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Coupling of capillary electrophoresis with reaction detection for the on-line evaluation of radical scavenging activity of analytes

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Abstract

The main task of this work was to create a rapid, simple and less material and time consuming method involving capillary electrophoretic separation in order to separate analytes and evaluate antioxidant activity within a single analytical run. Several interfaces were developed and used to couple CE to the reaction detector. The method developed enables simultaneous electrophoretic separation and evaluation of antioxidant activity of each separated compound in the mixture. The analysis was performed using DPPH (2,2-diphenyl-1-picrylhydrazyl) as synthetic radical reagent. The on-line capillary electrophoresis-reaction (antioxidant activity) detection method can be used for a rapid evaluation of individual antioxidants in complex mixtures, particularly extracts of natural products. Possibility to evaluate radical scavenging activity of extracts of natural products components is demonstrated on an example of aqueous propolis extract. Four phenolic acids where separated and their radical scavenging activity was on-line evaluated.

Keywords: Antioxidant activity, DPPH, capillary electrophoresis, on-line reaction detection;

1. Introduction

Numerous studies have linked the consumption of plant foods with improved health status and reduced risk of chronic disease. Advances in separation science, biology, and chemistry made progress to the fields of pharmacognosy and natural products chemistry research [1]. Relatively new methods for measuring radical scavenging detection ability have been created using stable free radical species like 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical or 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation. DPPH• can accept an electron or hydrogen radical from an antioxidant and therefore lose its absorbance at 517 nm [2]. Model radical tests, like the DPPH• scavenging test are simple, fast, reproducible and their mechanism of action is known. Therefore these tests are convenient, e.g. for a preliminary screening of many samples [3]. Spectrophotometric assay of DPPH• scavenging is commonly used to evaluate total radical scavenging activity of different extracts of natural products [4]. Any identification is improved with a mixture separation. In the most popular approach a colored, relatively stable radical such as DPPH• or $ABTS^{+}$•, is pumped at constant flow rate and is mixed with the effluent in the reaction coil. The signal of to the DPPH• or $ABTS^{+}$• colour bleaching is registered with a second photometric detector, so that only radical scavenging

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intensity for every separated compound can be evaluated. The antioxidants are detected by a decrease in absorbance at visible wavelengths due to the conversion of colored radicals to their non-colored form. Recently Teris van Beek and co-authors proposed HPLC method coupled with post column DPPH• reaction detector [5]. Total methanolic extract of thyme leaves was analysed and radical scavenging activity was evaluated using on-line assay involving single relatively stable reagent DPPH•. During the last five years this method became more widely spread [5]. The main advantages of the HPLC separation method are, that this is a traditional method with a good reproducibility, different detection possibilities and suitability to be coupled with different analytical methods. As possible drawbacks of HPLC can be mentioned: much solvent required, miniaturization is somewhat limited, high pressure pumps require routine maintenance etc.

Capillary electrophoresis (CE) has several advantages over the HPLC, such as high resolution, microformat analysis with a very little consumption of reagents, sample and solvents and rapid analysis. One of the main drawbacks is fluctuation of electroosmotic flow (EOF), which affects reproducibility of the results. On the other hand, this can be overcome using inter-run washes with sodium hydroxyde, voltage conditioning of the capillary before run or using internal standard. Recently Estonian scientists [6] introduced CE method with a "preanalysis" DPPH• reaction, which included comparative analysis of two samples DPPH• pretreated and original extract. Comparison of the electropherograms revealed the compounds which are DPPH• - active, their peaks were reduced or have disappeared. A comparison of the antioxidative capabilities of polyphenols contained in the artificial mixture of polyphenols and the extract of *S. melongena*, was carried out using capillary zone electrophoresis.

The main task of the present study was to create a rapid and effiecient method employing capillary electrophoresis and *on-line* reaction detection for evaluation of antioxidant activity of separated compounds. The on-line capillary electrophoresis-reaction (antioxidant activity) detection method is based on compound separation and post-column reaction with relatively stable free radical to evaluate antioxidant activity level of active compounds in natural products, such as plant extracts etc.

