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Separation of polyphenolic compounds extracted from plant matrices using capillary electrophoresis

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Abstract

Phenolic compounds constitute a large group of secondary plant products whose chemical structure may range from quite simple compounds to highly polymerized substances. The polyphenols content have been investigated in the alcoholic extract of the fruits of three different plants: sweet gale, sea buckthorn, hiprose. The *trans*-resveratrol content we have studied in roots, stems, leaves and flowers of Japanese knotweed grown in Estonia. Plant material was pre-treated in two different ways: by infusing with methanol and by supercritical fluid extraction with carbon dioxide modified with different alcohols. The relationship between variables (pressure, temperature, modifier amount) and yields are examined. The capillary zone electrophoresis methods were developed for the separation of polyphenolic anti-oxidative compounds. Using both water based borate buffer and acetonitrile based non-aqueous media it was possible to get reliable separation of several polyphenolic compounds. Based on that there has been identified such as flavone, *trans*-resveratrol, catechin, chlorogenic acid, quercetin and myricetin in plant extracts. Changes in the relative concentrations of *trans*-resveratrol in different parts of the knotweed have been established.
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1. Introduction

The use of synthetic antioxidants in the food industry is severely restricted as to both application and level. This is the reason for wider interest to natural antioxidants extracted from plants. Polyphenols are the major plant compounds with antioxidant activity. Research of the polyphenols occurring in plants has been raised because of their perceived health-beneficial effects, such as antimutagenic, anticarcenogenic, antiatherogenic etc. Primarily stibenes and flavonoids caused great interest after they were found to have effects in inhibiting the copper-catalyzed oxidation of low-density lipoprotein (LDL), inhibiting platelet clotting and arachidonate metabolism, reducing liver injure from peroxidized oil, and having cancer-chemopreventative activities [1]. Especially berry seeds could be very good source for antioxidant-rich oils [2]. Considering environmental friendliness of carbon dioxide, supercritical extraction technique is a potential choice for isolation of valuable constituents from berries and it deserves further investigation [3]. In this paper some berries from plants, which are well known in folk medicine, are extracted by supercritical carbon dioxide. The most influential variables on polyphenols recovery are supercritical CO_2 density,

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and nature and percentage of organic modifier [4]. Extraction at different pressures allows obtaining oils with different qualities. Ethanol would be the best choice for polar modifier if looking for food or pharmaceutical application of extracts.

Several methods have been developed to analyse the polyphenols in plant matrices: thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and HPLC–mass spectrometry (LC–MS) are the most powerful analytical separation methods [5].

Capillary electrophoresis has gained widespread interest as a favourable technique for the determination of pharmacologically interesting compounds in biological matrices such as plants [6]. The most attractive advantages of CE are rapidity of the method, small sample amounts required and a strictly limited solvent waste. Borate buffers with pH 8–11 and a concentration of 25–200 m*M* are commonly used, as borate can form complexes with ortho-dihydroxyl groups on the flavonoid nucleus and with vicinal *cis*-dihydroxyl groups of sugar and therefore ease the separation [7].

Specific complexation between borate ion and certain analytes containing hydroxyl groups results in negatively charged borate complexes. The borate buffer, under basic experimental conditions in which the polyphenols are negatively charged, could effect the separation either based on charge-to-mass ratios of the deprotonated polyphenols or through borate–phenol association [8]. One of the possibilities for using electrolyte additives that are easily ''tuneable'' is room-temperature molten salts or so-called ionic liquids. In some CE studies ionic liquids have been used as electrolytes to the CE separation of polyphenols [9].

Non-aqueous CE (NACE) is a method that can be used to separate compounds that are difficult to dissolve in aqueous systems. Various researches have successfully applied NACE for the analysis of natural compounds [10,11]. It is very easy to prepare ionic liquids with the same cationic part but with a different anionic part without significantly changing their gross physical properties. The chemical properties, in contrast, are more dependent on the anionic part. For example, the salts with the same cation are miscible in both water and/or organic solvents depending on anionic parts. This reason they are good candidates as electroosmotic-flow modifiers in organic solvents.

