ISSN 1330-9862
(FTB-2781)

original scientific paper

Characterization of Slovenian Apples with Respect to Their Botanical and Geographical Origin and Agricultural Production Practice

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Received: March 29, 2011
Accepted: October 24, 2011

Summary

The objective of this preliminary study is to demonstrate that the combination of multi-element analysis, several isotopic ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{2}H/^{1}H$) and selected chemical and physical parameters (fruit mass, antioxidant activity, content of ascorbic acid and total phenols) can be used to differentiate the varieties of Slovenian apples, the geographical location of their growth and agricultural practice. The stable isotope parameters in sugar, pulp, protein and water were shown to be the most significant variables in this regard. Botanical origin (cultivar) was found to have a major influence on the $\delta^{13}C$ and $\delta^{15}N$ values of proteins and the $\delta^{18}O$ and δD values of water. Geographical regions were well separated based on the $\delta^{18}O$ and δD values in water and the concentrations of Rb and S in fruit juice. The most significant variables to distinguish between organically and conventionally cultivated fruits were found to be $^{15}N/^{14}N$ ratio and antioxidant activity. In addition, significant differences were also observed in ascorbic acid content.

Key words: apples, stable isotopes, total reflection X-ray fluorescence (TXRF) spectrometry, botanical origin, geographical origin, agricultural practice, Slovenia

Introduction

The European Fruit Juice Directive of 2001 provides clear descriptions of fruit juices and their authenticity (*1*). These regulations are implemented by industry through a Code of Practice (CoP), which is established by the Association of the Industry of Juices and Nectars (AIJN), a Pan-European association for fruit juice producers and bottlers (*2*). As fruit juices are relatively easy to manipulate, new, sophisticated analytical tools to detect potential falsifications have to be established (*3*). A common theme of food authentication studies is the requirement for a database of genuine samples to which a sample can be compared to establish its authenticity. The same database could further be used to determine its geographical origin, which allows producers to be recognized and a premium price to be assigned.

The use of isotope ratio mass spectrometry (IRMS) provides information on botanical and geographical origins, as well as agricultural production practice, which

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are often considered, either by the consumer or by national and international regulations, important characteristics of many food products. This method is based on the measurement of stable isotope ratios (^{2}H, ^{13}C, ^{18}O, ^{15}N, *etc.*) of a product or of a specific component, such as an ingredient or a target molecule of the product (*4*).

Geographic indications are increasingly serving as a marketing tool that can add economic value to agricultural products by conveying a cultural identity through the region of origin (*5*). Measurements of the stable isotope ratios of hydrogen (^{2}H/^{1}H) and oxygen (^{18}O/^{16}O) are applicable for the characterization of geographical origin because they are strongly latitude dependent (*6*). While water is the only source of hydrogen for photosynthesis, oxygen is taken by plants from several sources, from atmospheric oxygen and carbon dioxide and, mainly, from water pools from the soil. Consequently, the ^{2}H- and ^{18}O-contents of fruit juices or wines should reflect the geographical origin of the product (*4*). In addition, it is well known that the content of selected minerals and trace elements in foods clearly reflects the environmental growing conditions. Evaluation of trace element content has therefore been proposed to assure the geographical origin of an agricultural product, particularly a fruit juice (*7,8*).

Local agricultural practices and animal diet also affect ^{15}N/^{14}N and ^{13}C/^{12}C ratios, respectively. In the case of carbon, the isotope ratio in foodstuffs is directly related to their botanical origin. C_3 plants use the Calvin photosynthetic pathway to assimilate CO_2. During this process, the plants discriminate against ^{13}C and therefore possess relatively lower ^{13}C/^{12}C ratios than C_4 plants, which utilize the more energy-efficient Hatch-Slack pathway. Since C_3 plants predominate at higher latitudes and C_4 plants are more common in warmer climates at lower latitudes (such as the tropics), there is a gradient of decreasing ^{13}C /^{12}C in plant material from the equator to the poles, which can also be used as a proxy for geographical origin determination. Factors other than CO_2-fixation pathway may also have some impact on the isotopic composition of plants. These include local atmosphere CO_2 concentrations, plant variety, and factors affecting plant physiology and the nutritional status of cells, enzyme levels, plant growth rate, water use efficiency and cultivation practice (*6,9–11*). On the other hand, the N isotopic composition could reflect the soil conditions and thus agricultural practices of the area, and could be used to determine the geographical origin of fruit juices (*12*). Nitrogen isotopes are also dependent on plant type, since N-fixing plants (*i.e.* legumes and some grasses) assimilate atmospheric N directly, whereas non-N-fixing plants assimilate ammonium or nitrate from the soil (*13*). In this case, N isotopic composition can also define the botanical origin of a plant.

Recently, organic farming has been widely adopted as an alternative agricultural practice to sustain economically viable production with minimal environmental impact (*14*). With the increasing economic benefits of organic farming, consumer concerns have grown about whether produce with organic labels is truly grown with organic inputs (*15*). There is, therefore, a need to find authentication tools that can distinguish whether organically certified supermarket produce is indeed organically grown. ^{15}N signatures can reflect the soil conditions and thus provide information about regional agricultural practices. Synthetic nitrogen fertilizers, extensively used in conventional farming, are not permitted in organic agriculture. Fertilizers that are permitted include animal manures, composts, and other products of plant and animal origin (*16,17*). It has recently been observed that organically produced crops, such as tomato, lettuce, carrots (*16,17*), maize (*18*), pepper (*19*), onion, cabbage (*20*) and citrus fruits (*21,22*), are significantly more enriched in ^{15}N than those growing on synthetic fertilizers, suggesting a possible use of δ^{15}N as a marker in labelling organic produce. Furthermore, current studies show that organically grown fruits or vegetables contain more bioactive compounds (vitamins, phenolics), minerals and antioxidants than conventionally grown ones (*23,24*).

