with the PLS-DA model only two samples of an independent test set were erroneously predicted. On the other hand, for peel samples excellent results were achieved by S-LDA and SIMCA models (all samples correctly classified), whereas for the PLS-DA model only one sample was erroneously assigned. Another significant achievement of the proposed method is represented by the observation that, for all of the chemometric models, the classification is unaffected by the harvesting period of the fruits.

It seems worth mentioning that the protocol here presented could be successfully applied to determine the origin of clementines produced in any other country and that an effort in this direction might offer consumers scientifically based

information, thus preventing fraudulent introduction in the market of foods of unknown origin.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information**

Mean concentrations and relative standard deviations of elements in clementine juice samples and peel samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**
The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

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