The aims of the present study were to investigate the influence of organic modifier on the supercritical fluid extraction (SFE) of plant material and the use of ionic liquid: 1-butyl-3-methylimidazolium-D-mandelate in buffer for separation of flavonoids as an alternative approach.

2. Experimental

There were fruits of three plants under the study: sweet gale (*Myrica gale* L.), sea buckthorn (*Hippophaë rhamnoides* L.) and hiprose (*Rosa majalis* L.) and knotweed (*Reynutria japonica* Houtt.) grown in Estonia. The plant material was collected from island of Saaremaa and knotweed near of Tallinn (Estonia). The plant materials were collected in summer and autumn when they were ripe, then dried and stored in an exicator. For each experiment weight of raw material was in range of 0.2–1.0 g.

Supercritical fluid experimentation was performed on Milton Roy sample preparation accessory (Milton Roy LDC Division, Riviera Beach, FL, USA) apparatus which allows the operating pressure up to 34.5 MPa and temperature up to 60 °C. Experiments were carried out at constant temperature 45 °C (318 K) using a pressure at range of 13.7–27.6 MPa. Extraction time was varied from 30 to 90 min. Collecting solvents were hexane or methanol. High purity carbon dioxide 99.5% from AS Eesti AGA was used.

CE separations were performed using an ISCO CV4 electropherograph (Isco, Lincoln, PA, USA) with an UV detector coupled to a personal computer, and controlled by in-house written software. All experiments were conducted with an applied voltage of +18 kV. The separation was monitored at 240 nm. An uncoated capillary (Polymicro Technologies, Phoenix, AZ, USA) with dimensions 75 cm (5 cm to detector)×50 μm, was used throughout the study. Before use, the capillary was rinsed with 1 *M* sodium hydroxide, water, methanol, and separation medium 10 capillary volumes of each. Between analyses the capillary was washed with a solvent and then with the one capillary volume of the separation medium.

1-Butyl-3-methyl imidazolium-D-mandelate (ionic

liquid) was prepared in the Institute of Chemistry at Tallinn Technical University, Estonia, following the procedure described elsewhere [12]. The starting materials were obtained from Sigma–Aldrich (Steinheim, Germany).

Polyphenols: *trans*-resveratrol, catechin, quercetin, rutin, myricetin, chlorogenic, acid, caffic acid, gallic acids were obtained from Merck (Darmstadt, Germany). *cis*-Resveratrol was obtained by 10-h exposure of *trans*-resveratrol methanol solution (0.1 mg/ml) to diffused daylight. At these conditions 80% of *trans*-resveratrol was converted into the *cis*-isomer.

Sodium tetraborate, sodium acetate and acetic acid were obtained from Merck (Darmstadt, Germany).

All solvents were chromatographic grade and were obtained from Sigma–Aldrich.

The separation media were water or acetonitrile and mixtures of acetonitrile and methanol. All analytes were dissolved separately in methanol (0.5 mg/ml) for identification purposes and as a mixture for selectivity studies.

3. Results and discussion

3.1. Supercritical extraction

It is quite natural that berries of different plants are very different on yield of extractable compounds. On extraction conditions at 30 MPa at 45 °C with 60 min of extraction time the total yield for sweet gale was 61 mg/g, for sea buckthorn 130 mg/g and for hiprose 6.3 mg/g.

The extraction pressure is important parameter to change the extract content. For extraction of essential oils the extraction pressure should not exceed the value of 12 MPa [13]. With increasing pressure, the density of fluid increases which in turn increases the solubility of high-molecular-mass compounds that do not contribute to fragrance formation but may have other important properties. In this study the attention was on high-pressure extraction, and this increased density, which resulted in higher extraction efficiencies of polyphenols.

