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Title: Parameters for discrimination between organic and conventional production: a case study for chicory plants (Cichorium intybus L.)

Article Type: VSI: ISOFOOD 2019

Keywords: bioactive compounds; fertility management; isotopic signature; multi-elemental profile; nitrogen assimilation.

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Abstract: Organic crop production has become a highly attractive way of production over the world and thus the need for robust analytical techniques for their authentication. The main aim of this study is to identify appropriate biomarkers to discriminate between organic and conventionally grown chicory. Chicory is an appreciated leafy vegetable among producers and consumers, especially due to its undemanding cultivation and content of bioactive substances. Six different fertility management practices (control, two organic, two mineral, and a combination of organic and mineral fertilizers) were used to produce five chicory cultivars in a glasshouse pot experiment. Analysis of bioactive compounds, nitrogen assimilation, multi-elemental profiling and stable isotope ratio determination of carbon (C), nitrogen (N) and sulphur (S) were performed to differentiate between organic and conventional production. In this study, nitrogen isotopes are found to be an excellent way of identifying organically produced chicory of a different variety with the highest δ15N values. Conversely, the same samples had the lowest δ34S values indicating that also stable isotopes of S could be used as a marker for the authentication of organic production.

Response to Reviewers: Manuscript Number FCT-D-19-02185
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Special Issue “The ISO-FOOD Symposium 2019”

Reviewers’ comments and authors’ replies

Reviewer#1

An interesting approach is presented that fit well into the scope of the journal. There are a few issues that needs to be improved: In the abstract the last sentence stating "The discrimination between

organically and conventionally produced chicory greatly improved when other parameters were included in the statistical evaluation of the data." Which other parameters? This needs to be more clearly explained in the abstract or omitted.
Our reply: We thank Reviewer #1 for the recognition of the value of our study and supportive comments. The last sentence in the abstract is omitted now as suggested.

The XRF approach was chosen for the analysis of microelements (P, S, Cl, K, and Ca), microelements (Zn, Mn, Rb, Br, Mo and Sr). Why these elements? What about trace elements? Some of them could clearly identify the difference as in the artificial nutrients some trace elements could be accumulated in the chicory (for example Cd…) … More discussion is needed in this part of the manuscript, indicating limitations.
Our reply: Energy dispersive X-ray fluorescence spectrometry (EDXRF) is non-destructive, fast and cheap method which offering also easy interpretation. Therefore it was chosen for multi-elemental analysis in macro and micro concentration range of chicory plant material studied within the present manuscript. Regarding trace elements, such as Cd, there is a limitation for analysis with XRF approach. Basically, XRF enable determination of elements down to few ppm level and above listed micro elements are in the range of few tens of ppm presented in chicory samples and therefore suitable for XRF analysis. On the other hand, Cd content is found in plant samples in the concentration around 1 ppm or lower. Because of this, Cd analysis by XRF is impossible.

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In the enclosed manuscript the authors identify appropriate biomarkers to discriminate between organic and conventionally grown chicory. Their study provides the first evaluation of the use of bioactive, stable isotope and elemental parameters to differentiate between different soil fertility management practices (control, two organic, two mineral, a combination of organic and mineral) for the production of five chicory cultivars. It is important subject and fits into the scope of the journal.
Our reply: We thank Reviewer #2 for the supportive comments, and we have tried to satisfactorily address the modifications requested, as detailed below.

Comments:
In the abstract the stable isotope ratio of C, N and S should be defined
Our reply: This has been defined as suggested.

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Our reply: Our apologies, this has been corrected as requested.

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Our reply: Our apologies for the mistake, this has been checked and changed to δ15N.

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Our reply: No delta is missing. The data could be expressed in two ways: we can use expression "13C-depleted or enriched", which results in "lower or higher δ13C values". The sentence was corrected.

In conclusions:
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Parameters for discrimination between organic and conventional production: a case study for chicory plants (*Cichorium intybus* L.)

Lovro Sinkovič[a,*], Marijan Nečemer[b], Nives Ogrinc[c,d], Dragan Žnidarčič[e], David Stopar[f], Rajko Vidrih[g] and Vladimir Meglič[a]

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[g]Department of Food Science and Technology, Biotechnical faculty, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

***Corresponding author.** *Address:* Crop Science Department, Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia. *Tel:* +386-3-15-821-68. *E-mail address:* lovro.sinkovic@kis.si (L.S.).

Highlights:

- Five chicory cultivars were grown in a pot experiment
- Six different fertility management practices were applied
- Differences in bioactive compounds content and nitrogen assimilation were evaluated
- Multi-elemental profiling and stable isotope ratios of light elements were studied
- Parameters to discriminate organic and conventionally grown chicory were identified

*Abstract

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Organic crop production has become a highly attractive way of production over the world and thus the need for robust analytical techniques for their authentication. The main aim of this study is to identify appropriate biomarkers to discriminate between organic and conventionally grown chicory. Chicory is an appreciated leafy vegetable among producers and consumers, especially due to its undemanding cultivation and content of bioactive substances. Six different fertility management practices (control, two organic, two mineral, and a combination of organic and mineral fertilizers) were used to produce five chicory cultivars in a glasshouse pot experiment. Analysis of bioactive compounds, nitrogen assimilation, multi-elemental profiling and stable isotope ratio determination of carbon (C), nitrogen (N) and sulphur (S) were performed to differentiate between organic and conventional production. In this study, nitrogen isotopes are found to be an excellent way of identifying organically produced chicory of a different variety with the highest δ^{15}N values. Conversely, the same samples had the lowest δ^{34}S values indicating that also stable isotopes of S could be used as a marker for the authentication of organic production. ~~The discrimination between organically and conventionally produced chicory greatly improved when other parameters were included in the statistical evaluation of the data.~~

Keywords: bioactive compounds; fertility management; isotopic signature; multi-elemental profile; nitrogen assimilation

1. Introduction

In recent years, there has been a growing interest in organic foodstuffs from consumers, which are produced sustainably and have a low environmental impact and command higher prices on the market (Vallverdú-Queralt and Lamuela-Raventós, 2016). The sphere of organic foods includes fruits, vegetables and cereals, which are often considered healthier, safer and better for the environment and animal welfare than conventional foods because synthetic pesticides and fertilisers are not used (Reganold and Wachter, 2016; de Souza Araújo et al., 2014). In Europe, agricultural products and foodstuffs of organic farming should be produced in accordance with the EU regulations on organic production and processing (No 834/2007 and No 889/2008), without the use of any synthetic plant protection products and soluble synthetic fertilizers, with a full list of the pesticides and fertilizers of natural origin. In 2018 a new EU regulation (No 2018/848) was adopted that will enter into force on January 1st 2021. Its main improvement is the introduction of uniform European rules that will apply to the entire organic production sector in the Union. However, since organic food production represents a significant market segment within the global food industry with products having higher market prices, fraud is expected to increase (Conti et al., 2014). Most food fraud is motivated by quick economic profit, and organic products are at a higher risk of such substitutions as they are sold at premium prices compared to conventional ones (Laursen et al., 2014). Thus, there is an urgent need to develop appropriate, effective and robust analytical approaches to distinguish between the two production systems (Tähkäpää et al., 2015; Mihailova et al., 2014).

At present, there is no universal analytical method that can be applied to differentiate organic and conventional produced plant-based foods. The use of nitrogen stable isotope ratio $^{15}N/^{14}N$ (expressed as $\delta^{15}N$) to discriminate organic from conventional production has been discussed in detail previously (Bateman et al., 2007; Rogers, 2008). It is based on the fact that

synthetic nitrogen fertilizers, used in conventional and integrated farming, have δ^{15}N values significantly lower than the animal manures and fertilizers permitted in organic agriculture. However, soil fertility in organic farming is maintained through the use of crop rotations that include legumes and green manures and also by the application of certain naturally derived fertilizers. Organically produced tomato, lettuce, carrots (Bateman et al., 2007; Bateman et al., 2005), maize (Choi et al., 2002), pepper (Flores et al., 2007), onion and cabbage (Georgi et al., 2005) are significantly enriched in ^{15}N than those ~~receiving~~ receiving synthetic fertilization. In general, the δ^{15}N ratios can be a useful discriminant tool for crops requiring intensive horticulture, but not for all cultivation typologies, especially in soil-grown crops with a long growth cycle. It is also reported that discrimination capability could be improved by including element profiling and applying appropriate statistical tools. Correlations between mode of production and δ^{13}C (except for greenhouse produced tomatoes warmed with natural gas) and δ^{34}S signatures have not been established. There are also a growing number of studies comparing organic and conventional farming vegetables concerning mineral profiles (Kapoulas et al., 2017; Krejčová et al., 2016), bioactive compounds (Ku et al., 2018; Orsini et al., 2016; Sinkovič et al., 2015), physicochemical contaminants (de Souza Araújo et al., 2014) and nutritional quality (Popa et al., 2019; Yu et al., 2018; Maggio et al., 2013; Herencia et al., 2011; Huber et al., 2011).

Although most publications concern crop production, to the best of our knowledge, no paper includes the stable isotope ratio of nitrogen in chicory plants. Chicory (*Cichorium intybus* L.) along with lettuce represents a fresh leafy vegetable crop with a total world production close to 27 million tons in 2017 (FAOSTAT, 2019). Although its production is quantitatively less abundant compared to lettuce, it amounts to around 30% of total production. Due to a range of advantages such as resistance to low temperatures and hence year-round supply, a broad palette of colours and potential health benefits, chicory has

become an economically attractive crop for many producers (Bergantin et al., 2017; D'Evoli et al., 2017). Also, the potential for organic and conventional production makes chicory a suitable vegetable crop for a variety of different case studies.

This study aimed to examine the effect of different fertility management practices on the biochemical composition of several chicory cultivars and to verify the possibility to differentiate between organic and conventional production. The study includes the following analyses: determination of bioactive compounds (total phenolics, antioxidant potential, total flavonoids), nitrogen assimilation, multi-elemental profiling and the measurement of stable isotope ratios of carbon, nitrogen and sulphur. Based on the obtained data, a robust model to control the type of fertility management practice was established.

