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Chemometric guidelines for selection of cultivation conditions influencing the antioxidant potential of beetroot extracts

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ABSTRACT

The estimation of influences of different cultivation conditions on chemical composition of crops can be quite complex task. Usually, the classical statistic approach (comparison of statistical characteristics of different populations) is applied. However, this study presents the analysis of the influence of different cultivation conditions (cultivation with or without foil cover, different preceding crops and fertilization) on the contents of dry matter, betalains and phenolic compounds in 64 beetroot (*Beta vulgaris* L. ssp. *vulgaris*) samples and their antioxidant potential (IC_{50}) by using powerful chemometric tools, such as Wald-Wolfowitz run test, cluster analysis and sum of ranking differences, in order to obtain groupings of the samples which share similar properties or to test if the samples come from the same population. Besides the aforementioned classification or pattern recognition analysis, the regression methods (linear, multiple linear and artificial neural network regressions) were carried out in order to establish reliable linear and non-linear relationships between IC_{50} and contents of betalains, phenolic compounds and dry matter. The results reveal certain influence of foil cover, used during the cultivation, on beetroot antioxidant potential and content of the determined betalains and phenolic compounds, as well as the strong non-linear relationship between the analyzed variables. Certain influence of preceding crops on phenolic compounds content is detected by Wald-Wolfowitz run test.

1. Introduction

Beetroot (*Beta vulgaris* L.) is a biennial plant with very significant features including an outstanding antioxidant capacity (Vinson et al., 1998; Čanadanović-Brunet et al., 2011; Kanner et al., 2002) and very high total phenolic compounds content (50–60 μmol/g dry matter) (Vinson et al., 1998). Beetroot is a rich source of betalains, red and yellow indole-derived pigments, red-violet betacyanins and yellow betaxanthins. The betacyanin and betaxanthin concentration ratio usually ranges between 1 and 3. It depends on beetroot varieties and applied extraction technology (Nemzer et al., 2011). Betanin (betanidin 5-*O*-β-glucoside) is the main betacyanin present in beetroot. Its concentration is decreasing in the following order: peel, crown and flesh (Kujala et al., 2000, 2002). The results by Kapadia et al. (2011) suggest that betanin, the major betacyanin constituent, may play an important role in the cytotoxicity exhibited by the red beetroot extract. Vulgaxanthin (I and II) belongs to the group of betaxanthins present in beetroot. Betanin and vulgaxanthin, isolated from beetroot, are widely used as natural food dyes.

Beetroot is a cool-weather crop and it tolerates some freezing. The optimal soil temperatures for beetroot seed germination is between 18 and 24 °C (Joubert, 1974; Hartmann et al. 1988). Deep, loose and well-drained soil is favorable for beetroot cultivation, especially of pH of 5.8–7.0 (Joubert, 1974; Hartmann et al., 1988). In order to obtain high yields and good quality of beetroot a continuous supply of nitrogen, potassium and phosphate should be provided.

In earlier studies it has been shown that the preceding crops have certain influence on growth, development and yield of components of different plant species (Christen and Sieling, 1995; Olofsson, 1993; Christen et al., 1992; Anderson, 2009). The aim of the present study was to examine the combined effects of preceding crops (cauliflower, broccoli, cabbage and kohlrabi), type of fertilization and the use of foil cover during the cultivation of beetroot on the experimental fields on their antioxidant activity and content of certain compounds. The impact of the aforementioned cultivation conditions on the contents of dry matter, phenolic compounds, betanin and vulgaxanthin I and antioxidant potential

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of beetroot extracts was studied using different chemometric tools: classification methods (cluster analysis and sum of ranking differences, including Wald-Wolfowitz run test) and regression methods (linear and multiple linear regression and artificial neural networks). Chemometrics is a multidisciplinary approach which can be used not only in chemistry (Kovačević et al., 2013, 2014), but in many other scientific fields, such as forensics and biotechnology (Gadzuric et al., 2014; Dodić et al., 2014). Chemometric methods can reveal some features of the analyzed objects that are not apparent. It can be used for pattern recognition and regression and correlation analysis between variables. Since the determination of the influence of different cultivation conditions on beetroot characteristics can be quite complex, chemometrics can make this task easier and can reveal which cultivation conditions are optimal for the growth of beetroot with desired characteristics.

2. Material and methods

2.1. Raw beetroot samples

The beetroot (*Beta vulgaris* L. ssp. *vulgaris*, *Chenopodiaceae*) samples were grown on the open experimental fields of Faculty of Agriculture, University of Novi Sad. These samples were grown on soil with different preceding crops (cabbage, cauliflower, kohlrabi and broccoli) and treated with fermented bovine manure 20 t/ha, swine composted manure 20 t/ha, fermented bovine manure 20 t/ha + $N_{33}P_{33}K_{63}$, swine composted manure 20 t/ha + $N_{33}P_{33}K_{63}$, fermented bovine manure 20 t/ha + $N_{55}P_{55}K_{105}$, swine composted manure 20 t/ha + $N_{55}P_{55}K_{105}$, $N_{33}P_{33}K_{63}$ and $N_{55}P_{55}K_{105}$. Some of the samples were grown with foil cover and some without. The collected samples were minced using laboratory blender device and stored in freezer (−25 °C). The total number of samples analyzed was 64.