In this paper, we present preliminary results on the characterization of Slovenian apples of different varieties and geographical origins. In addition, we have examined different parameters, including isotope ratios of apples grown under organic and conventional production systems, in order to determine differences that may result from the production system. Furthermore, the analyzed parameters of 19 samples of various sorts of apples, from ecological or conventional production of different geographical origin were statistically evaluated.

Materials and Methods

Plant material

Mature fruits (*N*=19) of 6 apple (*Malus domestica* Borkh.) cultivars (Topaz, Idared, Golden Delicious, Goldrush, Gala, Gloster) were collected in 2009 from different geographical regions of Slovenia (Alpine, Dinaric, Pannonian and Mediterranean) under organic (confirmed by certificate) and conventional orchard management. The number of samples for each cultivar, region and production system is presented in Table 1. Samples were collected directly from different orchards. Mean (average) fruit mass was (186±27) g per fruit for organically produced apples and (191±45) g per fruit for conventionally produced apples. Samples were stored in a controlled atmosphere at 4 °C and were squeezed within four days of picking with a juicer (Phillips, HR 1865, Amsterdam, The Netherlands) immediately prior to analysis. Replicate analyses were carried out on all individual samples.

Table 1. Number of samples for each cultivar, region and production system

Variety	*N*	Geographical origin	*N*	Agricultural practice	*N*
Gala	2	Alpine	5	conventional	12
Gloster	2	Dinaric	4	organic	7
Goldrush	2	Pannonian	9		
Topaz	5	Mediterranean	1		
Golden Delicious	4				
Idared	4				

N=number of samples

HPLC determination of ascorbic acid

The extraction method was adapted from Plestenjak and Golob (*25*). A juice sample (10 g) and 10 g of 2 % (by mass) metaphosphoric acid (MPA, Merck, Darmstadt, Germany) were mixed by vortexing, centrifuged at 14 000×*g* for 5 min and filtered (Millipore, 45 μm, Billerica, MA, USA). The supernatant was injected into an HPLC column. Ascorbic acid was determined using a Knauer (Berlin, Germany) high-performance liquid chromatograph equipped with a Bio-Rad Aminex® HPX (87H, 300×7.8 mm, Bio-Rad, Hercules, CA, USA) column fitted with the same guard column. System conditions were: injection volume 20 μL, detector (variable UV/VIS, Knauer) wavelength 245 nm and flow rate 0.6 mL/min. The mobile phase was 0.004 M H_2SO_4. Ascorbic acid was identified by comparing its UV spectrum and retention time with that of a standard. Ascorbic acid standard (Kemika, Zagreb, Croatia) solutions were stabilised with 2 % (by mass) MPA (Merck). Quantification was carried out by means of calibration curve using concentration range of ascorbic acid from 5 to 60 mg/L.

Determination of antioxidant activity

The antioxidant activity (AOA) of the apple juices was evaluated by the DPPH free radical scavenging method. A volume of 60 μL of juice sample (stabilized with 2 % (by mass) MPA in 50:50 ratio, centrifuged and filtered) or methanol (control) was added to 1.5 mL of methanolic solution of a 1,1-diphenyl-2-picrylhydrazyl radical (DPPH, >90 %; Sigma-Aldrich, St. Louis, MO, USA) having an absorbance of 1 at 517 nm. The mixture was shaken vigorously, left to stand in the dark at room temperature for 15 min, and the absorbance was then read at 517 nm, using an Agilent 8453 spectrophotometer (Agilent Technologies, Palo Alto, CA, USA).

In the case of juices having UV absorption overlapping that of $DPPH^{\cdot}$ at 517 nm, solutions without $DPPH^{\cdot}$ were used as blanks. Three separate determinations were carried out for each sample. AOA of apple juice was calculated from the Trolox calibration curve and expressed as Trolox equivalents (TE) in mM/L. Trolox standards (Sigma-Aldrich) were assayed under the same conditions as described for red wine (*26*).

Determination of total phenols in apple juice

The total phenolic content of the control and phenol-enriched apple juices was determined spectrophotometrically at 765 nm following the Folin-Ciocalteu method described by Singleton *et al.* (*27*). Immediately after pressing, apple juice was mixed with 2 % (by mass) MPA. An aliquot (500 μL of stabilised apple juice) was transferred into a test tube and 30 mL of water and 2.5 mL of Folin-Ciocalteu (Sigma-Aldrich) reagent (previously diluted tenfold with distilled water, the mixture should be golden green and discarded if it is olive green) were added and mixed. The mixture was allowed to stand at room temperature for 3 min. A volume of 7.5 mL of 20 % (by mass per volume) sodium carbonate (20 g made up to 100 mL using distilled water) was added to the mixture and mixed gently. A blank was made by mixing water and reagents. After allowing the mixture to stand at room temperature for 2 h, the absorbance was read at 765 nm using a UV/VIS spectrophotometer (Agilent Technologies 8453) with 1-cm path length disposable plastic cuvettes. The measurements were carried out in triplicate. A standard calibration curve was plotted using gallic acid (Merck) in the concentration range of 1–500 mg/L. The results are expressed as mass of gallic acid equivalents per volume of apple juice (in mg/L).