The effect of pressure on the yield of fruit oil was significant in extraction of sweet gale. The oil yield increased about three times with increasing the extraction pressure from 13.7 to 27.6 MPa at 40 °C (60 min extraction time). As very often the fruits or seeds are covered with hard scale, the effect of grinding was studied in detail. There was considerable difference between two runs. Extraction from ground fruits of sweet gale gave six times higher yield compared to non-ground fruits (40 °C, 20.7 MPa, and extraction time 30 min). Fluid penetrates the matrix easier and has direct contact with analyte. It is important to grind fruits immediately prior to extraction to avoid possible loss of volatile components. The same effect was seen in cases of hiprose and sea buckthorn, and all further experiments were carried out on ground berries.

The study of sweet gale extracts GC [14] showed that the extracts have very complex composition, however two used extraction methods—SFE (13.7 MPa, 40 °C, 60 min) and simultaneous distillation extraction (SDE)—gave rather similar results, only the amount of polyphenolic compounds (flavonoids) was higher in SFE extract (3.7%) compared with SDE extract (0.3%). Furthermore, at higher pressure (20.7 MPa) the amount of flavonoids was raised up to 13.2%. Also from these studies it became clear that GC is not the right technique to study the extracts made at high pressure and with modifier.

3.2. Capillary electrophoresis

3.2.1. The separation of standard mixtures of polyphenols

The separation of standard polyphenols mixtures both in aqueous buffer and in non-aqueous solutions has been investigated. As shown in Fig. 1 the migration order of flavonols (namely quercetin, rutin, myricetin) depends on the nature of the background electrolyte. It is also evident from the Fig. 1 that the separation selectivity is significantly improved in case of non-aqueous environment. The separation selectivity and mobility of anions is also influenced by methanol content of the background electrolyte (see Fig. 2).

The methods were optimised with the aim to obtain good resolution of phenolic compounds in real sample.

3.2.2. The analysis of extracts from plant matrices

Further analysis of extracts was performed by capillary electrophoresis, and for that some efforts

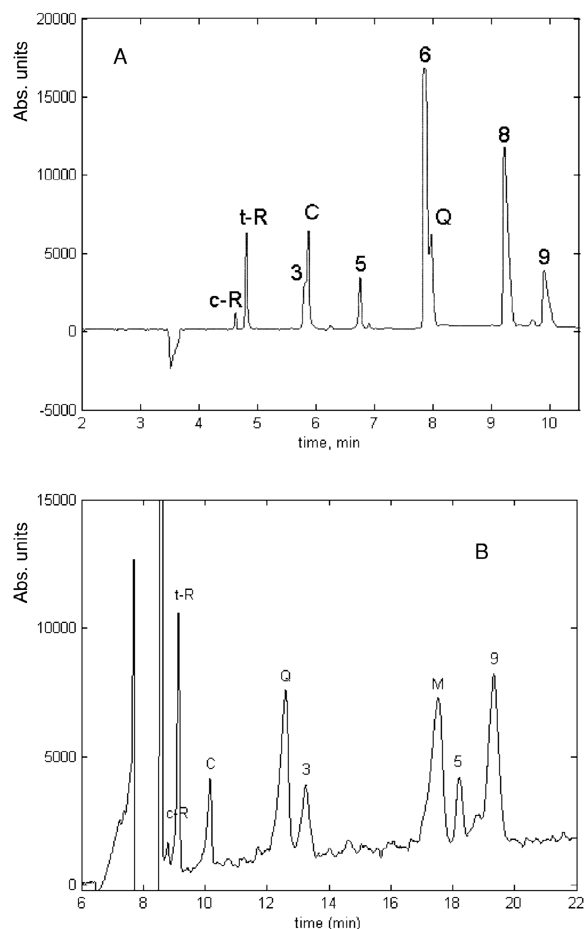

Fig. 1. Separation of standard mixture of polyphenols. (A) 25 m*M* disodium tetraborate buffer in water (pH 9.4); (B) 1.5 m*M* 1-butyl-3-methylimidazolium mandelate in acetonitrile (1% acetic acid added). Peak identification: F=flavone; R=resveratrol; 3=rutin; C=catechin; 5=chlorogenic acid; M or 6=myricetin; Q=quercetin; 8=caffeic acid; 9=gallic acid.

