THESIS ON NATURAL AND EXACT SCIENCES B121

Development of Methods for the CE Analysis of Plant Phenolic Compounds and Vitamins

JELENA GORBATŠOVA

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TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Chemistry

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Supervisor: Prof. Dr. Mihkel Kaljurand, Department of Chemistry, Faculty of Science, Tallinn University of Technology

Opponents: Prof. Dr. Tõnu Püssa, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Prof. Dr. Andrei R. Timerbaev, Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, Moscow, Russia

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

Jelena Gorbatšova

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LOODUS- JA TÄPPISTEADUSED B121

Kapillaarelektroforeesil põhinevate meetodite väljatöötamine taimsete fenoolsete ühendite ja vitamiinide analüüsiks

JELENA GORBATŠOVA

TTÜ KIRJASTUS

Моей семье посвящается

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LIST OF PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals.

I. J. Gorbatšova, T. Lõugas, R. Vokk, M. Kaljurand. - Comparison of the contents of various antioxidants of sea buckthorn berries using CE - *Electrophoresis* 2007, 28, 4136–4142.

II. K. Helmja, M. Vaher, J. Gorbatšova, M. Kaljurand. - Characterization of bioactive compounds contained in vegetable of Solanaceae family by capillary electrophoresis - *Proceedings of Estonian Academy of Sciences* 2007, 56, 4, 172-186.

III. J. Mazina, J. Gorbatšova. - Sample preparation for CE-DAD analysis of the water-soluble vitamins in food products - *Procedia Chemistry* 2010, 2, 46–53.

IV. J. Gorbatšova, M. Jaanus, M. Kaljurand. - Digital Microfluidic Sampler for a Portable Capillary Electropherograph - *Analytical Chemistry* 2009, 81, 8590–8595.

THE AUTHOR'S CONTRIBUTION

I. The author performed a substantial part of the experimental work and contributed to the preparation of the manuscript.

II. The author determined the vitamins concentration in plant extracts.

III. The author was the mostly responsible for planning the experimental part and participated in the writing of the article.

IV. The author optimized the experimental setup and performed the practical work. She also wrote a part of the manuscript. The electronics and software were implemented by co-author M. Jaanus.

ABBREVIATIONS

BGE	Background electrolyte
C^4D	Contactless conductivity detector
CE	Capillary electrophoresis
DAD	Diode array detector
DMF	Digital microfluidic sampler
EWOD	Electrowetting – on - dielectric
HPLC	High performance liquid chromatography
IUB	International union of biochemistry
IUPAC	International union of pure and applied chemistry
MS	Mass spectrometry
TDP	Thiamin diphosphate
TMP	Thiamin monophosphate
TTP	Thiamin triphosphate
USP	United states pharmacopeia

INTRODUCTION

Among the compounds naturally occurring in foods, plant phenolics and vitamins represent groups of substances that in last decades have aroused keen interest among the the food scientists and nutritionists as well as food producers and pharmacological manufacturers and consumers due to their beneficial effect on human health.

Most plant phenolics are reducing agents that act like antioxidants by scavenging free radicals and protecting against oxidative reactions. This is probably the main mechanism of action explaining the biological activity of plant flavonoids, however not the only one. According to literature flavonoids may play a role in the prevention of oxidative stress-related diseases like cardiovascular diseases, cancer, inflammatory disorders, neurological degeneration (Parkinson's and Alzheimer's diseases), premature ageing, etc [1-8].

Although the human organism requirs vitamins in relatively small quantities they cannot be synthesized in the body and have to be obtained from the diet. As a result, the analysis of vitamins in foods as a natural source of these compounds becomes critical. Besides, this analysis would allow to check the labeling compliance for quality control or study analytes bioavailability and stability, among others [9-11].

The development of simultaneous multianalyte methods for the analysis of bioactive compounds, like plant phenolics and water-soluble vitamins, in complex matrixes, such as biological fluids, nonfortified and fortified foods, is extremely important. The analytical methods must overcome difficulties of low natural levels, presence of multiple metabolic forms,which are sometimes specific to the matrix. Moreover, the analytes of interest can form complexes with macromolecules, such as transfer proteins or carbohydrates. Consequently, specific extraction procedures are needed to liberate ones from the complexes and at the same time to prevent the degradation of the other compounds. Additionally, the determination of analytes in the extracts becomes a challenge in itself, since the separation technique should minimize the interference of the matrix and detector to identify minor concentrations of analytes. Generally, multianalyte methods for the analysis of pharmaceuticals and nutritional supplements (addition of synthesized analytes) are easy to develop and more difficult to work out for biological samples such as tissues and serum, or natural samples like unfortified food. Comparing the first group (pharmaceuticals, supplements) assays number to the second one (biological, natural samples) only few multianalyte methods for analysis are available[12-14].