2. Materials and methods

2.1. Plant material and fertility management experiment

Five commercial cultivars of chicory (*Cichorium intybus* L.) were studied, namely 'Treviso', 'Verona', 'Anivip', 'Castelfranco' and 'Monivip', which are the most popular cultivars for production and consumption in Slovenia and neighboring countries. The seeds were purchased from commercial seed companies ('Treviso', 'Verona' and 'Castelfranco' from Semenarna, Ljubljana, Slovenia; 'Anivip' and 'Monivip' from L'Ortolano, Cesena, Italy).

Chicory plants were produced in the spring growing season in a glasshouse at the Biotechnical Faculty, University of Ljubljana, Slovenia (46° 04´ N, 14° 31´ W; 320 m a.s.l.). Along with the control (no added fertilizer), different types of fertilizers (two organic, two mineral), and a combination of organic and mineral fertilizer were tested. For each of these five cultivars, the same six fertility management practices were applied in a completely

randomized factorial design, namely: no added fertilizer/control (CONT); addition of the two single basal organic fertilizers, using Plantella Organic (ORG1; 3-3-2; 67.5 g/pot; Unichem, Slovenia) and Stallatico Pallettato (ORG2; 3-3-3; 45 g/pot; Fomet, Italy); a single water-soluble mineral fertilizer, using Kristalon Blue (MIN1; 19-6-20; watering with 9 g/100 L; Yara, Norway); a single basal mineral fertilizer, using Entec Perfect (MIN2; 14-7-17; 7.9 g/pot; EuroChem, Italy); and a combination of an organic (ORG1) and a mineral (MIN1) fertilizer (ORG1+MIN1; Plantella Organic+Kristalon Blue; 2.5 g/pot+after 1 month, watering once per week with 3.5 g/L).

Chicory seeds (n = 10) were sown in plastic pots containing 7 L of virgin soil without or with the addition of fertilizer according to the protocol. The pots were placed on rolling benches in a heated glasshouse compartment (18 ±2°C) and watered when required. After several weeks, the seedlings were thinned to give five plants per pot. The water-soluble fertilizer (MIN1) was applied during the watering of the plants when two fully expanded chicory leaves had grown. The harvesting of chicory leaves was performed 130 days after sowing.

2.2. Sample preparation

The uniform leaves of five chicory cultivars grown under the six fertility management regimes were collected between 06:00 to 08:00, solar time and prepared in three ways. First, 10 g of fresh leaves were extracted in a ploypropylene plastic vial with 15 g 50 % methanol. The tissue was homogenized using an Ultraturax T25 (20,500 rpm) for 5 min. The samples were then frozen and stored at –20 °C until analysis of total phenolics content (TPC), antioxidant potential (AOP) and total flavonoid content (TFC). Second, few uniform leaves were immediately frozen in liquid nitrogen and then lyophilized and homogenized to a fine powder using a laboratory ball mill (Retsch mm 301). The samples were stored in glass vials

in humidity-proof plastic bags filled with silica gel before analysis of nitrogen assimilation. Third, chicory leaves were dried in a laboratory oven at 80 °C for 28 h, ground up with a mortar and pestle. The ground leaves were then used in the determination of dry matter (DM), multi-elemental profiling and the determination of stable isotope ratios of C, N and S.

2.3. Bioactive compounds

The total phenolics content (TPC) was determining ~~ed using a~~ spectrophotometer~~r~~rically following the Folin-Ciocalteu method, as first described by Singleton and Rossi (1965), and slightly modified by Roura et al. (2006). Gallic acid was used for the construction of the calibration curve. Briefly, 1 mL of each centrifuged methanol fraction from the sample was mixed with 60 mL deionized water and 5 mL diluted (1:17) Folin-Ciocalteu reagent (Sigma-Aldrich, Saint Louis, MO, USA). The solutions were well mixed, then 15 mL of a 20% Na_2CO_3 solution was added. After incubation at room temperature for 30 min, the absorbances of the mixtures were measured on a spectrophotometer (Cecil Aurius Series CE 2021 UV/Vis; Cecil Instruments Limited, Cam-bridge, UK) at 765 nm, with each measurement carried out in triplicate. The eight-point calibration curve ranging from 3 mg GAE/L to 150 mg GAE/L (R^2 = 0.9998). The results are expressed as gallic acid equivalents (mg GAE/100 g fresh weight; FW). ~~using a calibration curve ranging from 3 mg/L to 150 mg/L (R^2 = 0.9998).~~ *The antioxidant potential (AOP)* was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (Nakajima et al., 2004). Trolox solution was used for the construction of calibration curve. Briefly, 60 µL of each centrifuged methanol extract was mixed with 1.5 mL DPPH solution. After 15 min of incubation at room temperature, the absorbance was measured at 517 nm, with each measurement carried out in triplicate. The results are expressed as Trolox equivalents (mg TE/g FW) using ~~a~~ the calibration curve ranging from 40 mg TE/L to 220 mg TE/L

(R^2 = 0.9900). *The total flavonoid content (TFC)* was measured according to the method of Lin and Tang (2007). Quercetin was selected for the construction of calibration curve. Briefly, 250 µL of each centrifuged methanol extract was mixed with 750 µL of 95% ethanol, 50 µL of 10% aluminium chloride hexahydrate, 50 µl of 1M potassium acetate and 1.4 mL of deionized water. After 40 min of incubation at room temperature, the absorbance was measured at 415 nm, with each measurement carried out in triplicate. ~~Quercetin was selected as the standard~~T. A~~a~~he seven-point standard curve ~~was constructed,~~ ranging from 0.3 mg quercetin equivalents (QE)~~QE~~/100 mL to 15 mg QE/100 mL (R^2 = 0.9924). The data are expressed as mg QE/100 g FW.

2.4. Nitrogen assimilation

Nitrate-nitrite nitrogen (NO_3-N+NO_2-N) was determined according to ISO 13395(1996) standard method and ammonium nitrogen (NH_4-N) according to EN-ISO 11732(1997) method. All three ions (NO_3^-, NO_2^-, NH_4^+) were measured in the water extracts of lyophilised leaf samples (Opatić et al., 2018) using the continuous flow analyser FLOWSYS (Systea Analytical Technologies). All measurements were made in triplicate, and the data are expressed in mg/kg FW.

2.5. Multi-elemental profile

A multi-elemental profile was obtained non-destructively using energy dispersive X-ray fluorescence spectroscopy. Samples (0.5 to 1.0 g) were prepared as pressed pellets using a pellet die and hydraulic press. As primary excitation sources, the annular radioisotope excitation sources of Fe-55 (10 mCi) and Cd-109 (25 mCi) were used, as obtained from Isotope Products Laboratories, USA (Nečemer et al., 2008; Nečemer et al., 2011). The emitted fluorescence radiation was measured using an energy dispersive X-ray spectrometer

composed of a Canberra Si(Li) detector (Canberra Industries, Meriden, U.S.A.), a Canberra M2024 spectroscopy amplifier (Canberra Industries, Meriden, U.S.A.), a Canberra M8075 ADC (Canberra Industries, Meriden, U.S.A.) and a PC based Canberra MCA S-100 (Canberra Industries, Meriden, U.S.A.). The spectrometer was equipped with a vacuum chamber. The energy resolution of the spectrometer was 175 eV at 5.9 keV. The analysis of complex X-ray spectra was performed using the AXIL (IAEA, Vienna, Austria) spectral analysis program (Nečemer et al., 2008). Quantification was performed utilizing the in-house developed QAES (Quantitative Analysis of Environmental Samples) software (Nečemer et al., 2011). The estimated uncertainty of the analysis was 5%.

2.6. Stable isotope ratios

Stable isotope ratios of C, N and S of dry bulk samples were determined using an Isotope Ratio Mass Spectrometer – IRMS (GV Instruments) (IsoPrime, Cheadle Hulme, UK). For analysis, 4 mg of dry leaf samples were weighed directly into a tin capsule (Sercon, Crewe, UK), closed with tweezers and put into the automatic sampler of the elemental analyser. All stable C and N analyses were performed separately on a Europa Scientific 20-20 continuous flow mass spectrometer with an ANCA-SL solid-liquid preparation module (Sercon, Crewe, UK). Samples for stable isotope ratio of S were analysed on an IsoPrime100-Vario PYRO Cube (OH/CNS) Pyrolyser/Elemental Analyser (IsoPrime, Cheadle, Hulme, UK). Isotope data were expressed using the conventional δ-notation (‰): δ (‰) = [(R_{sample} / $R_{standard}$) – 1] × 1000, where R is the ratio between the heavier and the lighter isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in the sample and standard. Values are reported relative to the following international standards: for carbon the Vienna Pee Dee Belemnite (VPDB), atmospheric N_2 (AIR) for nitrogen, and the Vienna Canyon Diablo Troilite (VCDT) for sulphur. To monitor precision and accuracy, the following reference materials were used: B2155 Protein Sercon

$\delta^{13}C$ = –26.98 ± 0.13‰, $\delta^{15}N$ = 5.94 ± 0.08‰, $\delta^{34}S$ = 6.32 ± 0.80‰ and Casein Protein CRP $\delta^{13}C$ = –20.34 ± 0.09‰, $\delta^{15}N$ = 5.62 ± 0.19‰, $\delta^{34}S$ = 4.18 ± 0.74‰, IAEA-N-1 $\delta^{15}N$ = 0.4 ± 0.2‰ and IAEA-N-2 $\delta^{15}N$ = 20.3 ± 0.2 ‰ and USGS43 $\delta^{13}C$ = –21.28 ± 0.10‰, $\delta^{15}N$ = 8.44 ± 0.10‰, $\delta^{34}S$ = 10.46 ± 0.22‰ and IAEA-SO-5 $\delta^{34}S$ = 0.5 ± 0.2‰, and NBS 127 with $\delta^{34}S$ = 20.3 ± 0.4‰. Each sample was analysed in triplicate, and the mean values calculated. The reproducibility was ± 0.2‰ for $\delta^{13}C$, and ± 0.3‰ for $\delta^{15}N$ and $\delta^{34}S$.