2.2. Preparation of beetroot extracts

The extraction procedure depends on which compound needs to be determined. Therefore, in this paper two different extraction methods were used.

The extract for determination of betalains (betanin and vulgaxanthin) was obtained by extraction with water. The sample amount needed for extraction was measured by analytical balance and was added into water. The obtained mixture was intensively stirred for 15 min. Filtration agent (Celite) was added. The mixture was filtered through the Bühner funnel with Whatman No. 1 filter paper. The sample remains on the filter paper were rinsed until discoloration. The collected extract was transferred to corresponding volumetric flask.

The extracts used for determination of phenolic compounds content and antioxidant activity assay were obtained by method introduced by Gonzalez-Gomez et al., 2010, with certain modifications. An amount of 10 g of the minced sample was dissolved in methanol (Merck, Germany) which was used as the extracting agent. The extraction was carried out in a dark place with constant stirring in a laboratory shaker during 24 h at room temperature. After extraction, the sample was quantitatively transferred in a volumetric flask (50 cm^3) which was then filled with methanol to calibration mark. Afterwards the obtained solution was filtered and kept in a cold and dark place.

2.3. Determination of dry matter and contents of betanin, vulgaxanthin I and phenolic compounds

Dry matter (DM) of the samples was determined by gravimetric method by oven drying at 105 °C to constant weight.

The contents of betanin and vulgaxanthin I in beetroot extracts were determined applying spectrophotometric method (spectrophotometer Bruker, Rheinstetten, Germany) (von Elbe, 2001). The absorbances of extracts were measured at 538, 476 and 600 nm. The corrected absorbances were calculated on the basis of the following formulas:

$$A_1 = 1.095 \cdot (a - c) \quad (1)$$

$$Z = a - A_1 \quad (2)$$

$$A_2 = b - Z - (A_1/3.1) \quad (3)$$

where: a – absorbance of the sample at 538 nm, b – absorbance of the sample at 476 nm, c – absorbance of the sample at 600 nm, Z – absorbance of impurities, A_1 – absorbance of betanin (corrected for the contribution of colored impurities), A_2 – absorbance of vulgaxanthin I (corrected for the contribution of betanin and colored impurities). The following formulas were used for calculation of betanin (B) and vulgaxanthin I (V) contents:

$$\mathrm{B} = (V_\mathrm{m} \cdot A_1 \cdot \mathrm{DF})/(\mathrm{m} \cdot 1120)[\mathrm{mg}/100\ \mathrm{g}] \quad (4)$$

$$\mathrm{V} = (V_\mathrm{m} \cdot A_2 \cdot \mathrm{DF})/(\mathrm{m} \cdot 750)[\mathrm{mg}/100\ \mathrm{g}] \quad (5)$$

V_m – extract volume (cm^3), m – mass of the sample taken for the analysis (g), DF – dilution factor.

Folin–Ciocalteu method was applied for determination of total content of phenolic compounds using chlorogenic acid as a standard (Kähkönen et al., 1999; Singleton and Rossi, 1965). The absorbance was measured at 765 nm. The results were expressed in mg equivalent of chlorogenic acid per 100 g of dry matter (mg CAE/100 g DM).

2.4. Antioxidant activity assay

Free radical scavenging activity (RSC%) of the examined beetroot extracts was determined spectrophotometrically. The hydrogen atom or electron donation ability of the extract was measured by so-called DPPH test (Espin et al., 2000) from the bleaching of a purple-colored methanol solution of stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·). This method is based on tracking the transformation of DPPH· radical into reduced yellow-colored DPPH-H form. The prepared extract was mixed with methanol (96%) and 90 μl of DPPH solution (18 mg in 50 cm^3 95% methanol prepared daily) up to concentrations of 2.0, 4.0 and 6.0 mg of sample per cm^3. After 60 min at room temperature, absorbance was measured at 517 nm. RSC% was calculated using the following equation:

$$\mathrm{RSC}\% = 100 - (A_\mathrm{sample}/A_\mathrm{blank}) \cdot 100 \quad (6)$$

A_sample – absorbance of the sample, A_blank – absorbance of control.

On the basis of linear dependence between the beetroot extract concentration and RSC% value, the IC_{50} values were calculated. Antioxidant activity was presented as IC_{50} value which is the beetroot extract concentration which expresses the 50% of antiradical activity. Lower IC_{50} is, higher antiradical activity of the extract.

The analytical reagents of exceptional purity (*pro analysi*) were used. All measures were replicated three times.

2.5. Wald-Wolfowitz run test, cluster analysis and sum of ranking differences

Wald-Wolfowitz run test (WWR) can be applied to examine if two random samples come from populations with the same distribution. WWR test can detect differences in averages or spread or any other important aspect between the two populations (Bhar, 2014). This test is efficient when each sample size is greater than