The special attention in present study was devoted to the development of a CE analysis method for the determination of bioactive compounds present in complex matrixes, like plant and fortified food extracts, and nutritional supplements. The research was also focused on the simultaneous sampling and

concentrating of vitamin solution droplets by using digital microfluidic (DMF) sampler for capillary electropherograph.

AIMS OF THE STUDY

The main goal of the present thesis was to work out a new and improve the current analytical methodology approaches applied for capillary electrophoretic (CE) analysis of bioactive compounds. The emphasis was placed on the following:

- improving extraction and capillary electrophoretic analysis procedures for a simplified simultaneous determination of water-soluble vitamins and plant phenolic compounds found in food matrices.
- optimizing the extraction and separation approaches to the CE analysis of bioavailable water-soluble vitamins present in complex food supplements and products matrixes.
- performing the simultaneous sampling and concentrating of vitamin solution droplets using digital microfluidic (DMF) sampler for capillary electropherograph.

1 LITERATURE OVERVIEW

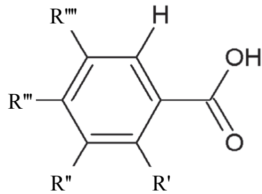
1.1 Plant phenolic compounds

"Plant phenolic" compounds[6,8,15] (with nearly 10,000 phenolic structures currently known) are considered as secondary metabolites that are synthesized by plants during normal development and in response to stress conditions such as infection, wounding, and UV radiation, among others. These compounds occur ubiquitously in plants and are a much-diversified group of phytochemicals derived from phenylalanine and tyrosine. The basic structure of plant phenolics is composed of one or more phenolic rings that are substituted with several hydroxyl groups and these are highly correlated with their strong antioxidant activity[16-20]. Plant phenolics include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. In plant, phenolics may act as phytoalexins, antifeedants, attractants for pollinators, contributors to the plant pigmentation, antioxidants, and protective agents against UV light. In food, phenolics may contribute to the bitterness, astringency, color, flavor, odor, and oxidative stability of products. In addition, the health-protecting capacity of ones and antinutritional properties of the others plant phenolics are of great importance to producers, processors and consumers[21,22].

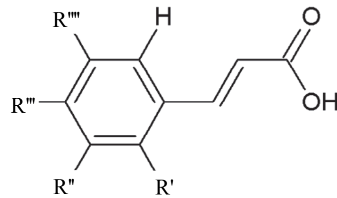
Phenolics are not uniformly distributed in plants at the tissue, cellular and subcellular levels. The insoluble (in berry juice) phenolics are the components of cell walls, while the soluble phenolics are compartmentalized within the plant cell vacuoles. At the tissue level, the outer layers of plants contain higher levels of phenolics than those located in their inner parts. Cell wall phenolics, such as lignins (the polymer of monolignol units) and hydroxycinnamic acids, are linked to various cell components These compounds contribute to the mechanical strength of cell walls, and play a regulatory role in plant growth and morphogenesis, as well as in the cell response to stress and pathogens. Ferulic and *p*-coumaric acids, the major phenolic acids, may be esterified to pectins and arabinoxylans or cross-linked to cell wall polysaccharides in the form of dimers such as dehydroferulates and truxillic acid. It has been suggested that these cross-links may play a significant role in cell–cell adhesion, serve as a site for the formation and contribute to the thermal stability of plant food texture[5,6,8,13-15,23-27].

The chemical structures of phenolic compounds under investigation are presented in Figure 1.

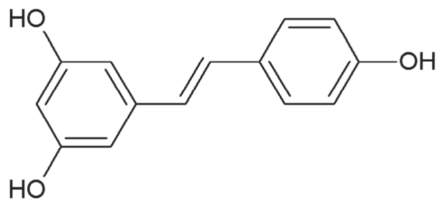
Nonflavonoids



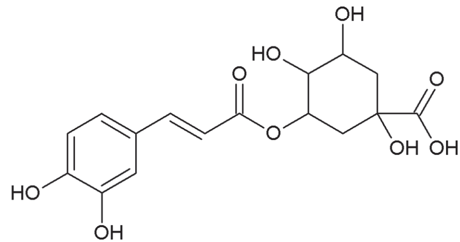
Phenolic acids
Gallic acid: R'=H;
R''=R'''=R''''=OH



Hydroxycinnamic acids
Cinnamic acid: R'=R''=R'''=R''''=H
p-Coumaric acid: R'=R''=R''''=H; R'''=OH
Caffeic acid: R'=R'''=R''''=H; R''=OH