Total reflection X-ray fluorescence (TXRF) spectrometry analysis of trace elements

Sample preparation

Approximately 1 g of apple juice was weighed in a 30-mL plastic tube (Sarstedt, Nümbrecht, Germany), and 1 mL of a solution of gallium (Ga) (CertiPUR®, Gallium ICP Standard, Merck) (0.01 g/L) was added as internal standard. The solution was then homogenized in an ultrasonic bath for 1 h. A volume of 10 μL of the solution was transferred onto a quartz (reflector) substrate and dried in a desiccator overnight, and the sample, as a very thin juice residue on the reflector, was then measured by TXRF.

TXRF analysis and quantification

The TXRF system was composed of a total reflection module, an X-ray spectrometer and X-ray tube excitation system with characteristics described in our previous papers (*28*,*29*). In this work the X-ray spectrometer was based on the silicon drift detector (SDD) from KETEK (München, Germany), with a resolution of about 140 eV at 5.9 keV. The complex X-ray spectra were analysed by the AXIL spectral analysis program (*30*). The uncertainty of this procedure included the statistical uncertainty of the measured intensities and the uncertainty of the mathematical fitting procedure. The overall uncertainty of spectral measurement and analysis was, in most cases, better than 1 %. Quantitative analysis based on an internal standard (Ga) of known concentration was straightforward because the sample was thin and matrix corrections could be neglected (*31*). Elemental contents of P, S, Cl, Ca, K, Mn, Fe, Cu, Ni, Zn, Br and Rb were determined in the analysed fruit samples.

Sample preparation for isotope ratio mass spectrometry (IRMS) measurements

The stable carbon isotope ratio ($^{13}C/^{12}C$) of pulp, sugars and proteins, stable nitrogen isotope ratio ($^{15}N/^{14}N$) of pulp and proteins and stable oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$) ratios of fruit juice were determined. Preparation steps for isotope measurements in apple juices are shown in Fig. 1. Pulp was separated from the liquid fraction (supernatant) of 50 mL of juice by centrifugation (3400×*g*) and purified. It was first resuspended in water, mixed and centrifuged (3400×*g*, 10 min), and the supernatant was discarded. The pulp was additionally washed with water, twice with acetone (Merck) and dried (*32*). The dried samples were homogenized with spatula and placed into tin capsules for isotope ratio measurement.

Sugars were separated from the supernatant and purified by the modified method of Koziet *et al.* (*33*). Soluble substances remaining in the supernatant liquid

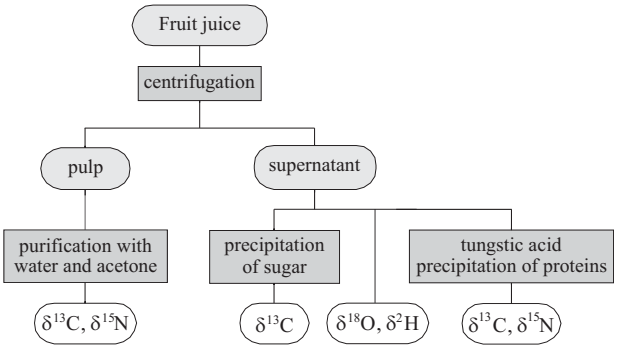

Fig. 1. Scheme of sample preparation for isotope analysis of apple juice samples

after the first centrifugation were purified by the addition of 2 mg of powdered calcium hydroxide (Merck) to the solution while stirring well and heating in a water bath at 90 °C for 3 min. The precipitate was separated by centrifugation of the hot solution (3400×*g*, 5 min) and the clear supernatant was decanted and acidified with 1 M sulphuric acid (Merck) to pH=5 when the colour of the solution became lighter. Residual calcium sulphate was then partially removed by storing the solution in a refrigerator at about 4 °C overnight. Just before stable isotope measurement, 100 μL of each sample were placed in a tin capsule, which was already filled with chromosorb (Chromosorb W 30–60 mesh, PDZ Europa Ltd, Northwick, Cheshire, UK).

Proteins were extracted from juice samples by tungstic acid precipitation according to the method of Jamin *et al.* (*12*). A volume of 5 mL of 10 % sodium tungstate solution (Sigma-Aldrich) was added to 200 mL of clarified juice, followed immediately by 15 mL of 1.34 M H_2SO_4. The mixture was placed in a water bath at 80 °C until flocks were formed. The suspension was centrifuged (3400×*g*, 15 min) and the supernatant was discarded. The solid was washed five times with water and then dried in an oven. The dried samples were homogenized with a spatula and placed in tin capsules for nitrogen and carbon isotope ratio measurements.