2. Materials and methods

All the experiments were performed using a standard automated Agilent CE System (Agilent Technologies, Waldbronn, Germany) with on - capillary DAD detector and on-line coupled to a model "Linear 200" variable UV/Vis reaction detector (Linear Instruments, USA). The apparatus automatically performed all steps of measurement protocols including capillary conditioning, sample introduction, voltage and pressure applications and detection.

Several interfaces were designed and used to couple CE to the reaction detection. The analysis was performed using 50 µg/ml DPPH (2,2-diphenyl-1-picrylhydrazyl) as synthetic radical reagent dissolved in 70% (v/v) methanol, 30% (v/v) run buffer pH 8.2.

The separation of honey bee propolis aqueous extract was performed in a fused silica capillary (50 µm I.D., Polymicro Technology, Phoenix, USA) of effective length of 22.5 cm, total length 67.5 cm. Prior to use, the capillary was rinsed with a 0.1 M NaOH solution for 5 min, water for 5min and separation buffer for 5min. As a background electrolyte 20 mM di-sodium tetraborate and 8 mM sodium di-hydro phosphate (pH 8.2) was used. High voltage of 25 kV was used for CE separation. To ensure transportation of the separated compounds in the interface a 50 mbar pressure was continuously applied at the inlet of the CE capillary during the analysis. For the construction of the interface and reaction coil different diameter fused silica capillaries were used, namely 75 µm I.D., 365 µm O.D., for a reaction coil (effective length till detection window 14 cm), 200 µm I.D., 365 µm O.D. for the CE capillary outlet adaptor, which vas connected to the reaction coil inlet with a piece of the Teflon 0.5 mm I.D. tubing starched by pulling till the appropriate inner diameter to fit outer diameter of fused silica capillary.

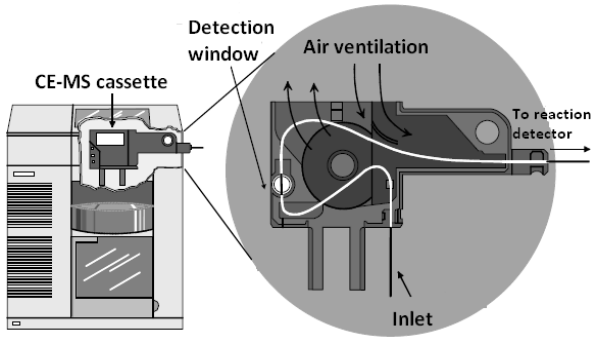

Fig.1. Scheme of capillary electrophoresis apparatus and standard mass spectrometry cassette (G1600-60013, Agilent Technologies, Germany), which was used for a development of on-line radical scavenging detection.