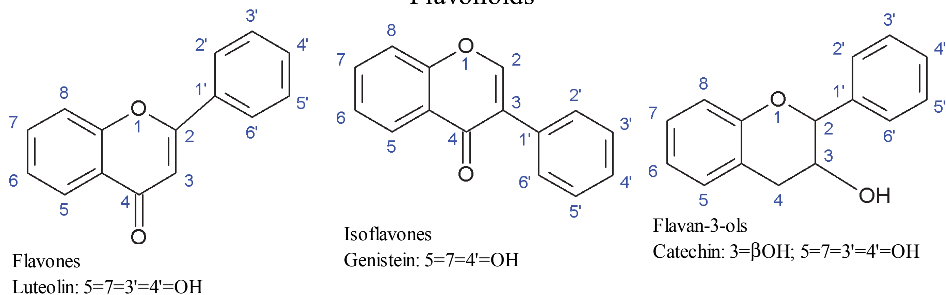
Stilbenes
Resveratrol

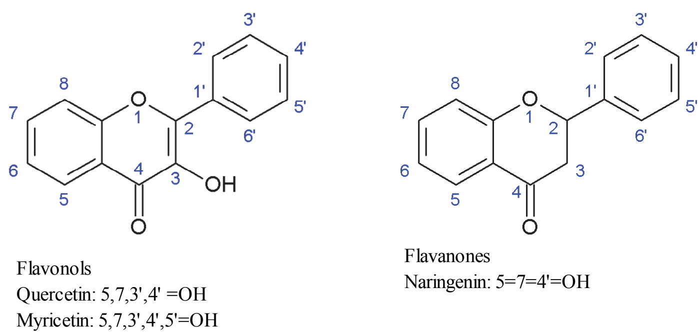


Cinnamic acid esters
Chlorogenic acid

Figure1 A. The chemical structure of plant phenolics (nonflavonoids)[6].

Flavonoids





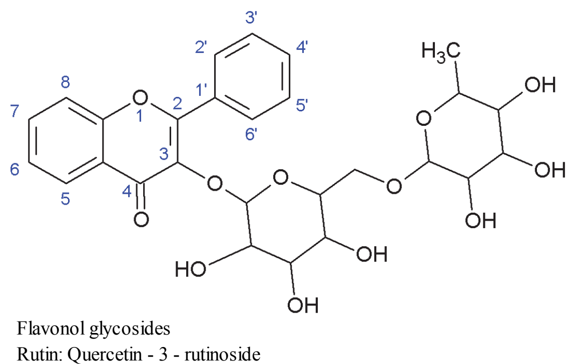


Figure1 B. The chemical structure of plant phenolics (flavonoids)[6].

1.2 Vitamins

Originally, the term “vitamine” came from the combination of a Latin word for “vita” (life) and "amine" (amine of life), as was suggested in 1912 by a Polish scientist Casimir Funk. He assumed that micronutrient food factors that prevent beriberi and perhaps other similar dietary-deficiency diseases might be chemicaly amines. However, for the class of micronutrient this assumption proved incorrect, and the word was shortened to vitamin. Today the term “vitamins” is used to characterize organic substances that are essential to the organism and have to be obtained from the diet on a regular basis because, with the exception of vitamin D, they cannot be produced within the body. Although a number of compounds were termed vitamins between the 1930s and 1950s, the nutritional science now recognizes only 13 substances, or groups of substances, as being true vitamins. Four of them are fat-soluble vitamins: A (retinol), D (calciferols), E (tocopherols) and K (phylloquinone and menaquinones); and the rest nine are water-soluble: C and the B-complex made up of vitamins B_1 (thiamin), B_2 (riboflavin), B_3 (niacin), B_6 (pyridoxine), B_{12} (cyanocobalamin), folic acid, biotin, and pantothenic acid[9-11].

In the view of the above, the qualitative and quantitative analysis of vitamins became essential for the food and pharmaceutical industries as well as for medical researchers. The food industry was mostly interested in the nutritional information about foodstuffs, while for the others it was important to clarify the protective and controlling rolls of vitamins played in human health[9,10]. Moreover, “good eating” habits became popular among the population together with an increased consumption of vitamin-supplemented and preserved foods. However, the concentration of vitamins in foods may change in time, during storage, or on processing, mostly because of possible chemical reactions. All together this led to the introduction of vitamin-labeling regulations[28 - 30]. As a result, the amount of samples to be analyzed increased drastically, as diid the need for rapid, accurate and sensitive analytical methods for simultaneous analysis. However, the investigation of such matrices is problematic due to the high variability of sample composition and number of interfering compounds and minor vitamin content[3,5,11-14].