IRMS measurement

Stable isotope analyses of sugars, pulp and proteins were performed on a Europa Scientific (PDZ Europa Ltd., Crewe, UK) 20–20 isotope ratio mass spectrometer (IRMS) with an automated nitrogen carbon analyzer solid/liquid (ANCA-SL) preparation module for solid and liquid samples. Stable oxygen and hydrogen isotope ratios were determined in fruit juice using an IsoPrime™ IRMS and MultiFlow preparation system (IsoPrime, Cheadle, UK). Oxygen stable isotope analysis was accomplished using the standard CO_2 equilibration method (*34*), while hydrogen isotopes were analysed by H_2 equilibration using Pt as a catalyst. The $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$ and $^{2}H/^{1}H$ ratios were expressed in the delta notation, $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, $\delta^{2}H$ as the deviation, in parts per million (‰), from the Vienna Pee Dee Belemnite (VPDB) standard for carbon, the atmospheric nitrogen (AIR) standard for nitrogen, and the Vienna Standard Mean Ocean Water (VSMOW) standard for oxygen and hydrogen (*35*). The analyses were calibrated against the International Atomic Energy Agency standards (*36*): IAEA-NBS22 (oil), IAEA-CH-7 (polyethylene) and IAEA-CH-6 (sucrose) with $\delta^{13}C$ values of (–29.7±0.2), (–31.8±0.2) and (–10.4±0.2) ‰ for carbon; IAEA-N1 (ammonium sulphate) and IAEA--N2 (ammonium sulphate) with $\delta^{15}N$ values of (0.4±0.2) and (20.3 ±0.2) ‰ for nitrogen; standard seawater and tap water with average values of (–0.25±0.08) and (–9.45± 0.06) ‰ for oxygen, and IAEA-OH-4 and IAEA-OH-1 with δD values of (–109±1.5) and (–3.9±1.5) ‰ for hydrogen, respectively. The precision of measurements was ±0.2 ‰ for $\delta^{13}C$, ±0.3 ‰ for $\delta^{15}N$, ±0.1 ‰ for $\delta^{18}O$ and 1 ‰ for δD. Data quality control charts were recorded throughout the study period. In addition, to ensure the validity and comparability of the stable isotope results, we participated in the inter-laboratory proficiency testing organized by Eurofins Scientific Analytics (Nantes, France) three times per year.

Statistical analysis

Statistical calculations were carried out using STATISTICA software package v. 7 (StatSoft Inc., Tulsa, OK, USA) and statistiXL (v. 1.8). Basic statistics included mean values (median and arithmetic mean), standard deviation (S.D.), minimum, maximum and Kruskal-Wallis one--way analysis of variance by ranks (Kruskal-Wallis test). Multivariate analysis involved linear discriminant analysis (LDA).

Results and Discussion

Botanical origin

Samples of the six most popular and frequently produced types of apple in Slovenia were taken for measurements. According to their predominant botanical origin, these were classified as Topaz, Idared, Golden Delicious, Goldrush, Gala and Gloster. The average values of measured parameters with standard deviations are listed in Table 2. The concentrations of total phenols ranged from 334 to 571 mg/L with the highest concentration observed in Idared, which also had the highest AOA. The highest average concentration of ascorbic acid (44.3 mg/100 mL) was observed in Topaz and the lowest (13.6 mg/100 mL) in Golden Delicious. The average mass fractions of the elements determined by TXRF followed the order: K>P>Ca>S>Cl>Rb>Zn>Mn. Gala apples had the highest average mass fractions of P and K and the lowest average mass fractions of Mn, Zn and Rb. Goldrush had the highest average mass fractions of S, Cl and Zn and the lowest average mass fraction of K. The highest average mass fractions of Ca, Mn and Rb were observed in Topaz, Gloster and Golden Delicious, respectively. The average $\delta^{13}C$ values in sugar ranged from –27.5 to –25.1 ‰; the AOAC average $\delta^{13}C$ value was –25.3 ‰ (*37*). A similar average $\delta^{13}C$ value of –25.4 ‰ for authentic apple juices was also reported by Doner *et al.* (*38*) and Jamin *et al.* (*12*), the values ranging from –27.9 to –22.5 ‰ and from –26.8 to –23.6 ‰ respectively. Brause and Raterman (*39*) reported a wider range of $\delta^{13}C$ values in apple juices, ranging from –28 to –22 ‰. Lower average $\delta^{13}C$ values were observed in pulp, ranging from –29.9 to –26.2 ‰

Table 2. Mean values and standard deviations of all analysed parameters related to different varieties from different orchards of Slovenian apples