2.7. Statistical analysis

Statistical calculations and multivariate analysis were carried out using the XLSTAT software package (Addinsoft, New York, USA). Basic statistics included mean values, standard deviation (SD), minimum (min) and maximum (max). Simple statistics, including analysis of variance of normally distributed data by ANOVA with Duncan's test for comparisons of means. For not normally distributed data, one-way analysis of variance by ranks (Kruskal-Wallis test) was performed to determine statistical differences among the different discriminating parameters for the 30 chicory samples. Further, to identify those parameters that can discriminate between the different fertility management practices, a multivariate discriminant analysis (DA) was used.

3. Results and discussion

The following 20 parameters were determined for the chicory leaf samples produced in the pot experiment using six different fertility management practices: dry matter (DM), bioactive compounds (TPC, AOP, TFC), nitrogen assimilation (NO_3-N+NO_2-N and NH_4-N), macroelements (P, S, Cl, K, and Ca), microelements (Zn, Mn, Rb, Br, Mo and Sr) and stable isotope ratios of C, N and S. The summary data of these parameters are presented in Tables 1 and 2 along with the minimum-maximums (Min-Max), means and standard deviations (SD).

First, simple statistics was performed and included the analysis of variance by ANOVA and the Kruskal-Wallis one-way analysis of variance by ranks to the values of the 20 parameters. Only variances of parameters of DM, $\delta^{13}C$, $\delta^{15}N$, Mn, Zn, Rb, Mo, P and Ca were normally distributed. According to ANOVA, $\delta^{13}C$, $\delta^{15}N$, Zn, Mo and P were statistically significant ($P < 0.05$) for possible discrimination between the different fertility management practices (Table 1, 2). Kruskal-Wallis test was performed on the parameters with not-normally distributed variance. The parameters NH_4-N, NO_3-N+NO_2-N, $\delta^{34}S$, Br, S and K were statistically significant for discriminating between the fertility managements.

3.1. Dry matter, bioactive compounds and nitrogen assimilation

The dry matter of the chicory leaves ranged from 6.8% to 14.8% (Table 1). The highest mean dry matter was observed for ORG2 (12.2%) and the lowest for MIN1 (10.6%). These results agree with previous reports for the leaves of chicory cultivar 'Treviso' (Nicoletto and Pimpini, 2009).

The data shows that the TPC, AOP and TFC content in the chicory leaves vary although the differences were not significant. The TPC ranged from 28.3 mg GAE/100 g FW to 127 mg GAE/100 g FW, AOP ranged from 0.26 mg TE/g FW to 1.65 mg TE/g FW, and TFC from 0.9 mg QE/100 g FW to 12.6 mg QE/100 g FW (Table 1). These results are in agreement with data reported for the 'Verona' and 'Treviso' cultivars (D'Acunzo et al., 2017; Montefusco et al., 2015; Koukounaras, 2014; Vanzani et al., 2011). The highest mean for AOP was obtained for MIN1 (0.98 mg TE/g FW), while ORG2 resulted in the lowest mean AOP (0.32 mg TE/g FW). A similar trend was observed for TPCs since the highest mean TPC levels were found in chicory leaves grown under MIN1 (93.9 mg GAE/100 g FW). A combination of organic and mineral fertilizers (ORG1+MIN1) resulted in a higher mean content of TFC (83.9 mg GAE/100 g FW) as compared to both ORG1 and ORG2, 57.3 mg

GAE/100 g FW and 44.5 mg GAE/100 g FW, respectively. The highest mean TFC was observed for ORG1+MIN1 (6.61 mg QE/100 g FW) and the lowest for ORG1 and ORG2, 1.64 mg QE/100 g FW and 1.80 mg QE/100 g FW, respectively. Generally, organic fertility management practices resulted in the lowest TPC, AOP and TFC as compared to control and other fertility management regimes.

Nitrate-nitrite nitrogen (NO_3-N+NO2-N) and ammonium nitrogen (NH_4-N) ranged from 0 to 1323 mg/kg FW and from 0 to 140 mg/kg FW, respectively and were statistically significant according to the Kruskal-Wallis test. The highest mean for NO_3-N+NO_2-N was determined in chicory leaves produced under MIN2 (1018 mg/kg FW). Similar contents of nitrate-nitrogen have been reported for head chicory (D'Acunzo et al., 2017; Koukounaras, 2014; Biesiada and Kołota, 2010; Ćustić et al., 2002). As was reviewed by Santamaria (2006), endive a close relative of chicory is classified among the "high nitrate content" vegetables and can accumulate up to 2.500 mg/kg FW of the nitrate ion. Although the highest levels were observed for MIN2, the data show no consistent differences in NO_3-N+NO_2-N content between the fertility management practices. In some cases, even higher NO_3-N+NO_2-N levels were observed where ORG1 and ORG2 minerals were used. These high levels can be explained by the fact that other factors such as light intensisty and physiological age besides fertility management can influence NO_3-N+NO_2-N levels in chicory. MIN1 resulted in the highest mean for NH_4-N (30.9 mg/kg FW).

3.2. Multi-elemental profile

A total of eleven different elements were determined in the 30 chicory leaf samples, which can be divided into two groups: the macroelements (> 1 g/kg DW) P, S, Cl, K, and Ca, and the microelements (>0.1 mg/kg DW) Fe, Zn, Mn, Rb, Br, and Sr (Table 2). The order of the elements by abundance, as determined by XRF spectroscopy, is

K>Ca>Cl>S>P>Br>Mn>Zn>Sr>Rb>Mo. As seen in Table 2, fertility management significantly affected only the content of P, Zn and Mo (P <0.05). Organic fertility management practices caused an increase in P. Significantly more of this element was present in the chicory leaves produced by ORG1 (3.6 g/kg DW) as compared to the others. The control contained significantly more Zn and Mo. The lowest content of Zn was found in the chicory leaves grown under MIN1 (22.0 mg/kg DW) and for Mo under MIN2 (0.6 mg/kg DW). MIN1 resulted in an increased content of S, while ORG1 produced the lowest S content. Both ORG1and ORG2 caused an increase in the levels of K and Cl, while MIN1 and MIN2 resulted in higher levels of Ca and Sr. The highest mean content of Br (77.4 mg/kg DW) was observed in the chicory leaves from MIN2. Discrimination between agricultural practices using the elemental profile in the leaves was also investigated; however, no systematic differences were noticed between the different fertility management practices when compared across plant species (Capuano et al., 2014). Laursen et al. (2011) investigated the potential of multi-element profiling for the authentication of organic winter wheat, spring barley, fava bean and potato. The authors demonstrated that no single element allowed discrimination between conventional and organic crops across locations, years and crop species. Kelly and Bateman (2010) analysed the trace element content of samples of organic and conventional tomatoes and lettuces and reported significantly higher concentrations of Ca, Cu, Zn and Rb in organic tomatoes and of Cu and Rb in organic lettuces and a significantly lower concentration of Mn in organic tomatoes. The authors reported that when trace elements were combined with δ^{15}N values and subjected to statistical evaluation, the correct classification of organic and conventional tomato samples was improved.

3.3. Stable isotope ratios of C, N and S

Significantly higher δ^{15}N values were found in the chicory leaves produced under ORG1 and ORG2 as compared to the control. The lowest δ^{15}N value was produced with using ORG1+MIN1 and with MIN1 and MIN2. Also, previous studies have demonstrated that plants grown in soils to which synthetic nitrogen fertilisers have been added have lower ~~δ^{13}N~~ δ^{15}N values than plants grown in the soil where organic fertilizers have been added (Capuano et al., 2012). However, different plant organs respond in different ways to the isotopic signature of fertilizers, where both the rate and mode of application are important. For instance, δ^{15}N values of lettuce (*Lactuca sativa*) tissues can reveal the use of synthetic fertilizers only when these are applied in a high single dose, and it is more challenging to detect the addition of synthetic fertilizer to basal organic fertilization (Sturm et al., 2011). Schmidt et al. (2005) reported that the δ^{15}N values in organic lettuce, cabbage, onions and Chinese cabbage from field cultivation were significantly higher than those of their conventional counterparts. Georgi et al. (2005) found no differences in the δ^{34}S values of the plants grown under the two production systems, while organically produced vegetables were depleted in ^{13}C and have lower δ^{13}C values compared to those grown under an integrated system. Other studies show no correlation between mode of production and δ^{13}C (except greenhouse tomatoes warmed with natural gas) values (Inácio et al., 2015). In our study, the highest δ^{34}S values were found for ORG1+MIN1, MIN1 and MIN2. Significantly lower δ^{34}S values were found in chicory leaves from CONT and ORG1 and ORG2. Both ORG1 and ORG2 and MIN2 resulted in higher δ^{13}C values (Table 1 and 3), whereas, CONT, MIN1 and ORG1+MIN1 produced lower δ^{13}C values compared to the other fertility management practices.

3.4. Multivariate analysis

Statistical evaluation of results was performed on the dataset by DA to identify the parameters responsible for differentiating chicory plants according to fertility management practices. The results of DA analysis are represented in Fig. 1 as (a) discriminant function score plot and (b) discriminant loadings plot. In plot (a) the observations and the multivariate means of each group (centroids) are shown as scatter plot, while in plot (b) the set of vectors are presented as a loadings plot which indicates the degree of association of the corresponding initial parameters with the first two discriminant functions. As seen from Fig. 1 (a) chicory leaf samples produced under ORG1 and ORG2 are located close to each other in the left part of plot (a) contrary to CONT, MIN1 and ORG1+MIN1 in the right part of plot (a). Chicory leaf samples produced under MIN2 are clearly distinguished from the others and is located in the upper part of the plot (a).