Despite the fact that the scientific literature is replete with descriptions of new analytical methods the enthusiasm for the development of better analysis methods is only growing. For illustration, the search through the SCIENCE DIRECT (http://www.sciencedirect.com/) website shows 339 articles that have been published in the year 2000 vs 967 in 2010 (the search phrase “qualitative and quantitative analysis of vitamins”). These descriptions reveal differences all stages of analysis like sample preparation, purification and introduction; analytes separation and detection; and data interpretation.

1.2.1 Characterization of water - soluble vitamins

1.2.1.1 Ascorbic acid

According to the IUPAC–IUB Commission on Biochemical Nomenclature the trivial name for vitamin C is L-ascorbic acid ($C_6H_8O_6$) whose structure is given in Figure 2. The name "L-ascorbic acid" is also the USP Convention standard. In biological samples L-ascorbic acid plays an important roll as a strong reducing agent that forms L-dehydroascorbic acid through oxidation process[9-12]. Vitamin C is more stable in the pH range 3.0–4.5 and unstable in alkaline solutions. At neutral or alkaline pH dehydroascorbic acid undergoes a nonreversible oxidation to form a biologically inactive, straight-chained compound, 2,3-diketogulonic acid. The half-life for this breakdown is 6 min at 37 °C, 2 min at 70 °C, and less than 1 min at 100 °C. However, under acidic conditions at pH 2.5–5.5, dehydroascorbic acid is stable for several days even at 48 °C [10-12, 31-33].

1.2.1.2 Thiamin (B_1)

According to the USP Convention thiamin hydrochloride ($C_{12}H_{18}ON_4SCl_2$) is used as a reference standard. The structure of the free - base thiamin is presented in Figure 2. The thiamin molecule contains a pyrimidine ring (4′-amino-2′-methylpyrimidinyl-5′-ylmethyl) linked through methylene bridge with 3-nitrogen atom in the substituted thiazole (5-(2-hydroxyethyl)-4-methylthiazole). Depending on the pH of the solution thiamin exists in monovalent or divalent cation form. In nature only phosphorylated forms of the vitamin occur, namely: thiamin mono (TMP)-, di- (TDP), and triphosphates (TTP). Of the other water-soluble vitamins, thiamin is the least stable of them. It is highly unstable at alkaline pH (above 6) and stable at acidic pH (between 2 and 4). The thermal degradation at neutral and alkaline pH also takes place primarily due to the scission of the methylene bridge, yielding pyrimidine and thiazole.

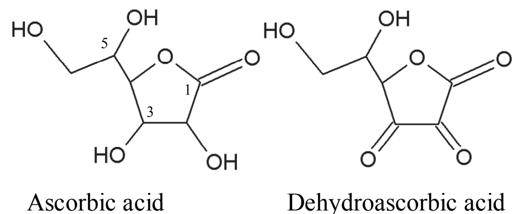
1.2.1.3 Niacin (B_3)

Both forms of niacin, namely nicotinic acid ($C_6H_5O_2N$) and nicotinamide ($C_6H_6ON_2$) have equal biological activity. However, nicotinic acid is the USP reference standard of niacin. The structures of the compounds is shown in Figure 2. The nicotinic acid molecule consists of pyridine 3-carboxylic acid, nicotinamide one of pyridine 3-carboxylic acid amide. The biological samples contain niacin mostly in forms of pyridine coenzymes

nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Among the other vitamins, niacin is the most stable to thermal processing, light, acid, alkali, or oxidation. In view of this, acid or alkaline hydrolysis can be used to free the niacin from coenzymes.

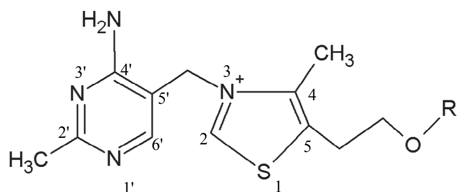
1.2.1.4 Pyridoxine (B_6)

Pyridoxine hydrochloride ($C_8H_{12}ClNO_3$) is the USP reference standard for vitamin B_6. (Figure 2) However, in biological samples other forms are distributed as well, namely pyridoxal and pyridoxamine with an aldehyde (–CHO) and aminoethyl (–CH_2NH_2) substituted at the 4-position of the pyridine ring, respectively. Since those molecules are metabolically interconvertible, they are considered biologically active equivalents. Moreover, all the above mentioned compounds can be phosphorylated by the pyridoxal kinase forming 5’-phosphates. Additionally, in nature they may exists in nature in protein and non–protein-bound forms. So, the primary role of extraction is to free such bound forms to ensure a complete analysis of the vitamin [4,11,33].