Variety	Gala	Gloster	Goldrush	Topaz	Golden Delicious	Idared
m/g	201±6	244±10	191±43	175±7	163±43	216±36
SS/°Brix	12.4±0.1	13.3±0.5	13.0±0.9	13.8±1.2	12.8±0.8	12.8±1.1
γ(total phenols)/(mg/L)	337±4	348±37	423±193	472±74	334±89	571±202
c(AOA of TE)/(mM/L)	2.8±0.12	3.3±0.02	3.3±0.25	3.6±0.12	3.0±0.05	4.1±0.15
γ(ascorbic acid)/(mg/100 mL)	14.0±2.9	18.4±0.5	20.7±1.1	44.3±11.1	13.6±4.0	29.7±8.6
$\delta^{13}C_{sug}$/‰	–26.1±0.4	–25.1±0.4	–25.3±0.7	–26.2±0.9	–27.5±1.6	–26.5±1.8
$\delta^{13}C_{pulp}$/‰	–28.7±0.7	–26.5±1.8	–26.2±0.5	–26.9±0.7	–29.9±0.3	–27.1±2.2
$\delta^{15}N_{pulp}$/‰	3.8±0.1	3.4±0.3	3.3±0.1	2.5±0.9	1.9±1.9	2.0±2.0
$\delta^{13}C_{prot}$/‰	–29.8±0.2	–26.3±0.6	–27.4±0.8	–27.9±0.6	–31.3±0.8	–28.4±1.7
$\delta^{15}N_{prot}$/‰	3.4±0.1	2.7±0.7	2.3±0.1	2.3±0.8	1.5±2.0	1.6±1.7
$\delta^{18}O$/‰	–4.13±0.09	–3.07±0.08	–2.74±0.02	–3.48±0.37	–3.24±0.23	–4.23±0.45
δD/‰	–45.4±0.3	–36.4±2.7	–32.9±0.3	–34.0±1.8	–28.5±2.6	–39.0±3.3
w(P)/(μg/g)	42.70±10.32	33.20±9.90	40.15±9.26	36.54±5.86	34.92±9.68	31.50±11.53
w(S)/(μg/g)	4.61±1.52	7.07±5.42	9.10±1.98	5.75±3.96	7.90±2.63	4.41±0.77
w(Cl)/(μg/g)	2.95±0.95	2.10±0.78	6.48±1.59	1.91±0.72	2.87±1.05	2.11±0.24
w(K)/(μg/g)	895±84	825±28	786±54	807±89	792±112	799±124
w(Ca)/(μg/g)	13.05±2.33	7.80±0.42	10.00±11.5	14.48±7.91	10.10±0.57	9.74±2.98
w(Mn)/(μg/g)	0.160±0.031	0.346±0.295	0.180±0.053	0.199±0.035	0.195±0.104	0.317±0.135
w(Zn)/(μg/g)	0.282±0.009	0.485±0.038	0.540±0.011	0.431±0.110	0.290±0.041	0.324±0.066
w(Rb)/(μg/g)	0.437±0.001	0.568±0.067	0.796±0.154	1.208±0.265	0.474±0.120	1.387±0.586

and, in protein from –31.3 to –26.3 ‰. The difference between $\delta^{13}C$ values of the sugars and pulp or proteins was always negative by an average of 1.6 ‰.

The average $\delta^{15}N$ values in pulp ranged from 1.9 to 3.8 ‰. In Italian authentic apples it was (2.4±1.8) ‰, but higher in German apples at (5.0±3.3) ‰ (*40*). The mean $\delta^{18}O$ values in the selected authentic Slovenian apple juices ranged from –4.23 to –2.74 ‰ and the mean δD values ranged from –45.4 to –28.5 ‰. These values are typical for fresh juices coming from a warm temperate zone (*4*). In our previous study of Slovenian apple fruit juices from the market the $\delta^{18}O$ values ranged from –10.7 to –5.3 ‰. The highest $\delta^{18}O$ value of –5.3 ‰ was observed in pure apple juice (*41*), lower than the values obtained in this study.

The data were not normally distributed, so only non-parametric tests were applied. The Kruskal-Wallis test showed statistically significant differences at $p<0.05$ in some of the analyzed parameters across these Slovenian apple types. For botanical origin, parameters like $\delta^{13}C$ values of pulp and proteins, $\delta^{15}N$ values of proteins, $\delta^{18}O$ values in water, δD values in water and mass fractions of Mn, Zn and Rb were crucial. LDA was performed with twelve parameters of AOA, $\delta^{13}C$ and $\delta^{15}N$ values of proteins, $\delta^{18}O$ and δD values in water and mass fractions of P, S, Cl, K, Ca, Mn, Zn and Rb. Some values approached the minimum tolerance limit, which indicates multi-co-linearity between the variables and therefore these were excluded from the LDA treatment. The relative magnitude of the standardised discriminant function scores for discriminant function 1 (88 % of total variance) indicates that $\delta^{13}C$ and $\delta^{15}N$ values of proteins and $\delta^{18}O$ and δD values in water could be the major contributors to this function. A graph of discrimination of botanical origin of six varieties of apple is shown in Fig. 2 where good separation between different apple varieties is seen.

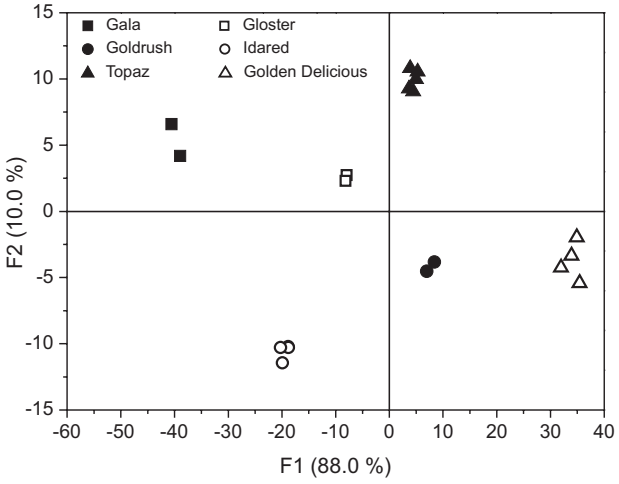

Fig. 2. LDA performed with twelve parameters: AOA, $\delta^{13}C$ values in proteins, $\delta^{14}N$ values in proteins, $\delta^{18}O$ and δD in water and concentrations of P, S, Cl, K, Ca, Mn, Zn and Rb of 19 samples from six different botanical origins. The plane constituted of the first two discriminant axes F1/F2 represents 98.0 % of the total variance for twelve variables

Geographical region

Apples from four different macroregions were selected for study: Alpine, Dinaric, Pannonian and Mediterranean (Fig. 3) (*42*). The ranges of the $\delta^{13}C$ values