A good separation among chicory leaf samples according to fertility management is evident. Comparison of graphs (a) and (b), correlation of position of different groups in plot (a) with the position, direction and length of each vector in plot (b) reveal crucial parameters responsible for separating the groups. The most influential parameters for discriminating chicory leaf samples produced under ORG1 and ORG2 in the left part of plot (a) correlate with the vectors of K, NO_3-N+NO_2-N and δ^{15}N in plot (b) (the highest mean values). Contrary, vectors AOP, TPC, TFC and δ^{34}S are connected with MIN1 and ORG1+MIN1, which means that these parameters reach their maximum mean values in these two groups and minimum mean values in the opposite direction (ORG1 and ORG2). Further, the higher levels of Br, Sr, K and Ca separates MIN2 from the other groups. Group MIN1 differs from ORG1+MIN1 in lower means of parameter NH_4-N but is opposite in the case of Zn. The parameters important for separating ORG1 and ORG2 and MIN1 were δ^{34}S, δ^{15}N, δ^{13}C and S. MIN2 is differentiated from MIN1 according to NO_3-N+NO_2-N and Br content, while MIN1

and ORG1+MIN1 are separated according to NO_3-N+NO_2-N and NH_4-N parameters. Organic fertility managements ORG1 and ORG2 are discriminated by the levels of Mn and P.

4. Conclusions

This study provides the first evaluation of the use of bioactive, stable isotope and elemental parameters to differentiate between different soil fertility management practices (control, two organic, two mineral, a combination of organic and mineral) for the production of five chicory cultivars. Organic fertility management practices increased the contents of P, Cl and K, while mineral fertility management practices increased the content of Sr and Ca. The highest δ^{15}N and the lowest δ^{34}S values were also produced under organic fertility management. This study has demonstrated that by combining isotopic, ~~quality~~ bioactive and elemental ~~markers~~ parameters and by applying multivariate discriminant statistics, it is possible to distinguish between organic and conventionally ~~produced~~ grown chicory. A minimum set of ten parameters, i.e. TPC, δ^{15}N, δ^{34}S, Zn, Br, Mo, P, S and K, is according to our study, needed to appropriately distinguish between organic and conventionally grown chicory. Such an approach should be proposed to be included in the EU regulation for organic farming system.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1. The summary data of dry matter, bioactive compounds, nitrogen assimilation and stable isotopes studied in chicory plants.

Parameter	Unit	n	Statistical parameter	Fertility management					
				CONT	ORG1	ORG2	MIN1	MIN2	ORG1+MIN1
Dry matter	%	5	Min - Max	10.1 - 14.8	7.54 - 13.0	10.8 - 13.5	8.18 - 13.4	8.09 - 13.6	6.82 - 12.5
			Mean	11.99	10.68	12.23	10.55	11.65	10.80
			SD	1.74	2.41	0.99	2.03	2.48	2.33
TPC	mg GAE/100 g FW	5	Min - Max	39.3 - 115	40.6 - 71.2	30.3 - 58.7	55.1 - 127	37.5 - 97.6	28.3 - 120.
			Mean	77.29	57.27	44.54	93.86	57.53	83.92
			SD	35.92	11.03	10.87	26.28	24.16	34.89
AOP	mg TE/g FW	5	Min - Max	0.31 - 1.65	0.28 - 0.85	0.27 - 0.42	0.48 - 1.56	0.30 - 1.07	0.26 - 1.57
			Mean	0.77	0.50	0.32	0.98	0.53	0.96
			SD	0.55	0.24	0.06	0.39	0.31	0.48
TFC	mg QE/100 g FW	5	Min - Max	1.89 - 9.22	0.94 - 2.29	1.02 - 2.76	2.24 - 10.5	1.90 - 6.8	1.25 - 12.6
			Mean	4.25	1.64	1.80	4.51	3.13	6.61
			SD	3.01	0.57	0.73	3.46	2.09	4.56
NO_3-N+NO_2-N	mg/kg FW	5	Min - Max	0 - 387.2	569 - 1080	600 - 1016	0 - 96.3	916 - 1323	41.7 - 624
			Mean	190	798	793	41	1018	184
			SD	176	190	196	47	173	249
NH_4-N	mg/kg FW	5	Min - Max	6.07 – 17.0	14.4 - 26.7	10.7 - 28.4	0 - 139	9.04 - 18.9	2.32 - 9.18
			Mean	10.79	19.48	17.79	30.93	15.48	6.93
			SD	4.85	4.46	7.08	60.79	4.23	2.70
δ^{13}C	‰	5	Min - Max	(–30.9) - (–30.1)	(–29.6) - (–28.1)	(–30.0) - (–28.5)	(–31.7) - (–29.9)	(–30.3) - (–28.3)	(–31.6) - (–29.1)
			Mean	-30.6 b	-28.9 a	-29.3 a	-31.0 b	-29.2 a	-30.3 b
			SD	0.4	0.6	0.7	0.7	0.9	1.0
δ^{15}N	‰	5	Min - Max	6.7 - 9.4	10.2 - 16.1	10.8 - 12.5	4.2 - 5.4	3.9 - 10.1	6.0 - 8.5
			Mean	8.3 b	13.1 a	11.7 a	4.9 c	5.5 c	7.1 bc
			SD	1.01	2.6	0.6	0.5	2.6	1.0
δ^{34}S	‰	5	Min - Max	6.1 - 7.3	3.9 - 5.9	3.1 - 5.7	7.4 - 8.4	6.4 - 8.3	6.3 - 7.5
			Mean	6.8	5.0	4.1	7.8	7.2	6.9
			SD	0.4	0.8	1.1	0.4	0.7	0.5

Abbreviations: FW, fresh weight; SD, standard deviation; CONT, control; ORG, organic; MIN, mineral. Means with different lower-case letters (a–c) within the rows are significantly different ($P < 0.05$).

Table 2. The summary data of multi-elemental profiles studied in chicory plants.

Parameter	Unit	n	Statistical parameter	Fertility management					
				CONT	ORG1	ORG2	MIN1	MIN2	ORG1+MIN1
P	g/kg DW	5	Min - Max	1.55 - 3.21	2.83 - 4.68	1.97 - 3.10	1.57 - 2.69	1.39 - 3.27	1.82 - 3.19
			Mean	2.31 b	3.59 a	2.62 b	1.95 b	2.44 b	2.45 b
			SD	0.68	0.78	0.47	0.47	0.72	0.49
S	g/kg DW	5	Min - Max	5.18 - 7.72	3.44 - 6.15	4.08 - 6.76	6.14 - 12.2	4.29 - 5.38	5.10 - 9.01
			Mean	6.64	4.31	5.27	8.08	4.74	6.81
			SD	1.04	1.08	0.96	2.45	0.47	1.47
Cl	g/kg DW	5	Min - Max	9.14 - 15.5	11.7 - 25.8	17.1 - 23.8	7.76 - 17.8	7.60 - 38.5	8.02 - 18.4
			Mean	12.2	19.2	20.0	12.1	17.4	10.70
			SD	2.96	5.77	3.04	4.07	12.70	4.44
K	g/kg DW	5	Min - Max	20.6 - 35.6	45.5 - 76.3	61.3 - 77.0	28.3 - 43.9	45.4 - 78.9	22.2 - 39.8
			Mean	25.5	63.2	65.9	34.2	61.1	31.3
			SD	6.0	11.4	6.6	7.3	13.6	6.3
Ca	g/kg DW	5	Min - Max	17.0 - 22.2	12.3 - 24.8	13.0 - 26.6	16.9 - 30.8	14.3 - 29.1	16.0 - 23.2
			Mean	19.6	18.6	19.0	22.6	22.0	18.8
			SD	2.29	4.46	5.26	5.69	6.45	3.02
Mn	mg/kg DW	5	Min - Max	23.4 - 57.2	28.5 - 93.5	14.8- 60.1	32.6 - 70.0	20.6 - 60.8	38.1 - 50.2
			Mean	36.3	59.5	40.4	51.2	39.1	43.8
			SD	13.1	23.7	18.3	14.4	14.8	4.9
Zn	mg/kg DW	5	Min - Max	27.3 - 46.6	22.6 - 36.6	29.3 - 38.0	18.4 - 24.5	24.0 - 37.4	26.7 - 30.2
			Mean	37.3 a	28.7 bc	33.7ab	22.00 c	30.6 ab	28.5 bc
			SD	7.73	5.70	3.77	2.33	5.58	1.57
Br	mg/kg DW	5	Min - Max	29.4 - 79.8	31.2 - 53.0	34.2 - 56.6	17.1 - 47.5	33.1 - 155	23.6 - 48.3
			Mean	46.3	44.9	44.6	33.02	77.7	31.3
			SD	20.8	8.97	8.01	11.9	47.2	10.3
Rb	mg/kg DW	5	Min - Max	10.2 - 17.8	11.1 - 13.0	8.85 - 12.6	11.7 - 13.8	7.88 - 16.0	9.39 - 13.1
			Mean	12.8	12.2	10.35	12.6	11.98	11.4
			SD	3.00	0.79	1.76	0.92	3.06	1.39
Sr	mg/kg DW	5	Min - Max	13.7- 17.9	8.79 - 23.0	10.9 - 21.3	11.4- 26.9	14.1 - 32.6	10.2 - 19.7
			Mean	15.62	14.9	14.96	17.26	22.1	13.9
			SD	1.85	5.24	4.85	6.19	8.05	3.76
Mo	mg/kg DW	5	Min - Max	0.92 - 2.86	0.48 - 1.81	0.65 - 1.74	0.80 - 2.45	0.45 - 0.86	1.38 - 1.92
			Mean	1.88 a	0.95 cd	1.10 bcd	1.69 ab	0.58 d	1.64 abc
			SD	0.73	0.54	0.49	0.65	0.16	0.26

Abbreviations: DW, dry weight; SD, standard deviation; CONT, control; ORG, organic; MIN, mineral. Means with different lower-case letters (a–d) within the rows are significantly different ($P < 0.05$).