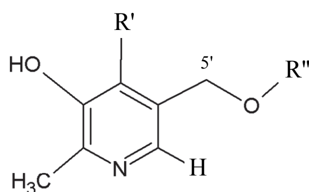


Ascorbic acid Dehydroascorbic acid

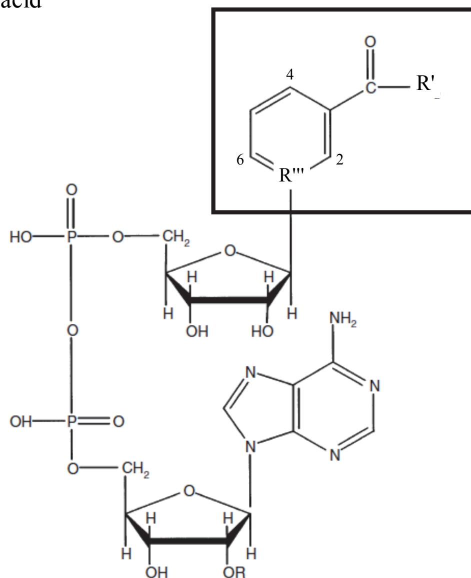
Nicotinamide: R'=NH_2; R'''=N
Nicotinic acid: R'=OH; R'''=N



Thiamin: R=H
Thiamin monophosphate: R=PO_3H_2
Thiamin diphosphate: R=$P_2O_6H_3$
Thiamin triphosphate: R=$P_3O_9H_4$



Pyridoxine: R'=CH_2OH; R''=H
Pyridoxine-5'-phosphate:
R'=CH2OH; R''=PO_3H_2
Pyridoxal: R'=CHO; R''=H
Pyridoxal-5'-phosphate:
R'=CHO; R''=PO_3H_2
Pyridoxamine: R'=CH_2NH_2; R''=H
Pyridoxamine-5'-phosphate:
R'=CH_2NH_2; R''=PO_3H_2
Pyridoxic acid: R'=COOH; R''=H
Pyridoxic acid-5'-phosphate:
R'=COOH; R''=PO_3H_2



Nicotinamide adenine dinucleotide
(NAD): R'=NH_2; R''= H; R'''=N^+
Nicotinamide adenine dinucleotide
phosphate
(NADP): R'=NH_2; R''= PO_3H_2; R'''=N^+
Nicotinic acid adenine dinucleotide
phosphate
(NAADP): R'=OH; R''= PO_3H_2; R'''=N^+

Figure 2. Structures of the analytes (water - soluble vitamins).

1.3 Methods for determination of bioactive compounds in complex matrixes.

The new multianalyte methods to be developed for a simultaneous analysis of bioactive compounds in food products and biological samples must eliminate several difficulties such as low concentration of analytes, presence of multiple metabolic forms, substation differences in chemical properties and ability to form complexes. Furthermore, different food samples vary significantly in structural constituents as well as the content of macronutrients, like proteins, oils and carbohydrates and micronutrients, such as plant phenolics and vitamins.

For natural products, the sample preparation includes the following steps: leaching[15], hydrolysis, combined multiple extraction steps involving acid protein precipitation (with perchloric acid[34,35], hydrogen chloric acid[36] or trichloroacetic acid[37]), enzymatic digestion[38] to release protein-bound sample, and centrifugation prior to the separation analysis[39]. However, enzymes are expensive and sometimes only small amounts are available. Recently a CE system combained with in-capillary enzyme reaction method (mixing, reacting, separation and detection occurs within the separation capillary) was employed allowing the use of nL amounts of enzyme[40]. Different types of novel extractions methods, like supercritical and ultrasound-assisted extractions[14,25,36] have found their application as well.

Fluorometric and spectrophotometric methods are considered rapid and sensitive techniques for measuring the total phenolic content in extracts or for antioxidative studies[23,24]. One of those is the Folin- Ciocalteu method that is not specific but detects all phenolic groups found in extracts, including those found in the extractable proteins. A disadvantage of this assay is the interference of reducing substances such as ascorbic acid with the determinations[19]. This method is based on the reduction of the phosphotungstic-phosphomolybdic reagent coupled with the oxidation of the phenolate ion forming a blue color phosphotungstic-phosphomolybdic chromophore complex of undefined structure since the chemistry behind the reaction is not clear[4].

The bioassay and microbiological methods described in the review articles by Blake[41] and Eitenmiller[11] are able to detect the smallest amounts of vitamins in serum, plasma, and food samples, however, used only for a single vitamin or vitamer Like in case of *Saccharomyces carlsbergensis* AOAC Method 961.15[42] or *Saccharomyces cerevisiae*[43] where the growth response depended on the form of pyridoxine.