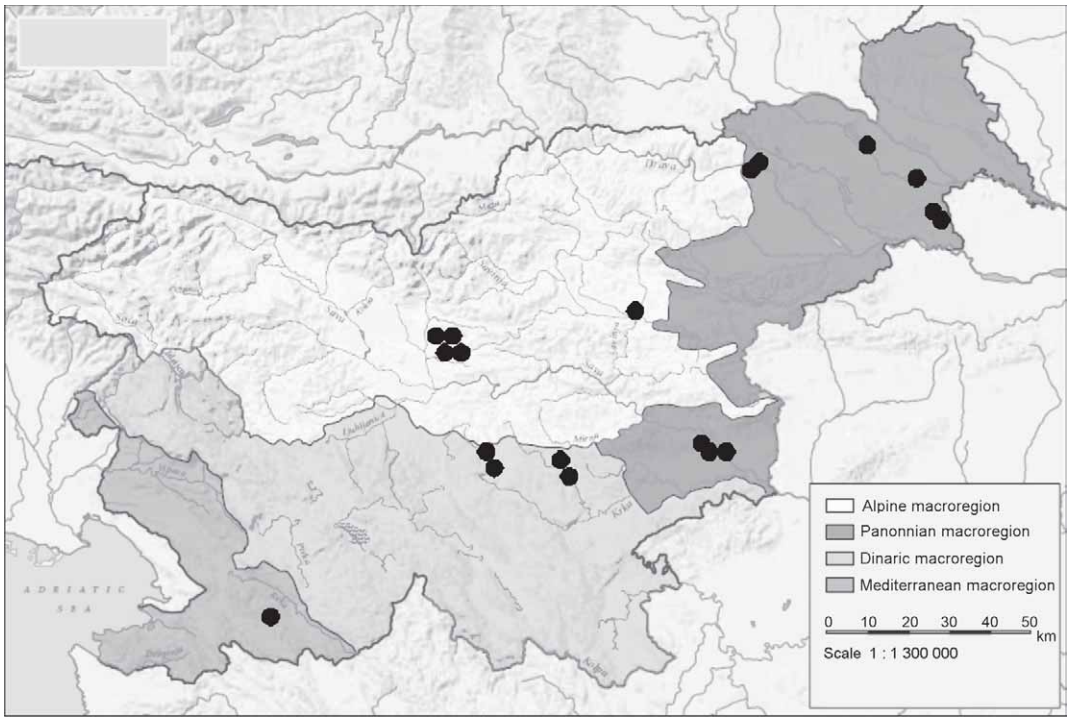

Fig. 3. A map of natural geographical regions of Slovenia (according to Perko (*42*)) showing geographical origins of the Slovenian apple samples (as indicated)

among apples from the four Slovenian regions differ, with the lowest value observed in apples from Pannonian (average $\delta^{13}C=(-28.2\pm1.5)$ ‰) followed by Dinaric (average $\delta^{13}C=(-27.4\pm0.9)$ ‰) and Alpine region (average $\delta^{13}C=(-26.9\pm0.9)$ ‰). The highest $\delta^{13}C$ values of (-25.2 ± 0.9) ‰ were found in the sample from the Mediterranean region. These differences were statistically significant when only Pannonian, Alpine and Dinaric regions were taken into account. In practice, higher $\delta^{13}C$ values between the fruits coming from warmer and dry compared to cold and humid areas were observed as a result of different growing conditions (temperature, humidity, light intensity) (*3*). The $\delta^{13}C$ and $\delta^{15}N$ values of pulp were found to be an additional tool for identifying the origin of juices (*40*). Jamin *et al.* (*12*) indicated that $\delta^{13}C$ and $\delta^{15}N$ values of proteins could be used to separate pineapple juices from different geographical regions in Africa due to the restricted location of the production area of the fruit. Statistical and multivariate analyses demonstrated differences between types of honey of various geographical origins in Slovenia, based on the isotopic parameters including $\delta^{13}C$ and $\delta^{15}N$ values in honey protein (*43*). However, in our study the combination of $\delta^{13}C$ and $\delta^{15}N$ values for pulp and proteins could not be used to differentiate among different regions, since the data overlap.

It appears from isotopic data that the $\delta^{18}O$ and δD values would be better parameters to differentiate among different regions. Water is the only source of hydrogen for photosynthesis, while oxygen is taken by plants from several sources, mainly from atmospheric oxygen and from the water pool in the soil. Consequently, the isotopic composition of hydrogen and oxygen reflects the place where the product comes from and thus could be related to geographical origin. In addition, evapotranspiration during maturation period causes isotopic fractionation and enrichment in heavy isotopes of both hydrogen and oxygen. The isotope ratio observed in plant water is positive relative to that in the corresponding ground water. It is seen from Fig. 4 that samples of apple fruit juices are clearly separated from the global meteoric water line defined by Craig (*44*):

$$\delta D=8\cdot\delta^{18}O+10 \qquad /1/$$

and, from the local meteoric water line determined from Slovenian precipitation data (*45*):