3. Results and discussion

Several interfaces were designed and used to connect CE with reaction detector. First configuration is shown in Fig. 2(A). The function of the stainless steel cross connection is to mix analytes with synthetic radical. Another function is to serve as a ground electrode. Cross connection is often used in the chips to introduce the sample. It was also used by Kulp et al. [7] for sampling for the reaction monitoring by CE. Therefore the cross configuration is very useful technique for the "pre-column" sample introduction or derivatization in the miniaturized separation techniques. For the "post-column reaction" often tee connection is used, however we preferred a cross connection for coupling of CE with the DPPH• reaction detection. With this interface it is easy to supply or exchange the reagent, since lifting up one reservoir and setting down another reservoir flushes the cross connection interior removing bubbles and replacing the reagent solution with a fresh. It is, however, somewhat difficult to control and reproduce the flow rate in the reaction coil and CE capillary, due to a difficulty to adjust a gap between an outlet of CE capillary and inlet of the reaction coil, when resetting the stainless steel cross joint. To ensure the quantitative transport of the analytes to the reaction coil a coaxial position of both capillaries should be fixed and hold during the experiments. It is advisable to adjust a gap between silica capillaries and hold it constant during all the experiments. Exact readjusting of the gap, which should be less than 50 µm, is always difficult. To supply reagent to the stream of working buffer coming into the reaction coil, the hydrostatic pressure is created lifting up the DPPH• reservoirs and setting down the reaction coil outlet vial. Too high level of DPPH• hinders the injection and resists to the transportation of the analytes by the CE apparatus internal pressure (10 - 50 mbar) applied to the inlet vial and the EOF generated in the CE capillary, since it generates a counter flow in the separation capillary. Therefore, the DPPH reservoirs should be kept at a level of the inlet vial. In case of the cross interface the coaxial alignment of the capillaries is critical for proper operation and reproducibility of the results. However, to achieve the alignment of the capillaries in opaque stainless steel cross connection is always difficult. Accumulation of gas due to the electrolysis taking place in the stainless steel cross connection hindered the separation, although the connection was flushed by the hydrostatic pressure generated flow of DPPH• reagent solution. Reproducibility of the results with this interface was low. Second interface (Fig. 2(B)) design is more reliable and robust due to its funnel form, which enables to ensure coaxiality of the CE capillary outlet and reaction coil inlet. The gap between the capillaries, which is needed to enter the reagent solution, is formed in any case due to nonperfect cut of the capillary ends. The demounting and mounting the joint does not dramatically change the flow rates. To supply the DPPH• solution the capillaries are bent into a loop and the joint is immersed in a 10 ml vial with DPPH• solution. The vial is capped with a rubber cap, which is inserted with the capillaries, platinum electrode and prepunched with a syringe needle to avoid pressure fluctuations in the DPPH• reagent container headspace.

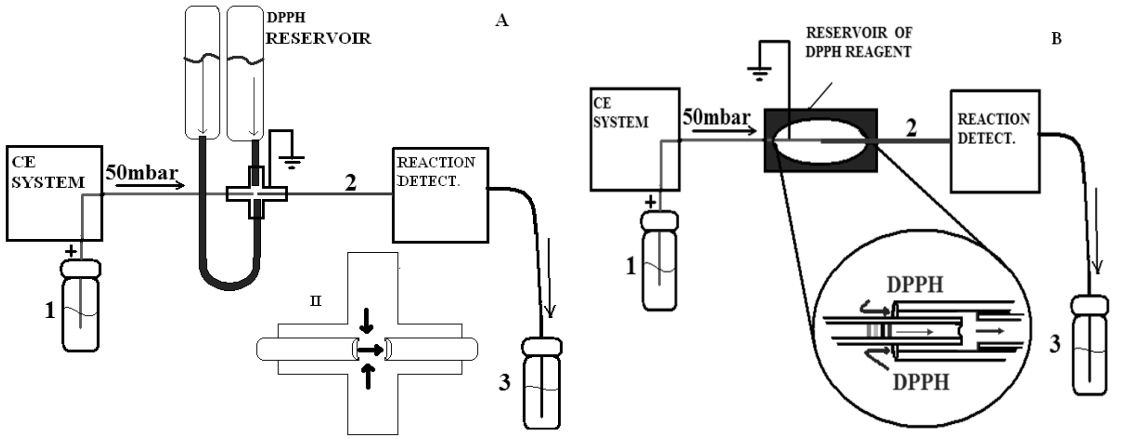

Fig. 2. The scheme of capillary electrophoresis – reaction detection setup: (A) with a stainless-steel cross connection interface and (B) funnel type interface. 1 – inlet vial (sample), 2 – reaction coil, 3 – outlet vial (waste). Sample is injected using 50 mbar pressure. This pressure is kept during the electrophoretic run.

The flow rate of DPPH• reagent and the reagent to CE capillary effluent volume ratio is controlled by hydrostatic pressure, which is set positioning the outlet vial at appropriate height. The DPPH• reservoir is kept at the same level as inlet vial, so that the injected sample is not exerted during the period, when sample vial is replaced with a buffer vial.