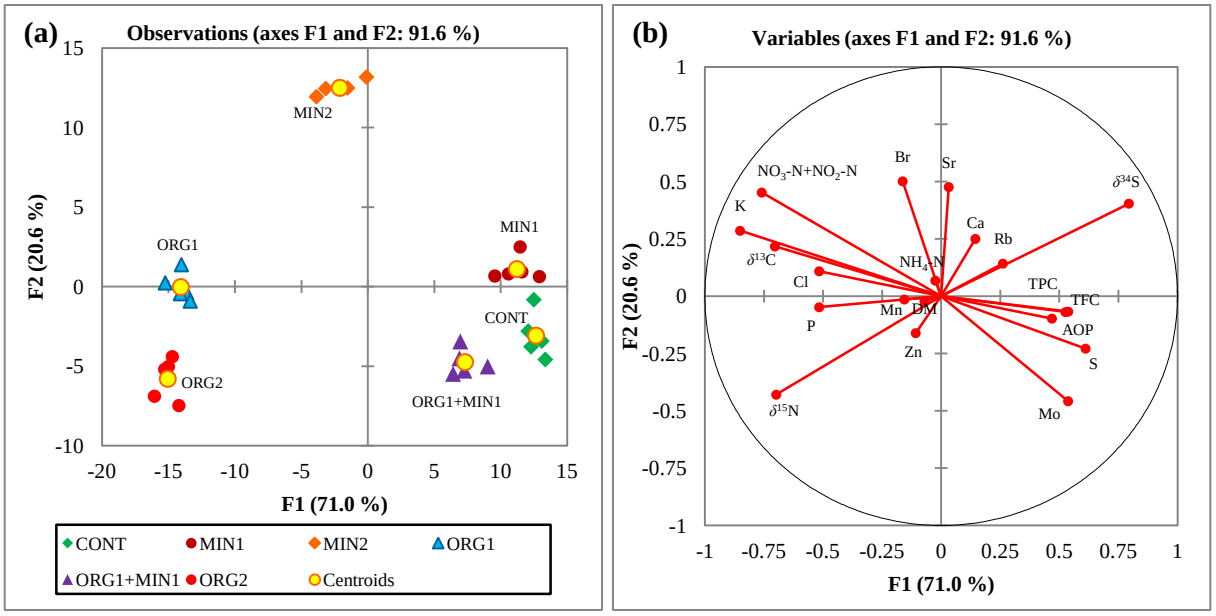

Fig. 1. (a) Discriminant function score plot and (b) discriminant loadings plot based on differences according to six fertility management practices (CONT, ORG1, ORG2, MIN1, MIN2, and ORG1+MIN1) for all chicory samples. Function 1 (F1) explained 71.0% and function 2 (F2) 20.6% of the total variance. Discriminant analysis performed with the 20 parameters including DM, TPC, AOP, TFC, NO_3-N+NO_2-N, NH_4-N, P, S, Cl, K, Ca, Mn, Zn, Br, Rb, Sr, Mo, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, of the 30 samples originated from five chicory cultivars.

1. Introduction

In recent years, there has been a growing interest in organic foodstuffs from consumers, which are produced sustainably and have a low environmental impact and command higher prices on the market (Vallverdú-Queralt and Lamuela-Raventós, 2016). The sphere of organic foods includes fruits, vegetables and cereals, which are often considered healthier, safer and better for the environment and animal welfare than conventional foods because synthetic pesticides and fertilisers are not used (Reganold and Wachter, 2016; de Souza Araújo et al., 2014). In Europe, agricultural products and foodstuffs of organic farming should be produced in accordance with the EU regulations on organic production and processing (No 834/2007 and No 889/2008), without the use of any synthetic plant protection products and soluble synthetic fertilizers, with a full list of the pesticides and fertilizers of natural origin. In 2018 a new EU regulation (No 2018/848) was adopted that will enter into force on January 1st 2021. Its main improvement is the introduction of uniform European rules that will apply to the entire organic production sector in the Union. However, since organic food production represents a significant market segment within the global food industry with products having higher market prices, fraud is expected to increase (Conti et al., 2014). Most food fraud is motivated by quick economic profit, and organic products are at a higher risk of such substitutions as they are sold at premium prices compared to conventional ones (Laursen et al., 2014). Thus, there is an urgent need to develop appropriate, effective and robust analytical approaches to distinguish between the two production systems (Tähkäpää et al., 2015; Mihailova et al., 2014).

At present, there is no universal analytical method that can be applied to differentiate organic and conventional produced plant-based foods. The use of nitrogen stable isotope ratio $^{15}N/^{14}N$ (expressed as $\delta^{15}N$) to discriminate organic from conventional production has been discussed in detail previously (Bateman et al., 2007; Rogers, 2008). It is based on the fact that

synthetic nitrogen fertilizers, used in conventional and integrated farming, have δ^{15}N values significantly lower than the animal manures and fertilizers permitted in organic agriculture. However, soil fertility in organic farming is maintained through the use of crop rotations that include legumes and green manures and also by the application of certain naturally derived fertilizers. Organically produced tomato, lettuce, carrots (Bateman et al., 2007; Bateman et al., 2005), maize (Choi et al., 2002), pepper (Flores et al., 2007), onion and cabbage (Georgi et al., 2005) are significantly enriched in ^{15}N than those receiving synthetic fertilization. In general, the δ^{15}N ratios can be a useful discriminant tool for crops requiring intensive horticulture, but not for all cultivation typologies, especially in soil-grown crops with a long growth cycle. It is also reported that discrimination capability could be improved by including element profiling and applying appropriate statistical tools. Correlations between mode of production and δ^{13}C (except for greenhouse produced tomatoes warmed with natural gas) and δ^{34}S signatures have not been established. There are also a growing number of studies comparing organic and conventional farming vegetables concerning mineral profiles (Kapoulas et al., 2017; Krejčová et al., 2016), bioactive compounds (Ku et al., 2018; Orsini et al., 2016; Sinkovič et al., 2015), physicochemical contaminants (de Souza Araújo et al., 2014) and nutritional quality (Popa et al., 2019; Yu et al., 2018; Maggio et al., 2013; Herencia et al., 2011; Huber et al., 2011).

Although most publications concern crop production, to the best of our knowledge, no paper includes the stable isotope ratio of nitrogen in chicory plants. Chicory (*Cichorium intybus* L.) along with lettuce represents a fresh leafy vegetable crop with a total world production close to 27 million tons in 2017 (FAOSTAT, 2019). Although its production is quantitatively less abundant compared to lettuce, it amounts to around 30% of total production. Due to a range of advantages such as resistance to low temperatures and hence year-round supply, a broad palette of colours and potential health benefits, chicory has

become an economically attractive crop for many producers (Bergantin et al., 2017; D'Evoli et al., 2017). Also, the potential for organic and conventional production makes chicory a suitable vegetable crop for a variety of different case studies.

This study aimed to examine the effect of different fertility management practices on the biochemical composition of several chicory cultivars and to verify the possibility to differentiate between organic and conventional production. The study includes the following analyses: determination of bioactive compounds (total phenolics, antioxidant potential, total flavonoids), nitrogen assimilation, multi-elemental profiling and the measurement of stable isotope ratios of carbon, nitrogen and sulphur. Based on the obtained data, a robust model to control the type of fertility management practice was established.

2. Materials and methods

2.1. Plant material and fertility management experiment

Five commercial cultivars of chicory (*Cichorium intybus* L.) were studied, namely 'Treviso', 'Verona', 'Anivip', 'Castelfranco' and 'Monivip', which are the most popular cultivars for production and consumption in Slovenia and neighboring countries. The seeds were purchased from commercial seed companies ('Treviso', 'Verona' and 'Castelfranco' from Semenarna, Ljubljana, Slovenia; 'Anivip' and 'Monivip' from L'Ortolano, Cesena, Italy).

Chicory plants were produced in the spring growing season in a glasshouse at the Biotechnical Faculty, University of Ljubljana, Slovenia (46° 04´ N, 14° 31´ W; 320 m a.s.l.). Along with the control (no added fertilizer), different types of fertilizers (two organic, two mineral), and a combination of organic and mineral fertilizer were tested. For each of these five cultivars, the same six fertility management practices were applied in a completely

randomized factorial design, namely: no added fertilizer/control (CONT); addition of the two single basal organic fertilizers, using Plantella Organic (ORG1; 3-3-2; 67.5 g/pot; Unichem, Slovenia) and Stallatico Pallettato (ORG2; 3-3-3; 45 g/pot; Fomet, Italy); a single water-soluble mineral fertilizer, using Kristalon Blue (MIN1; 19-6-20; watering with 9 g/100 L; Yara, Norway); a single basal mineral fertilizer, using Entec Perfect (MIN2; 14-7-17; 7.9 g/pot; EuroChem, Italy); and a combination of an organic (ORG1) and a mineral (MIN1) fertilizer (ORG1+MIN1; Plantella Organic+Kristalon Blue; 2.5 g/pot+after 1 month, watering once per week with 3.5 g/L).

Chicory seeds (n = 10) were sown in plastic pots containing 7 L of virgin soil without or with the addition of fertilizer according to the protocol. The pots were placed on rolling benches in a heated glasshouse compartment (18 ±2°C) and watered when required. After several weeks, the seedlings were thinned to give five plants per pot. The water-soluble fertilizer (MIN1) was applied during the watering of the plants when two fully expanded chicory leaves had grown. The harvesting of chicory leaves was performed 130 days after sowing.

2.2. Sample preparation

The uniform leaves of five chicory cultivars grown under the six fertility management regimes were collected between 06:00 to 08:00, solar time and prepared in three ways. First, 10 g of fresh leaves were extracted in a ploypropylene plastic vial with 15 g 50 % methanol. The tissue was homogenized using an Ultraturax T25 (20,500 rpm) for 5 min. The samples were then frozen and stored at –20 °C until analysis of total phenolics content (TPC), antioxidant potential (AOP) and total flavonoid content (TFC). Second, few uniform leaves were immediately frozen in liquid nitrogen and then lyophilized and homogenized to a fine powder using a laboratory ball mill (Retsch mm 301). The samples were stored in glass vials

in humidity-proof plastic bags filled with silica gel before analysis of nitrogen assimilation. Third, chicory leaves were dried in a laboratory oven at 80 °C for 28 h, ground up with a mortar and pestle. The ground leaves were then used in the determination of dry matter (DM), multi-elemental profiling and the determination of stable isotope ratios of C, N and S.

