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Zwitterionic hydrophilic interaction liquid chromatography coupled to mass spectrometry for analysis of beetroot juice and antioxidant interactions between its bioactive compounds

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ABSTRACT

Hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC – MS) was employed to determine selected bioactive compounds (polyphenolics and vitamins B) in beetroot *(Beta vulgaris* L.) juice. Appropriate optimization of the method allowed to select the separation conditions and to increase the sensitivity of the method in comparison with RP mode. The limits of detection on ZIC HILIC column were 0.01 mg L^{-1} for flavonoids and phenolic acids, and 0.03 mg L^{-1} for vitamins B. The investigation of interaction between the major phenolic acids (protocatechuic and caffeic) and B vitamins (nicotinamide and panthotenic acid) found in beet juice was evaluated. Antioxidant activities of single components and their mixtures were determined by the mostly used spectrophotometric methods. Additive effect between protocatechuic acid and B vitamins were observed in DPPH assay, while synergistic effect is observed between caffeic acid and B3 vitamin. In CUPRAC and Folin-Ciocalteu assays only antagonistic effects were observed.

1. Introduction

During the recent years, there has been significant growing customer interest in healthy nutrition and natural foods without artificial additives. Several studies have shown that a diet rich in fruits and vegetables can effectively protect against many chronic diseases such as cardiovascular disease, cancer and stroke (Slavin and Lloyd 2012; Shahidi & Ambigaiplan, 2015). Among the bioactive compounds in vegetables and fruits, polyphenols are good antioxidants, which can effectively scavenge the excess of free radicals in body and inhibit lipid oxidation (Costa et al., 2017; Oroian & Escriche, 2015).

Beetroots (*Beta vulgaris* L.), also known as red beets, are popular vegetables grown throughout the Americas, Europe and Asia. They have been gaining in popularity as a new super food due to recent studies claiming that beetroot juice can improve athletic performance, lower blood pressure, and increase blood flow (Eggebeen et al., 2016; Wruss et al., 2015). In contrast to fruits, the main sugar in beetroots is sucrose with only small amounts of glucose and fructose. Because fructose reduces human exercise capacity, a low fructose and a high sucrose content is preferable, for example in sports drinks (Eggebeen et al., 2016). Beetroots have also received increasing attention due their antioxidant and anti-inflammatory activities (Georgiev et al., 2010) and chemo-preventive effects (Zhang, Pan, Wang, Lubet, & Yo, 2013). Beetroots are a great source of betalains, a group of chromoalkaloids, which can be devided into betacyanins (red-violet pigments) and betaxanthins (yellow pigments) (Wruss et al., 2015). Due to their properties, they are often used as natural colorants by the food industry. It was reported that beetroots are also the source of some hydroxycinnamic acids as well as flavonoids (Wruss et al., 2015; Georgiev et al., 2010; Kujala et al., 2002; Kazimierczak et al., 2014). No information was found regarding the content of vitamins, except vitamin C (Kazimierczak et al., 2014). Beetroots can be eaten boiled or grilled, however, every heat treatment of these vegetables affect their antioxidant content (Ravichandran et al., 2013). Thus, it is better to serve them fresh in salads or drink fresh beet juice.

Hydrophilic interaction liquid chromatography (HILIC) is a relatively new chromatographic mode developed in response to the lack of retention of polar compounds in conventional reversed-phase (RP) mode (McCalley, 2017; Sentkowska, Biesaga, & Pyrzyńska, 2015). Recent studies showed that the mechanism of separation in HILIC results not only from the partition between the aqueous layer accumulated close to the solid surface and a highly organic mobile phase (Guo, 2015; Jandera & Janás, 2017; Salas, Borrull, Fontanals, & Marc, 2017). Other interaction such as adsorption via hydrogen bonding, dipole-dipole and

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electrostatic interactions with bonded ionic groups have also a great impact (Sentkowska, Biesaga, & Pyrzyńska, 2013; Zuo, Zhou, Zuo, & Deng, 2015).

The aim of this work was to evaluate the potential of HILIC in the chromatographic analysis of beet juice for determination of some bioactive compounds such as polyphenols and vitamins from B group. The ZIC column was applied in the analysis of B vitamins for the first time. Some information regarding the use of diol and amide stationary phases under HILIC conditions for this purpose can be found in the literature data (Yang, Boysen, & Hearn, 2013, Katapranis et al., 2010; Chatterjee, 2017). Higher content of organic solvent in the mobile phase under HILIC conditions enhances the efficiency of the ionization in the ion source of mass spectrometer and significantly higher sensitivity can be obtained (Katapranis, Fiamegos, & Stalikes, 2010; Pyrzyńska & Sentkowska, 2015; Sentkowska et al., 2015). Additionally, the interactions between the individual phenolics and vitamins B found in the analyzed beet juice were examined to evaluate their impact on the antioxidant capacity of the sample.

2. Materials and methods

2.1. Reagents and sample

Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade from Merck (Darmstadt, Germany). Pure ammonium formate as well as formic acid and all reagents used in reducing power assays were purchased from Merck (Darmstadt, Germany).

The commercial standards of flavonoids, phenolic acids and vitamins B were purchased from Sigma (Steinheim, Germany). The stock solutions of these individual compounds were prepared at 100 mg L^{-1} concentration level in acetonitrile.