were made to develop an efficient buffer system for the analysis of raw extracts of berries. The electrolyte solutions for preliminary capillary electrophoresis work were prepared using disodium hydrogen phosphate, sodium dihydrogen phosphate, ammonium acetate and borate, which were of different concentrations. At last the borate buffers were selected for optimisation experiments. 25 m*M* disodium tetraborate for work at pH 9.4 was used to optimise conditions, which gave the optimal separation of

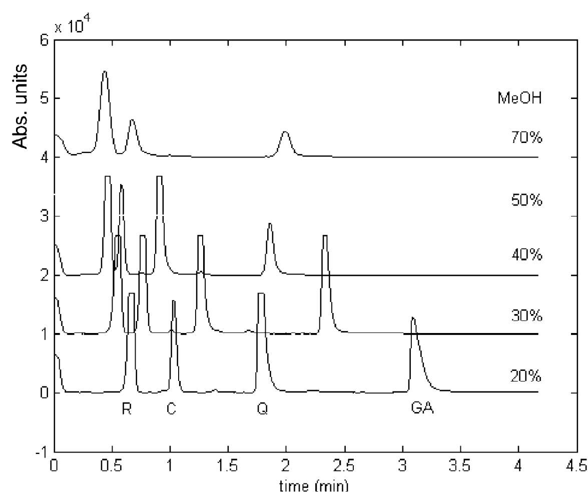

Fig. 2. The influence of methanol content in buffer on separation of polyphenols. Separation buffer: 25 m*M* sodium acetate (1% acetic acid added) in acetonitrile (or mixture of acetonitrile and methanol). Peaks: R=resveratrol; C=catechin; Q=quercetin; GA=gallic acid.

flavone, resveratrol, D-catechin, quercetin and myricetin.

The identification of target analytes was done by addition of pure compound of known concentration. Example of separation of extract components is shown in Fig. 3. Identification of analytes is done based on comparison with standard.

Ethanol was used as modifier and different amounts were added to the sample (2.5, 5 and 7.5%, w/w). As expected, the addition of ethanol increased the yield of compounds in the extract (including the polyphenols as seen in Fig. 4).

The experiments demonstrated in case of knotweed that resveratrol in this plant is mainly accumulated in roots (Fig. 5). No significant seasonal dependence was observed.

4. Conclusions

Several polyphenolic compounds have been identified and measured in the supercritical CO_2 extracts. The results show the importance of experimental studies for the evaluation of the quantitative influence from process parameters especially the