2.3. Bioactive compounds

The total phenolics content (TPC) was determining spectrophotometrically following the Folin-Ciocalteu method, as first described by Singleton and Rossi (1965), and slightly modified by Roura et al. (2006). Gallic acid was used for the construction of the calibration curve. Briefly, 1 mL of each centrifuged methanol fraction from the sample was mixed with 60 mL deionized water and 5 mL diluted (1:17) Folin-Ciocalteu reagent (Sigma-Aldrich, Saint Louis, MO, USA). The solutions were well mixed, then 15 mL of a 20% Na_2CO_3 solution was added. After incubation at room temperature for 30 min, the absorbances of the mixtures were measured on a spectrophotometer (Cecil Aurius Series CE 2021 UV/Vis; Cecil Instruments Limited, Cam-bridge, UK) at 765 nm, with each measurement carried out in triplicate. The eight-point calibration curve ranging from 3 mg GAE/L to 150 mg GAE/L ($R^2 = 0.9998$). The results are expressed as gallic acid equivalents (mg GAE/100 g fresh weight; FW). *The antioxidant potential (AOP)* was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay (Nakajima et al., 2004). Trolox solution was used for the construction of calibration curve. Briefly, 60 μL of each centrifuged methanol extract was mixed with 1.5 mL DPPH solution. After 15 min of incubation at room temperature, the absorbance was measured at 517 nm, with each measurement carried out in triplicate. The results are expressed as Trolox equivalents (mg TE/g FW) using the calibration curve ranging from 40 mg TE/L to 220 mg TE/L ($R^2 = 0.9900$). *The total flavonoid content (TFC)* was measured according to the method of Lin and Tang (2007).

Quercetin was selected for the construction of calibration curve. Briefly, 250 µL of each centrifuged methanol extract was mixed with 750 µL of 95% ethanol, 50 µL of 10% aluminium chloride hexahydrate, 50 µl of 1M potassium acetate and 1.4 mL of deionized water. After 40 min of incubation at room temperature, the absorbance was measured at 415 nm, with each measurement carried out in triplicate. The seven-point standard curve ranging from 0.3 mg quercetin equivalents (QE)/100 mL to 15 mg QE/100 mL (R^2 = 0.9924). The data are expressed as mg QE/100 g FW.

2.4. Nitrogen assimilation

Nitrate-nitrite nitrogen (NO_3-N+NO_2-N) was determined according to ISO 13395(1996) standard method and ammonium nitrogen (NH_4-N) according to EN-ISO 11732(1997) method. All three ions (NO_3^-, NO_2^-, NH_4^+) were measured in the water extracts of lyophilised leaf samples (Opatić et al., 2018) using the continuous flow analyser FLOWSYS (Systea Analytical Technologies). All measurements were made in triplicate, and the data are expressed in mg/kg FW.

2.5. Multi-elemental profile

A multi-elemental profile was obtained non-destructively using energy dispersive X-ray fluorescence spectroscopy. Samples (0.5 to 1.0 g) were prepared as pressed pellets using a pellet die and hydraulic press. As primary excitation sources, the annular radioisotope excitation sources of Fe-55 (10 mCi) and Cd-109 (25 mCi) were used, as obtained from Isotope Products Laboratories, USA (Nečemer et al., 2008; Nečemer et al., 2011). The emitted fluorescence radiation was measured using an energy dispersive X-ray spectrometer composed of a Canberra Si(Li) detector (Canberra Industries, Meriden, U.S.A.), a Canberra M2024 spectroscopy amplifier (Canberra Industries, Meriden, U.S.A.), a Canberra M8075

ADC (Canberra Industries, Meriden, U.S.A.) and a PC based Canberra MCA S-100 (Canberra Industries, Meriden, U.S.A.). The spectrometer was equipped with a vacuum chamber. The energy resolution of the spectrometer was 175 eV at 5.9 keV. The analysis of complex X-ray spectra was performed using the AXIL (IAEA, Vienna, Austria) spectral analysis program (Nečemer et al., 2008). Quantification was performed utilizing the in-house developed QAES (Quantitative Analysis of Environmental Samples) software (Nečemer et al., 2011). The estimated uncertainty of the analysis was 5%.

2.6. Stable isotope ratios

Stable isotope ratios of C, N and S of dry bulk samples were determined using an Isotope Ratio Mass Spectrometer – IRMS (GV Instruments) (IsoPrime, Cheadle Hulme, UK). For analysis, 4 mg of dry leaf samples were weighed directly into a tin capsule (Sercon, Crewe, UK), closed with tweezers and put into the automatic sampler of the elemental analyser. All stable C and N analyses were performed separately on a Europa Scientific 20-20 continuous flow mass spectrometer with an ANCA-SL solid-liquid preparation module (Sercon, Crewe, UK). Samples for stable isotope ratio of S were analysed on an IsoPrime100-Vario PYRO Cube (OH/CNS) Pyrolyser/Elemental Analyser (IsoPrime, Cheadle, Hulme, UK). Isotope data were expressed using the conventional δ-notation (‰): δ (‰) = [(R_{sample} / $R_{standard}$) – 1] × 1000, where R is the ratio between the heavier and the lighter isotope ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in the sample and standard. Values are reported relative to the following international standards: for carbon the Vienna Pee Dee Belemnite (VPDB), atmospheric N_2 (AIR) for nitrogen, and the Vienna Canyon Diablo Troilite (VCDT) for sulphur. To monitor precision and accuracy, the following reference materials were used: B2155 Protein Sercon $\delta^{13}C = –26.98 \pm 0.13$‰, $\delta^{15}N = 5.94 \pm 0.08$‰, $\delta^{34}S = 6.32 \pm 0.80$‰ and Casein Protein CRP $\delta^{13}C = –20.34 \pm 0.09$‰, $\delta^{15}N = 5.62 \pm 0.19$‰, $\delta^{34}S = 4.18 \pm 0.74$‰, IAEA-N-1 $\delta^{15}N = 0.4 \pm$

0.2‰ and IAEA-N-2 $\delta^{15}N$ = 20.3 ± 0.2 ‰ and USGS43 $\delta^{13}C$ = –21.28 ± 0.10‰, $\delta^{15}N$ = 8.44 ± 0.10‰, $\delta^{34}S$ = 10.46 ± 0.22‰ and IAEA-SO-5 $\delta^{34}S$ = 0.5 ± 0.2‰, and NBS 127 with $\delta^{34}S$ = 20.3 ± 0.4‰. Each sample was analysed in triplicate, and the mean values calculated. The reproducibility was ± 0.2‰ for $\delta^{13}C$, and ± 0.3‰ for $\delta^{15}N$ and $\delta^{34}S$.

2.7. Statistical analysis

Statistical calculations and multivariate analysis were carried out using the XLSTAT software package (Addinsoft, New York, USA). Basic statistics included mean values, standard deviation (SD), minimum (min) and maximum (max). Simple statistics, including analysis of variance of normally distributed data by ANOVA with Duncan's test for comparisons of means. For not normally distributed data, one-way analysis of variance by ranks (Kruskal-Wallis test) was performed to determine statistical differences among the different discriminating parameters for the 30 chicory samples. Further, to identify those parameters that can discriminate between the different fertility management practices, a multivariate discriminant analysis (DA) was used.

3. Results and discussion

The following 20 parameters were determined for the chicory leaf samples produced in the pot experiment using six different fertility management practices: dry matter (DM), bioactive compounds (TPC, AOP, TFC), nitrogen assimilation (NO_3-N+NO_2-N and NH_4-N), macroelements (P, S, Cl, K, and Ca), microelements (Zn, Mn, Rb, Br, Mo and Sr) and stable isotope ratios of C, N and S. The summary data of these parameters are presented in Tables 1 and 2 along with the minimum-maximums (Min-Max), means and standard deviations (SD).

First, simple statistics was performed and included the analysis of variance by ANOVA and the Kruskal-Wallis one-way analysis of variance by ranks to the values of the 20

parameters. Only variances of parameters of DM, $\delta^{13}C$, $\delta^{15}N$, Mn, Zn, Rb, Mo, P and Ca were normally distributed. According to ANOVA, $\delta^{13}C$, $\delta^{15}N$, Zn, Mo and P were statistically significant ($P < 0.05$) for possible discrimination between the different fertility management practices (Table 1, 2). Kruskal-Wallis test was performed on the parameters with not-normally distributed variance. The parameters NH_4-N, NO_3-N+NO_2-N, $\delta^{34}S$, Br, S and K were statistically significant for discriminating between the fertility managements.

3.1. Dry matter, bioactive compounds and nitrogen assimilation

The dry matter of the chicory leaves ranged from 6.8% to 14.8% (Table 1). The highest mean dry matter was observed for ORG2 (12.2%) and the lowest for MIN1 (10.6%). These results agree with previous reports for the leaves of chicory cultivar 'Treviso' (Nicoletto and Pimpini, 2009).

The data shows that the TPC, AOP and TFC content in the chicory leaves vary although the differences were not significant. The TPC ranged from 28.3 mg GAE/100 g FW to 127 mg GAE/100 g FW, AOP ranged from 0.26 mg TE/g FW to 1.65 mg TE/g FW, and TFC from 0.9 mg QE/100 g FW to 12.6 mg QE/100 g FW (Table 1). These results are in agreement with data reported for the 'Verona' and 'Treviso' cultivars (D'Acunzo et al., 2017; Montefusco et al., 2015; Koukounaras, 2014; Vanzani et al., 2011). The highest mean for AOP was obtained for MIN1 (0.98 mg TE/g FW), while ORG2 resulted in the lowest mean AOP (0.32 mg TE/g FW). A similar trend was observed for TPCs since the highest mean TPC levels were found in chicory leaves grown under MIN1 (93.9 mg GAE/100 g FW). A combination of organic and mineral fertilizers (ORG1+MIN1) resulted in a higher mean content of TFC (83.9 mg GAE/100 g FW) as compared to both ORG1 and ORG2, 57.3 mg GAE/100 g FW and 44.5 mg GAE/100 g FW, respectively. The highest mean TFC was observed for ORG1+MIN1 (6.61 mg QE/100 g FW) and the lowest for ORG1 and ORG2,

1.64 mg QE/100 g FW and 1.80 mg QE/100 g FW, respectively. Generally, organic fertility management practices resulted in the lowest TPC, AOP and TFC as compared to control and other fertility management regimes.