An on-line method to evaluate the antioxidant activity of phenolic compounds from honey bee propolis, was developed coupling CE - reaction detection. Analytes are separated according to their charge to mass ratio. The most polar components migrated last. Four phenolic compounds were separated with CE system (Fig. 3A) and after reaction with DPPH• showed radical scavenging activity (Fig. 3B).

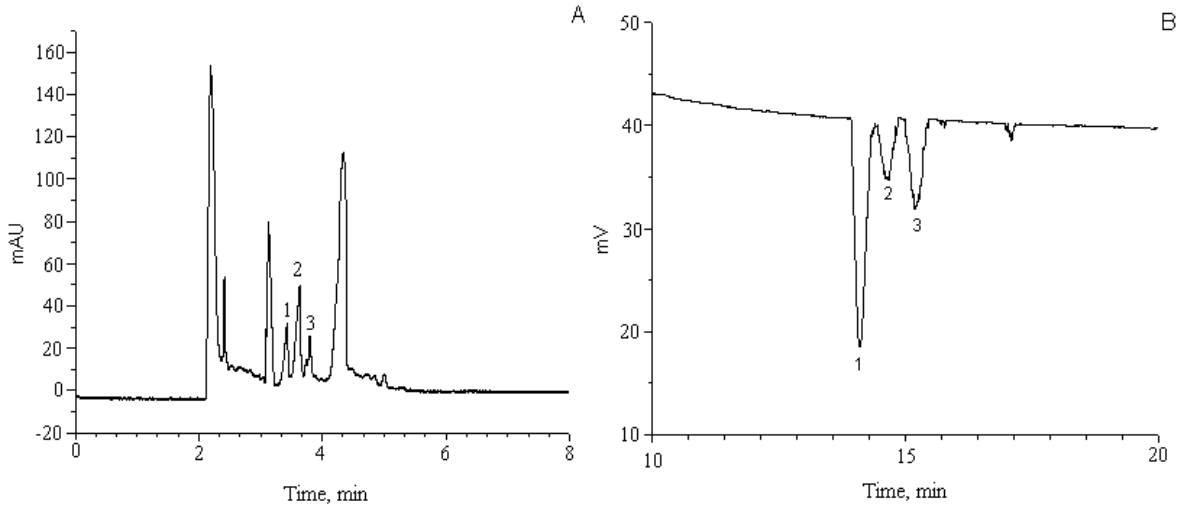

Fig. 3 (A) Electropherogram of the separated compounds of honey bees propolis aqueous extract obtained at UV wavelength 200 nm. (B) The DPPH• reaction detection signal profile at 517nm. Detector coupled on-line to the CE system. Reagent concentration 50µg/ml DPPH. Peaks1- ferulic acid; 2 - trans-p-coumaric acid; 3 - caffeic acid.

The developed interfaces allow a quantitative analysis of radical scavenging activity, which is reflected in the reaction detector peak areas. The reproducibility of the results was acceptable for the second type connection of CE to the DPPH• reaction detection (funnel type interface Fig.2 (B)). For the peak area of the identified compounds in CE electropherogram SSN was 1.63% and for reaction detector peak area of identified

compounds 1.31%, for the migration time 5.22% and 9.43% correspondingly (within the consecutive injections n=5).

Conclusion

Capillary electrophoresis coupled with direct antioxidant activity detection technique was developed and tested. Antioxidant activity was investigated by the reaction with a stable free radical DPPH•. Original funnel type interface for introduction of the free radical solution between CE and reaction detector was developed. Several phenolic acids and other phenolic compounds found in the honey bees propolis aqueous extract were separated and three phenolic acids were identified. This method was found to be suitable for the on-line monitoring of the radical scavenging effect of individual compounds in the complex mixture.

Acknowledgements
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