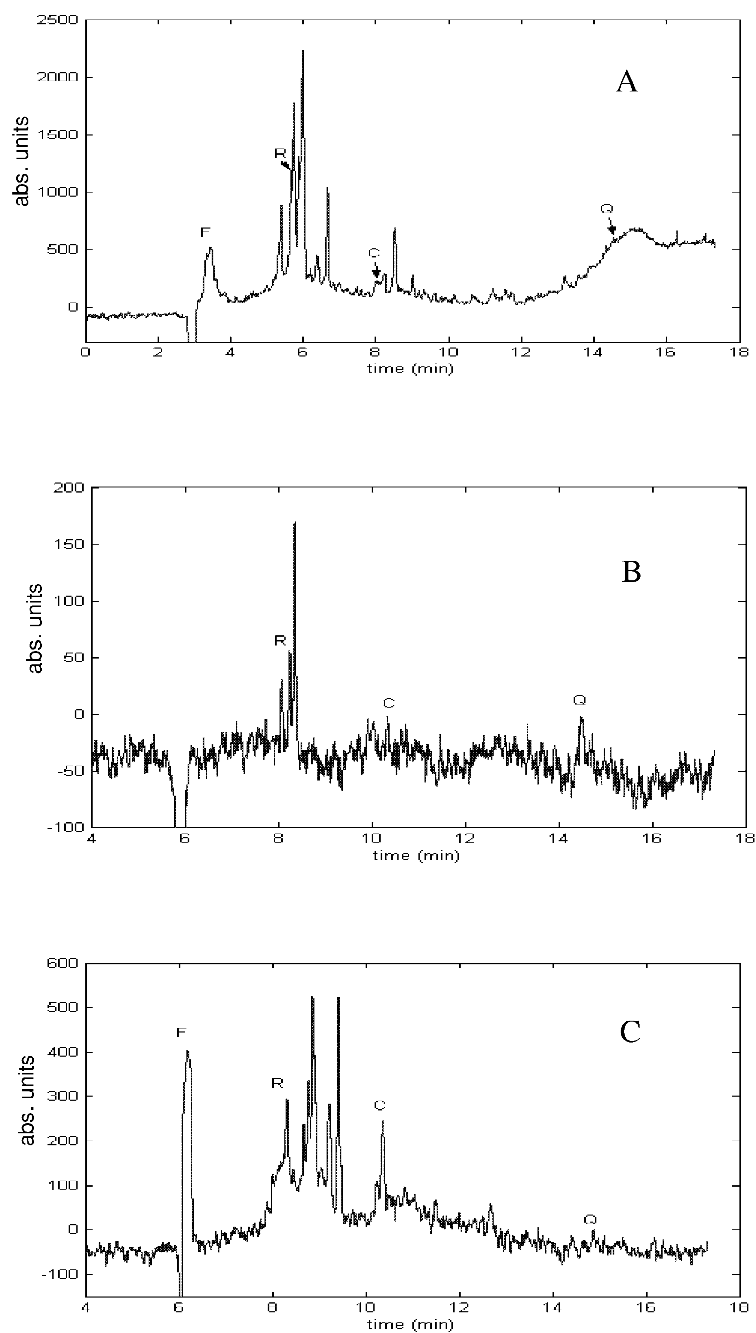

Fig. 3. CE separation of plant extracts. (A) Hiprose; (B) Sea buckthorn; (C) Sweet gale. Separation buffer: 25 m*M* disodium tetraborate in water (pH 9.4).

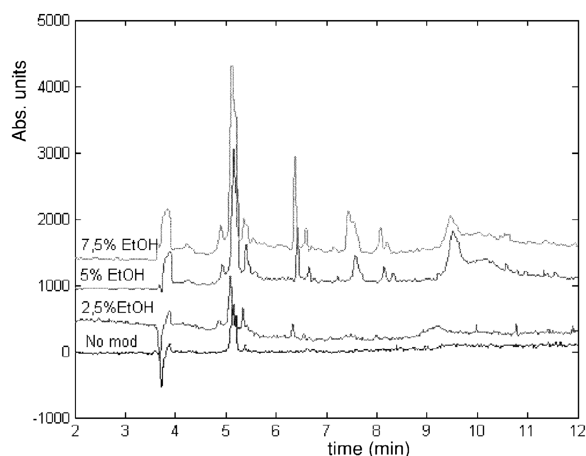

Fig. 4. The pherograms of hiprose SFE extracts (extraction pressure: 15 MPa, temperature: 45 °C, time: 1 h) with different amounts of ethanol as modifier.

amount of modifier. By using of fractioning extraction at different pressure values it is possible to achieve the separation of different chemical components.

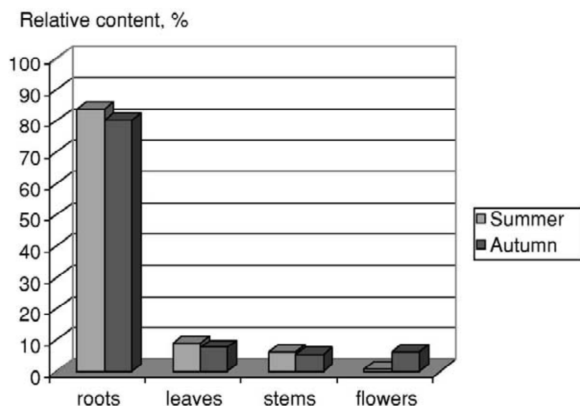

Fig. 5. Seasonal dependence of trans-resveratrol content in different parts of the knotweed grown in Estonia.

By using different electrolytes in CE allows altering separation selectivity of polyphenols

Acknowledgements

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