$$\delta D=(8.06\pm0.08)\cdot\delta^{18}O+(9.84\pm0.71) \qquad /2/$$

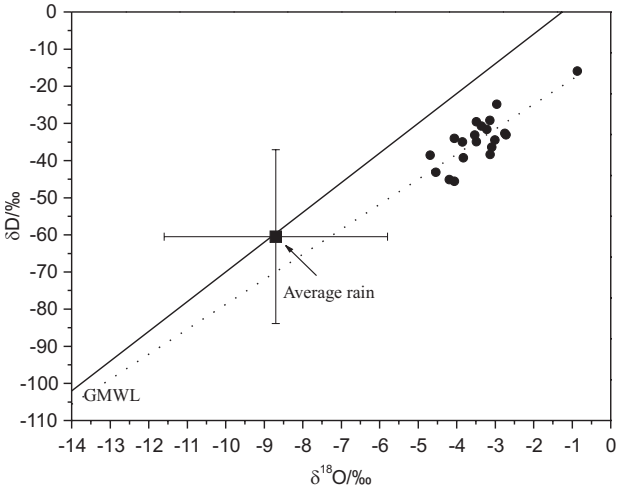

Fig. 4. Global meteoric water line (GMWL) with the average isotopic composition of precipitation in Ljubljana, Slovenia, and stable isotope data applied to fresh apple fruit juices together with linear regression line

The evaporation line obtained from our apple samples (Fig. 3) is:

$$\delta D=(6.73\pm1.16)\cdot\delta^{18}O-(11.38\pm4.06);\ R^2=0.63 \qquad /3/$$

and the resulting slope is higher than that found by Bong *et al.* (*46*), probably due to lower evaporation.

The geographical discrimination of the original data set for four geographical regions is shown in Fig 5. LDA was carried out with fifteen parameters: AOA, ascorbic

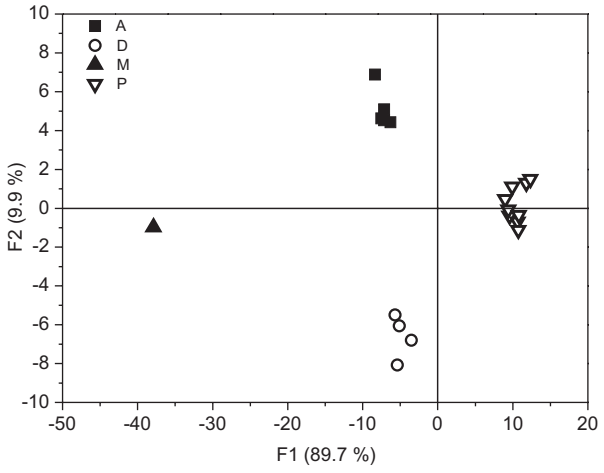

Fig. 5. LDA performed with AOA, ascorbic acid, $\delta^{13}C$ value in sugars, $\delta^{13}C$ value in proteins, $\delta^{14}N$ value in proteins, $\delta^{18}O$ and δD in water and mass fractions of P, S, Cl, K, Ca, Mn, Zn and Rb with 19 samples from four different geographical origins. The plane constituted of the first two discriminant axes F1/F2 represents 99.6 % of the total variance for fifteen variables. Symbols: A – Alpine, D – Dinaric, M – Mediterranean, P – Pannonian

acid, $\delta^{13}C$ values in sugars, $\delta^{13}C$ values in proteins, $\delta^{14}N$ values in proteins, $\delta^{18}O$ and δD values in water and mass fractions of P, S, Cl, K, Ca, Mn, Zn and Rb. The relative magnitude of the standardised discriminant function scores for discriminant function 1 (89.7 % of total variance) indicated the tendency of $\delta^{18}O$ and δD values in water, mass fraction of S, and $\delta^{13}C$ in sugar to be the major contributors to this function, while for discriminant function 2, $\delta^{18}O$ values in water, and mass fractions of S, Ca and Rb were the major contributors.

Agricultural practice

The average values for all defined parameters, standard deviations, together with statistical p-values between organically and conventionally grown apples are presented in Table 3. The different production methods affect the levels of ascorbic acid in apple fruits. The average ascorbic acid content of 32.2 mg/100 mL in the organically grown fruits was higher than the average content of 23.0 mg/100 mL in the conventional ones. Even the average ascorbic acid content overlaps when introducing the S.D. values; there is a statistical difference observed between two management practices (Table 3). Higher ascorbic acid content was also found in organically grown citrus and other fruits (*21,47*). Since ascorbic acid content is thought to be inversely correlated

Table 3. Mean values and standard deviations of all analysed parameters related to soil management with p-values

Treatment	Conventional	Organic	p-value
m/g	197±42	201±6	0.0671
SS/°Brix	12.8±0.8	12.4±0.1	0.5043
γ(total phenols)/(mg/L)	420±166	337±4	0.3102
c(AOA of TE)/(mM/L)	3.4±0.70	2.8±0.50	0.0952
γ(ascorbic acid)/(mg/100 mL)	23.0±12.7	32.2±1.5	**0.0157**
$\delta^{13}C_{sug}$/‰	–26.5±1.5	–26.0±0.9	0.3283
$\delta^{13}C_{pulp}$/‰	–28.1±1.8	–26.9±1.3	**0.0493**
$\delta^{15}N_{pulp}$/‰	2.5±1.6	3.0±0.9	0.4486
$\delta^{13}C_{prot}$/‰	–29.2±2.0	–27.8±1.2	**0.0181**
$\delta^{15}N_{prot}$/‰	1.9±1.5	2.4±0.7	0.4801
$\delta^{18}O$/‰	–3.58±0.63	–3.46±0.53	**0.0152**
δD/(‰)	–35.5±6.1	–36.4±4.3	**0.007**
w(P)/(μg/g)	33.40±8.6	39.90±7.5	0.744
w(S)/(μg/g)	5.46±2.45	7.40±3.87	0.3781
w(Cl)/(μg/g)	2.89±1.07	2.56±2.25	0.127
w(K)/(μg/g)	807±89	819±96	0.7794
w(Ca)/(μg/g)	9.64±3.31	14.0±7.20	0.1968
w(Mn)/(μg/g)	0.223±0.115	0.248±0.140	0.4903
w(Zn)/(μg/g)	0.343±0.098	0.443±0.107	**0.0289**
w(Rb)/(μg/g)	0.845±0.525	0.992±0.470	**0.0217**