1.3.1 Separation methods

For a multianalyte, simultaneous analysis of bioactive compounds in a complex matrix the traditional identification methods should be replaced by more potent ones based on the use of advanced separation techniques.

1.3.1.1 High Performance Liquid Chromatography (HPLC)

HPLC is widely used separation method for the analysis of bioactive compounds in food and plant matrices[43-48]. In recent years the identification of compounds by mass spectrometry (MS) has become more and more important resulting in a hyphenated HPLC-MS [49,50] analytical technique.

1.3.1.2 Capillary electrophoresis

While HPLC-MS has now become an almost routine technique in the analysis of bioactive compounds, CE is still a novel technique in this field. Due to their low sample consumption and phenomenal separation efficiency CE[51,52] and CE-MS[53,54] have gained in popularity among the plant extract researchers. However, much remains to be done to make CE an acceptable and routinely used technique in plant and food extract research[55-58] and erase all doubt of analysts based on the biased belief of disadvantages of the CE method (based mainly on the poor reproducibility of CE). This work attempts to contribute to that goal.

Basic principles and theory of CE

Separation by CE is based on two electrokinetic phenomena, namely electrophoresis and electroosmosis. Where electrophoresis is the movement of a charged species (q) under the influence of an electric field (E). According to the Debye-Hückel-Henry theory under steady-state conditions, two opposite forces (the electrostatic force $F_{el}=qE=qV/L$ and the friction force of a spherical ion in the surrounding media $F_{fr}= -6\pi\eta r\nu$) balance each other and a final electrophoretic velocity, ν_{ep}, is reached.

$$\nu_{ep} = \frac{qE}{6\pi\eta r} = \frac{qV}{6\pi\eta rL} = \mu_{ep}E \tag{1}$$

where $6\pi r$ describes the size of specie, η is the viscosity of a specie surrounding media, V is applied voltage and L is the length of the capillary.

Electroosmosis is the movement of a liquid inside the capillary when an electric field is applied, producing an electroosmotic flow (EOF). When silica is in contact with an aqueous solution on the inner surface of the capillary, the silanol surface groups are form. These groups may be positively charged as $SiOH_2^+$, neutral as SiOH or negatively charged as SiO^-, depending on the pH value of the surrounding electrolyte solution. Above a pH of 2 the walls of untreated fused silica capillaries are negatively charged, because the silanol groups are deprotonated. Therefore, the steady-state constant electroosmotic velocity, ν_{eof} , is reached in the liquid outside of the electrical double layer.

For theoretical approach the Helmholtz-Smoluchowski equation has been used

$$\nu_{eof} = \frac{\varepsilon \xi E}{4\pi\eta} = \mu_{eof} E \tag{2}$$

where ε is the electric permittivity of the surrounding medium, ξ is the electrokinetic potential at the surface of the charged wall and μ_{eof} is the electroosmotic mobility.

For practical applications (3) equation is used, where l corresponds to the effective lenth of the capillary and t_{eofm} migration time for a neutral marker (usually acetone, mesityl oxide or benzyl alcohol).

$$\nu_{eof} = \frac{l}{t_{eofm}} \tag{3}$$

As a concequence, the observed velocity (apparent velocity), ν_{ap}, of a specie in surrounding media under influence of the electric field, will be due to the sum of both velocities.

$$\nu_{ap} = \nu_{ep} + \nu_{eof} = (\mu_{ep} + \mu_{eof})E = \mu_{ap} E \tag{4}$$

where, μ_{ap} is apperant mobility, that could be easily calculated from the electropherogram as well (Equation 5). Using l the effective length of the capillary, and t_{an}, the migration time of the analyte.

$$\mu_{ap} = \frac{lL}{t_{an} V} \tag{5}$$

As a result, only neutral species move with the EOF. Anions move towards a positively charged anode in the opposite direction to the EOF and cations advance to cathode influence are accelerated with the EOF.

There certain strategies could be used to influence the resolution and efficiency of the separation process. As seen from the Equation (1), the electrophoretic mobility of analytes is strongly dependent on the pH (affecting

the analyte ionization) and concentration (affecting the bulk solution viscoccity) of the separation media. The direction and velocity of the electroosmotic flow, Equation (2), depend on the surface charge and buffer parameters. Consequently the easiest way to modify the EOF is by changing the ionic strength and pH of the buckground electrolyte (BGE) as well as adding the organic solvents (methanol, ethanol, acetonitril) or modifiers (e.g. micelles, cyclodextrines, neutral polymers)[14, 59-61].

2 Experimental

2.1 Reagents and Materials

2.1.1 Reagents

The purity of chemicals used was either 98% or higher. All the samples, including standard solutions, were filtered through a 0.45 μM PTFE filter before analysis.