Nitrate-nitrite nitrogen (NO_3-N+NO2-N) and ammonium nitrogen (NH_4-N) ranged from 0 to 1323 mg/kg FW and from 0 to 140 mg/kg FW, respectively and were statistically significant according to the Kruskal-Wallis test. The highest mean for NO_3-N+NO_2-N was determined in chicory leaves produced under MIN2 (1018 mg/kg FW). Similar contents of nitrate-nitrogen have been reported for head chicory (D'Acunzo et al., 2017; Koukounaras, 2014; Biesiada and Kołota, 2010; Ćustić et al., 2002). As was reviewed by Santamaria (2006), endive a close relative of chicory is classified among the "high nitrate content" vegetables and can accumulate up to 2.500 mg/kg FW of the nitrate ion. Although the highest levels were observed for MIN2, the data show no consistent differences in NO_3-N+NO_2-N content between the fertility management practices. In some cases, even higher NO_3-N+NO_2-N levels were observed where ORG1 and ORG2 minerals were used. These high levels can be explained by the fact that other factors such as light intensisty and physiological age besides fertility management can influence NO_3-N+NO_2-N levels in chicory. MIN1 resulted in the highest mean for NH_4-N (30.9 mg/kg FW).

3.2. Multi-elemental profile

A total of eleven different elements were determined in the 30 chicory leaf samples, which can be divided into two groups: the macroelements (> 1 g/kg DW) P, S, Cl, K, and Ca, and the microelements (>0.1 mg/kg DW) Fe, Zn, Mn, Rb, Br, and Sr (Table 2). The order of the elements by abundance, as determined by XRF spectroscopy, is K>Ca>Cl>S>P>Br>Mn>Zn>Sr>Rb>Mo. As seen in Table 2, fertility management significantly affected only the content of P, Zn and Mo (P <0.05). Organic fertility

management practices caused an increase in P. Significantly more of this element was present in the chicory leaves produced by ORG1 (3.6 g/kg DW) as compared to the others. The control contained significantly more Zn and Mo. The lowest content of Zn was found in the chicory leaves grown under MIN1 (22.0 mg/kg DW) and for Mo under MIN2 (0.6 mg/kg DW). MIN1 resulted in an increased content of S, while ORG1 produced the lowest S content. Both ORG1and ORG2 caused an increase in the levels of K and Cl, while MIN1 and MIN2 resulted in higher levels of Ca and Sr. The highest mean content of Br (77.4 mg/kg DW) was observed in the chicory leaves from MIN2. Discrimination between agricultural practices using the elemental profile in the leaves was also investigated; however, no systematic differences were noticed between the different fertility management practices when compared across plant species (Capuano et al., 2014). Laursen et al. (2011) investigated the potential of multi-element profiling for the authentication of organic winter wheat, spring barley, fava bean and potato. The authors demonstrated that no single element allowed discrimination between conventional and organic crops across locations, years and crop species. Kelly and Bateman (2010) analysed the trace element content of samples of organic and conventional tomatoes and lettuces and reported significantly higher concentrations of Ca, Cu, Zn and Rb in organic tomatoes and of Cu and Rb in organic lettuces and a significantly lower concentration of Mn in organic tomatoes. The authors reported that when trace elements were combined with δ^{15}N values and subjected to statistical evaluation, the correct classification of organic and conventional tomato samples was improved.

3.3. Stable isotope ratios of C, N and S

Significantly higher δ^{15}N values were found in the chicory leaves produced under ORG1 and ORG2 as compared to the control. The lowest δ^{15}N value was produced with using ORG1+MIN1 and with MIN1 and MIN2. Also, previous studies have demonstrated that

plants grown in soils to which synthetic nitrogen fertilisers have been added have lower $\delta^{15}N$ values than plants grown in the soil where organic fertilizers have been added (Capuano et al., 2012). However, different plant organs respond in different ways to the isotopic signature of fertilizers, where both the rate and mode of application are important. For instance, $\delta^{15}N$ values of lettuce (*Lactuca sativa*) tissues can reveal the use of synthetic fertilizers only when these are applied in a high single dose, and it is more challenging to detect the addition of synthetic fertilizer to basal organic fertilization (Sturm et al., 2011). Schmidt et al. (2005) reported that the $\delta^{15}N$ values in organic lettuce, cabbage, onions and Chinese cabbage from field cultivation were significantly higher than those of their conventional counterparts. Georgi et al. (2005) found no differences in the $\delta^{34}S$ values of the plants grown under the two production systems, while organically produced vegetables were depleted in ^{13}C and have lower $\delta^{13}C$ values compared to those grown under an integrated system. Other studies show no correlation between mode of production and $\delta^{13}C$ (except greenhouse tomatoes warmed with natural gas) values (Inácio et al., 2015). In our study, the highest $\delta^{34}S$ values were found for ORG1+MIN1, MIN1 and MIN2. Significantly lower $\delta^{34}S$ values were found in chicory leaves from CONT and ORG1 and ORG2. Both ORG1 and ORG2 and MIN2 resulted in higher $\delta^{13}C$ values (Table 1 and 3), whereas, CONT, MIN1 and ORG1+MIN1 produced lower $\delta^{13}C$ values compared to the other fertility management practices.

3.4. Multivariate analysis

Statistical evaluation of results was performed on the dataset by DA to identify the parameters responsible for differentiating chicory plants according to fertility management practices. The results of DA analysis are represented in Fig. 1 as (a) discriminant function score plot and (b) discriminant loadings plot. In plot (a) the observations and the multivariate means of each group (centroids) are shown as scatter plot, while in plot (b) the set of vectors

are presented as a loadings plot which indicates the degree of association of the corresponding initial parameters with the first two discriminant functions. As seen from Fig. 1 (a) chicory leaf samples produced under ORG1 and ORG2 are located close to each other in the left part of plot (a) contrary to CONT, MIN1 and ORG1+MIN1 in the right part of plot (a). Chicory leaf samples produced under MIN2 are clearly distinguished from the others and is located in the upper part of the plot (a).

A good separation among chicory leaf samples according to fertility management is evident. Comparison of graphs (a) and (b), correlation of position of different groups in plot (a) with the position, direction and length of each vector in plot (b) reveal crucial parameters responsible for separating the groups. The most influential parameters for discriminating chicory leaf samples produced under ORG1 and ORG2 in the left part of plot (a) correlate with the vectors of K, NO_3-N+NO_2-N and δ^{15}N in plot (b) (the highest mean values). Contrary, vectors AOP, TPC, TFC and δ^{34}S are connected with MIN1 and ORG1+MIN1, which means that these parameters reach their maximum mean values in these two groups and minimum mean values in the opposite direction (ORG1 and ORG2). Further, the higher levels of Br, Sr, K and Ca separates MIN2 from the other groups. Group MIN1 differs from ORG1+MIN1 in lower means of parameter NH_4-N but is opposite in the case of Zn. The parameters important for separating ORG1 and ORG2 and MIN1 were δ^{34}S, δ^{15}N, δ^{13}C and S. MIN2 is differentiated from MIN1 according to NO_3-N+NO_2-N and Br content, while MIN1 and ORG1+MIN1 are separated according to NO_3-N+NO_2-N and NH_4-N parameters. Organic fertility managements ORG1 and ORG2 are discriminated by the levels of Mn and P.

4. Conclusions

This study provides the first evaluation of the use of bioactive, stable isotope and elemental parameters to differentiate between different soil fertility management practices

(control, two organic, two mineral, a combination of organic and mineral) for the production of five chicory cultivars. Organic fertility management practices increased the contents of P, Cl and K, while mineral fertility management practices increased the content of Sr and Ca. The highest δ^{15}N and the lowest δ^{34}S values were also produced under organic fertility management. This study has demonstrated that by combining isotopic, bioactive and elemental parameters and by applying multivariate discriminant statistics, it is possible to distinguish between organic and conventionally grown chicory. A minimum set of ten parameters, i.e. TPC, δ^{15}N, δ^{34}S, Zn, Br, Mo, P, S and K, is according to our study, needed to appropriately distinguish between organic and conventionally grown chicory. Such an approach should be proposed to be included in the EU regulation for organic farming system.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1. The summary data of dry matter, bioactive compounds, nitrogen assimilation and stable isotopes studied in chicory plants.