Freshly squeezed beet juices (supplied by different manufacturers) were purchased at a local market. According to the information obtained by the Authors, beetroots (*Beta vulgaris* L.) from conventional cultivation were used for the production of that juice. For the comparison beet juice obtained from ecologically cultivated vegetables was also analyzed. Each juice was analyzed just after it purchased without storage them. In accordance with the manufacturer's assurance the juices were delivered not longer than 12 h after the preparation. During this time they were storage by manufacturer at 2 °C. The samples was filtered through PTFE 0.45 μm membrane filters (Alchem, Poland) and injected to the HPLC system.

2.2. Instrumentation

Chromatographic analysis was performed with the Shimadzu LC system consisted of binary pumps LC20-AD, degasser DGU-20A5, column oven CTO-20AC, autosampler SIL-20AC and 8030 triple quadrupole Mass Spectrometer (Shimadzu, Japan) equipped with an ESI source operated in negative-ion or in positive mode, according to the determined species. The ESI conditions were as followed: the capillary voltage 4.5 kV, temperature 400 °C, the source gas flow 3 L min^{-1}, drying gas flow 10 L min^{-1}. For each compound the optimum conditions of Multiple Reaction Mode (MRM) were determined. Continuous mass spectra was obtained by scanning *m/z* from 50 to 1100.

Compounds were separated on SeQuant™ ZIC-HILIC column (100 × 2.1 mm, 3.5 μm) from Merck (Darmstadt, Germany). For the separation of polyphenolic compounds mobile phase containing 5 mM ammonium formate at pH 7 (eluent A) and ACN (eluent B) was used in a gradient mode. The gradient profile was as follows: 0–4 min 98% B, 6–7 min 90% B, 8–8.4 min 80% B, 8.4–12 min 50% B, and 13–28 min 98% B. The mobile phase was delivered at 0.2 mL min^{-1}. The separation of B vitamins were performed using ACN/8 mM HCOOH (97:3 v/v) eluent at pH 2.8 (value refers to the water component) with isocratic elution. For RP chromatographic analysis C18 Kinetex column was used (100 × 2,1 mm; 2,6 μm). Eluents used in experiment were as followed: for panthotenic acid ACN/HCOOH (5/95% v/v); for caffeic acid and rhamnetin: gradient elution was as followed: 0–5 min. 20% B, 10–15 min 25% B, 20–25 min 30% B, 30–31 min 90% B, 32 min 20% B, where B is ACN and A is 8 mM HCOOH (pH 2.8).

All the spectrophotometric measurements were performed with a double-beam UV–Visible spectrophotometer (Lambda 20 Parkin Elmer).

2.3. Total phenolic content and antioxidant assays

The antioxidant properties of single bioactive compounds found in beetroot juice and their mixtures was evaluated using cupric reducing antioxidant capacity (CUPRAC) assay and Folin-Ciocalteau (FC) methods. In CUPRAC method 1 mL of $CuCl_2$ solution (0.01 mol L^{-1}) was mixed with 1 mL of neocuproine alcoholic solution (7.5×10^{-3} mol L^{-1}) and 1 mL of 1 mol L^{-1} CH_3COONH_4, followed by adding 0.5 mL of juice sample and 0.6 mL of water according to the method previously described by Dróżdż, Sentkowska, and Pyrzyńska (2017). The tube containing sample and reagents was incubated in a water bath at a temperature of 50 °C for 20 min, after which was cooled under running water. Absorbance against the blank reagent was measured at 450 nm. The calibration curve was drown with trolox (water-soluble analog of vitamin E) and the antioxidant activity of the samples was expressed as trolox equivalent (TRE) in mmol L^{-1}.

The FC assay was conducted according to Singleton, Orthofer, and Lamuela-Ravwntos (1999) with small modification. Briefly, 1 mL of a sample solution was mixed with 0.1 mL of FC reagent and 0.9 mL of water. After 5 min, 1 mL of sodium carbonate (7%, w/v) and 0.4 mL of water were added and 30 more min was allowed for stabilization of the blue color formed. The absorbance against a reagent blank was measured at 765 nm and the results were expressed as gallic acid in mg of gallic acid per L.

For DPPH assay 0.1 mL of sample was added to 2.4 mL of DPPH solution (3.0×10^{-5} mol L^{-1}) in methanol (Dróżdż, Sentkowska, & Pyrzyńska, 2016). After 30 min absorbance was measured at 539 nm. Trolox was used for calibration curve and the results were expressed in trolox equivalent (TRE) in mmol L^{-1}. Analysis were run in triplicates.

In every case the standard solution of single compounds (phenolic compounds or vitamins B) as well as their mixtures were used as a samples in antioxidant assays. The exception from this rule are the kinetic curves for DPPH radical scavenging. In all standard solutions analytes were in concentration 10 mg L^{-1}.

2.4. Statistical analysis

The results were expressed as mean ± standard deviation for at least three independent determinations. Statistical comparison of the means was performed using one-way ANOVA, followed by Turkey's test and differences at $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Chromatographic analysis

Mobile phase pH plays key role in the retention process due to its impact on ionization state of the analytes and the functional groups onto the stationary phase. The effect of the pH of the mobile phase on the retention of rhamnetin, caffeic acid and vitamin B6 is shown in Fig. 1. These compounds (belonging to different chemical classes) were chosen as the test analytes and were preliminary detected in beetroot juice in significant quantities. In the used ZIC-HILIC column, two active groups are present - strongly acidic sulfonic acid group and strongly basic quaternary ammonium group, both at 1:1 M ratio. The positive charge is close to the surface, while the negative charge is in the