Vitamins: Three of the vitamin standards, namely, thiamin hydrochloride (B1) (Sigma-Aldrich, Japan), pyridoxine (B6) (Sigma-Aldrich, Germany) and L-Ascorbic acid (C) (Riedel-de Haën Sigma-Aldrich Laborchemikalien Gmbh, Seelze, Germany) were obtained from Sigma Chemicals. Nicotinamide (B3), and nicotinic acid (B3) were purchased from Fluka Chemie Gmbh (Biochemika, Switzerland).

Phenolic compounds: Gallic acid (trihydroxybenzoic acid), *p*-coumaric acid (4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), chlorogenic acid (1,3,4,5-tetrahydroxycyclo-hexanecarboxylic acid 3-(3,4-dihydroxycinnamate)), *trans*-resveratrol (3,5,4'-trihydroxy-trans-stilbene), luteolin (3′,4′,5,7-tetrahydroxuflavone), genistein (4′,5,7-trihydroxyisoflavone), catechin (5,7,3′,4′-tetrahydroxyflavane), naringenin (4′,5,7-trihydroxyflavanone), quercetin (3′,4′,5,7-tetrahydroxyflavonol), myricetin (2,3′,4′,5,5′,7-pentahydroxyflavone), and rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside) were purchased from Sigma -Aldrich Chemie.

Chemicals for sample treatment procedures: Hydrochloric acid and sodium acetate were purchased from Sigma-Aldrich. Methanol and ethanol were of HPLC grade from Fluka Chemie (Switzerland). ACN was of HPLC grade from Rathburn Chemicals, Walkerburn (Scotland). Deionized water was prepared by a Milli-Q Water Purification system (Millipore, MA, USA). Takadiastase (amylase, phosphortase and protease activity) from *Aspergillus oryzae* (No 86250) was obtained from Fluka (Biochemika, Switzerland).

Background electrolytes and chemicals for CE procedures: Sodium tetraborate, phosphoric acid, sodium phosphate, boric acid, sodium dodecyl sulfate (SDS), acetic acid and sodium hydroxide were purchased from Sigma-Aldrich

Samples: Three different varieties of sea buckthorn were investigated in first publication, namely "Trofimovskaja" (TR), "Podarok Sadu" (PS), and "Avgustinka" (AV). The tomato (Solanum lycopersicum), eggplant (Solanum melongena), chilli pepper (Capsicum annuum), and potato (Solanum tuberosum) were under investigation in the second research (Publication II).

The samples used in the third study were following: natural products yeast "Veski-Mati" and beer "Saku Kuld", which are the rich source of vitamins B, food supplements enriched with synthetic vitamins "Doppelhertz® cardiovascular energy tonic" and syrup "Floradix Kindervital for Children".

2.1.2 Materials

Substrate used for fabrication of DMF chips was Epoxy single-sided copper clad laminate (copper layer 17.5 µm, Elfa Elektroonika AS). Other fabrication materials included food wrap (Lindner Haushaltsprodukte GmbH, D-51149, Köln) with estimated thickness of 10 µm, silicone oil (Valvoline Europe, a division of Ashland Inc., Netherlands), car windscreen water repellents Rain Away (Motip Dupli BV, Netherlands) and Nano Vitro (Motip Dulpi GmbH, Germany).

Materials used for coating of the outside wall of an uncoated capillary (Polymicro Technologies, Phoenix, AZ) with dimensions of 150 µm o.d. and 50 µm i.d. were Gold paint (Glanzgold NF 12% 2 g, Schjerning, Denmark) and silver paint (silver conductive paint, Electrolube, England) were applied for capillary coating.

2.2 Sample preparation

2.2.1 Sample preparation procedure for determination of the ascorbic acid content in sea buckthorn berries

5 g of frozen sea buckthorn berries were homogenized with mortar and pestle with extraction solution (0.0095 mol/L KH_2PO_4 buffer pH 3.0 with 5% methanol), and transferred to a 50 mL volumetric flask, and then 10 mL of extracts were centrifuged at 27 000 g for 5 min at 20 °C. The supernatant was filtered through a Whatman no. 1 and used for further investigation.

2.2.2 Simultaneous extraction procedure for ascorbic acid and phenolic compounds from sea buckthorn berries

10 g of frozen berries was squeezed and extracted one time during 1 h with 20 mL of a CH_3OH/H_2O (70:30) mixture and stored in the refrigerator overnight. Extracts were filtered and kept in the refrigerator at -14 °C. The extract was used only for CE analysis.

2.2.3 *Sample treatment for B-group vitamins analysis*

The classical sample treatment protocols described by Eitenmiller *et al.*[11] and [42] were modified or adapted as follows.