Parameter	Unit	n	Statistical parameter	Fertility management					
				CONT	ORG1	ORG2	MIN1	MIN2	ORG1+MIN1
Dry matter	%	5	Min - Max	10.1 - 14.8	7.54 - 13.0	10.8 - 13.5	8.18 - 13.4	8.09 - 13.6	6.82 - 12.5
			Mean	11.99	10.68	12.23	10.55	11.65	10.80
			SD	1.74	2.41	0.99	2.03	2.48	2.33
TPC	mg GAE/100 g FW	5	Min - Max	39.3 - 115	40.6 - 71.2	30.3 - 58.7	55.1 - 127	37.5 - 97.6	28.3 - 120.
			Mean	77.29	57.27	44.54	93.86	57.53	83.92
			SD	35.92	11.03	10.87	26.28	24.16	34.89
AOP	mg TE/g FW	5	Min - Max	0.31 - 1.65	0.28 - 0.85	0.27 - 0.42	0.48 - 1.56	0.30 - 1.07	0.26 - 1.57
			Mean	0.77	0.50	0.32	0.98	0.53	0.96
			SD	0.55	0.24	0.06	0.39	0.31	0.48
TFC	mg QE/100 g FW	5	Min - Max	1.89 - 9.22	0.94 - 2.29	1.02 - 2.76	2.24 - 10.5	1.90 - 6.8	1.25 - 12.6
			Mean	4.25	1.64	1.80	4.51	3.13	6.61
			SD	3.01	0.57	0.73	3.46	2.09	4.56
NO_3-N+NO_2-N	mg/kg FW	5	Min - Max	0 - 387.2	569 - 1080	600 - 1016	0 - 96.3	916 - 1323	41.7 - 624
			Mean	190	798	793	41	1018	184
			SD	176	190	196	47	173	249
NH_4-N	mg/kg FW	5	Min - Max	6.07 – 17.0	14.4 - 26.7	10.7 - 28.4	0 - 139	9.04 - 18.9	2.32 - 9.18
			Mean	10.79	19.48	17.79	30.93	15.48	6.93
			SD	4.85	4.46	7.08	60.79	4.23	2.70
δ^{13}C	‰	5	Min - Max	(–30.9) - (–30.1)	(–29.6) - (–28.1)	(–30.0) - (–28.5)	(–31.7) - (–29.9)	(–30.3) - (–28.3)	(–31.6) - (–29.1)
			Mean	-30.6 b	-28.9 a	-29.3 a	-31.0 b	-29.2 a	-30.3 b
			SD	0.4	0.6	0.7	0.7	0.9	1.0
δ^{15}N	‰	5	Min - Max	6.7 - 9.4	10.2 - 16.1	10.8 - 12.5	4.2 - 5.4	3.9 - 10.1	6.0 - 8.5
			Mean	8.3 b	13.1 a	11.7 a	4.9 c	5.5 c	7.1 bc
			SD	1.01	2.6	0.6	0.5	2.6	1.0
δ^{34}S	‰	5	Min - Max	6.1 - 7.3	3.9 - 5.9	3.1 - 5.7	7.4 - 8.4	6.4 - 8.3	6.3 - 7.5
			Mean	6.8	5.0	4.1	7.8	7.2	6.9
			SD	0.4	0.8	1.1	0.4	0.7	0.5

Abbreviations: FW, fresh weight; SD, standard deviation; CONT, control; ORG, organic; MIN, mineral. Means with different lower-case letters (a–c) within the rows are significantly different ($P < 0.05$).

Table 2. The summary data of multi-elemental profiles studied in chicory plants.

Parameter	Unit	n	Statistical parameter	Fertility management					
				CONT	ORG1	ORG2	MIN1	MIN2	ORG1+MIN1
P	g/kg DW	5	Min - Max	1.55 - 3.21	2.83 - 4.68	1.97 - 3.10	1.57 - 2.69	1.39 - 3.27	1.82 - 3.19
			Mean	2.31 b	3.59 a	2.62 b	1.95 b	2.44 b	2.45 b
			SD	0.68	0.78	0.47	0.47	0.72	0.49
S	g/kg DW	5	Min - Max	5.18 - 7.72	3.44 - 6.15	4.08 - 6.76	6.14 - 12.2	4.29 - 5.38	5.10 - 9.01
			Mean	6.64	4.31	5.27	8.08	4.74	6.81
			SD	1.04	1.08	0.96	2.45	0.47	1.47
Cl	g/kg DW	5	Min - Max	9.14 - 15.5	11.7 - 25.8	17.1 - 23.8	7.76 - 17.8	7.60 - 38.5	8.02 - 18.4
			Mean	12.2	19.2	20.0	12.1	17.4	10.70
			SD	2.96	5.77	3.04	4.07	12.70	4.44
K	g/kg DW	5	Min - Max	20.6 - 35.6	45.5 - 76.3	61.3 - 77.0	28.3 - 43.9	45.4 - 78.9	22.2 - 39.8
			Mean	25.5	63.2	65.9	34.2	61.1	31.3
			SD	6.0	11.4	6.6	7.3	13.6	6.3
Ca	g/kg DW	5	Min - Max	17.0 - 22.2	12.3 - 24.8	13.0 - 26.6	16.9 - 30.8	14.3 - 29.1	16.0 - 23.2
			Mean	19.6	18.6	19.0	22.6	22.0	18.8
			SD	2.29	4.46	5.26	5.69	6.45	3.02
Mn	mg/kg DW	5	Min - Max	23.4 - 57.2	28.5 - 93.5	14.8- 60.1	32.6 - 70.0	20.6 - 60.8	38.1 - 50.2
			Mean	36.3	59.5	40.4	51.2	39.1	43.8
			SD	13.1	23.7	18.3	14.4	14.8	4.9
Zn	mg/kg DW	5	Min - Max	27.3 - 46.6	22.6 - 36.6	29.3 - 38.0	18.4 - 24.5	24.0 - 37.4	26.7 - 30.2
			Mean	37.3 a	28.7 bc	33.7ab	22.00 c	30.6 ab	28.5 bc
			SD	7.73	5.70	3.77	2.33	5.58	1.57
Br	mg/kg DW	5	Min - Max	29.4 - 79.8	31.2 - 53.0	34.2 - 56.6	17.1 - 47.5	33.1 - 155	23.6 - 48.3
			Mean	46.3	44.9	44.6	33.02	77.7	31.3
			SD	20.8	8.97	8.01	11.9	47.2	10.3
Rb	mg/kg DW	5	Min - Max	10.2 - 17.8	11.1 - 13.0	8.85 - 12.6	11.7 - 13.8	7.88 - 16.0	9.39 - 13.1
			Mean	12.8	12.2	10.35	12.6	11.98	11.4
			SD	3.00	0.79	1.76	0.92	3.06	1.39
Sr	mg/kg DW	5	Min - Max	13.7- 17.9	8.79 - 23.0	10.9 - 21.3	11.4- 26.9	14.1 - 32.6	10.2 - 19.7
			Mean	15.62	14.9	14.96	17.26	22.1	13.9
			SD	1.85	5.24	4.85	6.19	8.05	3.76
Mo	mg/kg DW	5	Min - Max	0.92 - 2.86	0.48 - 1.81	0.65 - 1.74	0.80 - 2.45	0.45 - 0.86	1.38 - 1.92
			Mean	1.88 a	0.95 cd	1.10 bcd	1.69 ab	0.58 d	1.64 abc
			SD	0.73	0.54	0.49	0.65	0.16	0.26

Abbreviations: DW, dry weight; SD, standard deviation; CONT, control; ORG, organic; MIN, mineral. Means with different lower-case letters (a–d) within the rows are significantly different ($P < 0.05$).

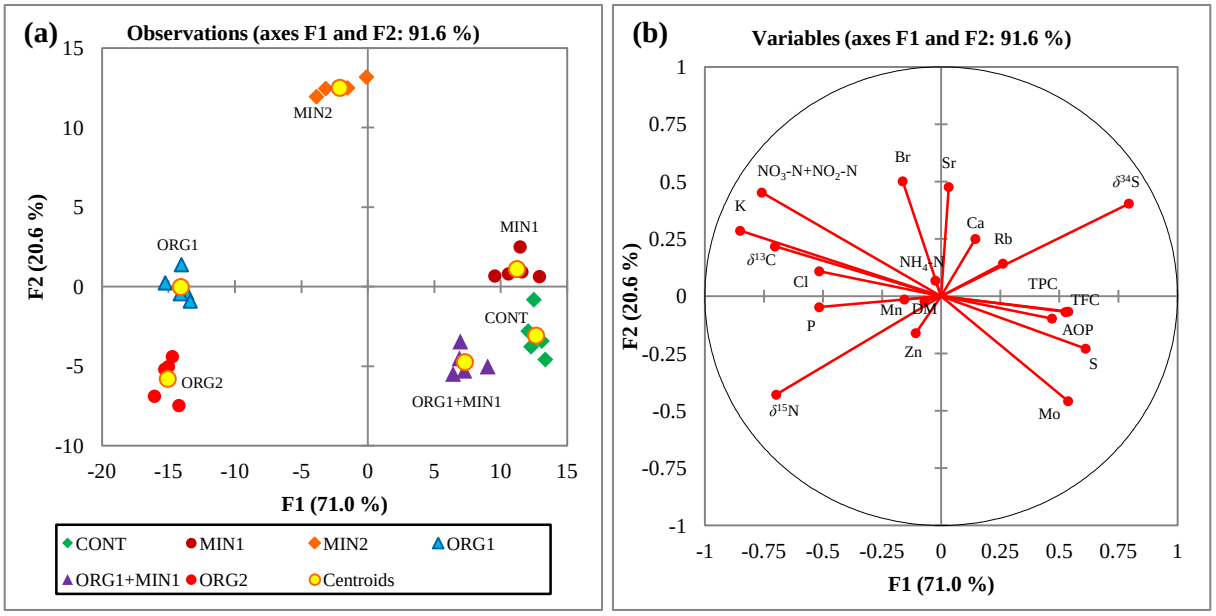

Fig. 1. (a) Discriminant function score plot and (b) discriminant loadings plot based on differences according to six fertility management practices (CONT, ORG1, ORG2, MIN1, MIN2, and ORG1+MIN1) for all chicory samples. Function 1 (F1) explained 71.0% and function 2 (F2) 20.6% of the total variance. Discriminant analysis performed with the 20 parameters including DM, TPC, AOP, TFC, NO_3-N+NO_2-N, NH_4-N, P, S, Cl, K, Ca, Mn, Zn, Br, Rb, Sr, Mo, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$, of the 30 samples originated from five chicory cultivars.

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

***Credit Author Statement**

Lovro Sinkovič: Conceptualization, Formal analysis, investigation, Data curation, Writing - Original draft, Visualisation, Project administration. **Marijan Nečemer:** Methodology, Software, Formal analysis, Investigation, Resources, Data curation, Writing - Review & Editing. **Nives Ogrinc:** Methodology, Resources, Data curation, Writing - Review & Editing, Supervision. **Dragan Žnidarčič:** Validation, Writing - Review & Editing. **David Stopar:** Formal analysis, Writing - Review & Editing. **Rajko Vidrih:** Methodology, Resources. **Vladimir Meglič:** Validation, Investigation, Writing - Review & Editing, Supervision, Project administration.