Statistically significant differences ($p<0.05$) are marked bold

with the nitrogen supply, the lower values found in conventionally grown fruits could be due to the use of synthetic fertilizers (*48*). Fruit phenolic compounds and AOA were the same in the two systems, as also reported by Roussos and Gasparatos (*49*). On the other hand, some reports support phenolic accumulation under organic management (*50,51*). As reported by Stracke *et al.* (*52*), organically grown apples showed a 7–27 % higher AOA and significantly higher concentrations of polyphenols. Inconsistent influence of farming management type on phenolic concentration has been found in many cases (*53–55*). In the apple fruit juices, concentrations of S and Ca were higher in organically grown fruits. The latter could be related to longer storage since Ca has nutrient apple fruit storage potential (*49*). However, since S.D. values overlap and there were no 'direct' statistical differences observed among these two parameters (Table 3), they could not be used to determine the difference between organically and conventionally grown fruits.

The values of $\delta^{13}C$ in pulp were found to differ between organically and conventionally grown apples, with lower values in the latter. Usually, the lower $\delta^{13}C$ values found in organic fruits have been due to higher microbial activity (*47*). However, other factors, such as variety or site of production, could be more significant parameters influencing $\delta^{13}C$ values than agricultural regime, as discussed in the section on botanical origin. Slightly higher average values of $\delta^{15}N$ in pulp and protein were observed in organically grown fruits than in conventional ones. The relative magnitude of the standardised discriminant function scores for discriminant function 1 in LDA analysis indicated that $\delta^{15}N$ in pulp (average (3.0±0.9)

‰) and $\delta^{13}C$ and $\delta^{15}N$ values in proteins (average (–27.8± 1.2) and (2.4±0.7) ‰, respectively) could be, besides AOA, P, Zn and Cl mass fractions, the major contributors to this function and could be used as a marker of organic apple fruits. A similar conclusion has been reached from studies of other fruits (*21,22,47*). The other isotopic parameters were shown to be less significant in the separation of organic from conventional fruits.

This calculation additionally supports our previous conclusions based on the difference between the average values obtained for organic and conventional production, except for AOA and ascorbic acid. It should be again stressed that the data set is limited and that the LDA results provide only a tendency on variables which might be related to either the geographical or agricultural practice.

Conclusions

Apples are an important fruit product in Slovenia. In this preliminary study the possibility of differentiating among different varieties, geographical origin and management practice was investigated, using multi-element analysis, stable isotope parameters and selected chemical parameters. Botanical origin was found to have a major influence on $\delta^{13}C$ and $\delta^{15}N$ values of proteins and on $\delta^{18}O$ and δD values in water, while geographical regions were well separated on the basis of $\delta^{18}O$ and δD values in water and the mass fractions of Rb and S. The greatest differences in the average values between the two different management treatments were observed in ascorbic acid content, S and Ca mass fractions and $\delta^{13}C$ values in pulp. In contrast to the ascorbic acid content and $\delta^{13}C$ values in pulp the S and Ca mass fractions do not determine the statistical differences between organically and conventionally grown fruits. Furthermore, the LDA analysis showed that $\delta^{14}N$ values in proteins and pulp, $\delta^{13}C$ values in proteins and AOA could be important variables for differentiating between organically and conventionally grown fruits. In the case of $\delta^{13}C$ in proteins, other factors such as variety and year or site of production could influence the values more significantly than agricultural regime. On the other hand, it is well known that $\delta^{14}N$ values are the only isotopic parameter that can be reliably used as a marker of organic fruits.

In this preliminary investigation a small set of nineteen samples of various botanical, geographical and production origin of apples and twenty analyzed parameters was used in an identification model. We were aware that the data set used contained a smaller number of samples than is ideal of both geographical and botanical diversity. It is obvious that a larger data bank of characteristic parameters would improve and extend the proposed identification model. However, based on the obtained data, we were able to determine the number and types of apples and the minimum number of samples that has to be taken from the same region for determining the geographical origin. Higher number of systematically collected samples equally distributed both botanically and geographically all over Slovenia in different years of production would encourage further activities in this direction. In the case of management treatments, a systematic research applying different fertilizers in the experimental orchards will be performed. We are, however, able to confirm that the proposed LDA model is effective and that valuable information about important parameters for good separation of botanical, geographical and production origin was obtained.

Acknowledgement

The authors are grateful to Mr Stojan Žigon and Barbara Petrović for technical support and assistance. The research was partially financed by the European Union, European Social Fund. Special thanks are given to Prof Roger H. Pain for linguistic corrections. The authors also thank Alenka Caf and apple producers for providing samples from their orchards.

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