Dilution: Doppelhertz and Floradix were diluted twice and three times with 40 mmol/L boric acid, pH 4.5 in order to decrease viscosity. For sample treatment 10 mL of a fluid (beer, Doppelhertz or Floradix) or yeast mixture (2 g of yeast and 8 mL of water) was weighted in a 15 mL polypropylene (further PP) centrifuge tube.

Hydrochloric acid hydrolysis: 100 µL of a 37% hydrochloric acid was added to the sample in a 15 mL PP centrifuge tube. The solution in the closed tube was placed in an oven at 100 °C for 30 min. After having been allowed to cool, it was adjusted to pH 4.5 with a 2.5 mol/L sodium acetate and diluted to 15 mL by Milli-Q water. The supernatant was separated by centrifugation for 10 min at maximum speed and filtered through a 0.45 µm cellulose acetate filter. The filtrates were used for further investigation.

Enzymatic treatment: The sample mixture was adjusted to pH 4.5 with a 2.5 mol/L sodium acetate. 0.666 g of Takadiastase was added to the sample in a 15 mL PP centrifuge tube. The solution was sonicated at 37 °C for 90 min and diluted to 15 mL by Milli-Q water, then thoroughly shaken and cooled. The supernatant was separated by centrifugation for 10 min at maximum speed and filtered through a 0.45 µm cellulose acetate filter. The filtrates were used for further investigation.

Two-step extraction: 100 µL of a 37% hydrochloric acid was added to the sample mixture in a 15 mL PP centrifuge tube. The solution in the closed tube was placed in an oven at 100 °C for 30 min. After being allowed to cool, it was adjusted to pH 4.5 with a 2.5 mol/L sodium acetate. 0.666 g of Takadiastase was added to the sample in a 15 mL PP centrifuge tube. The solution was then sonicated at 37 °C for 90 min and diluted to 15 mL by Milli-Q water. Then thoroughly shaken and cooled. The supernatant was separated by centrifugation for 10 min at maximum speed and filtered through a 0.45 µm cellulose acetate filter. The filtrates were used for further investigation.

2.3 Instrumentation

2.3.1 Capillary electrophoresis

Experiments were performed using commercial and in-house built instruments.

In-house built CE system used in first research (Publication I) was equipped with high voltage power supply (Spellmann, Hauppauge, NY), UV detector (Linear 1000, Prince Technologies), and a fused-silica capillary (Polymicro Technologies, Phoenix, AZ) 70 cm (effective length 35 cm), 360 µm o.d. and 50 µm i.d.. The UV detector was coupled to a personal computer. Data acquisition was done by the software written in-house, using a LabView program (National Instruments, Austin, TX).

In the first and second publications, a commercial instrument Agilent CE System instrument equipped with the ChemStation software (Agilent Technologies, Waldbronn, Germany) with DAD was used. The recording of electropherograms was performed at various wavelengths depending on the UV absorption spectra of the analytes. The total length of the capillary (360 µm o.d. and 50 µm i.d.) was 60 cm and length to the detector was 52 cm.

2.3.2 Software

If needed, the electropherograms were edited using software tools available at the Department of Chemistry, Tallinn University of Technology. The edinting included spike and baseline removal and calculation of the peak areas. To compensate for migration time variations, (for comparising of electropherograms, and help identification of peaks electropherograms were subjected to the simple linear migration axis compression/stretching procedure which preserves the peak area value. The procedure implements a pair of peaks (first and last) on two electropherograms to be aligned. The procedure (written in-house in Matlab, Mathworks, MA, USA and Agilents ChemStation) assigned the same migration time value to the same first compound peak in all electropherograms, and similarly the same migration time value to the same last compound peak and aligned rest of the electropherograms between first and last peaks.

The analytes in complex matrices were identified by analyzing spectropherograms, a two-dimensional plot of pherograms, recorded at different wavelengths, provided by CE-DAD. Also, specific ChemStation programs were applied to the CE-DAD signal interpretation, namely *peak purity* to the confirmation of peak selectivity and *similarity* to identify analytes in the sample

solution. The latter is based on the match of peak spectra with one another and could be defined by the equations (6) [62,63].

$$\text{SIMILARITY}=1000*r^2 \tag{6}$$

where r is the correlation coefficient between two spectra as follows:

$$r=\frac{\sum_{i=l}^{i=n}\left[(A_i-A_{av})\times(B_i-B_{av})\right]}{\sqrt{\sum_{i=l}^{i=n}(A_i-A_{av})^2\times\sum_{i=l}^{i=n}(B_i-B_{av})^2}}$$

where A_i and B_i are the absorbencies measured at the first and second spectrum at the same wavelength, and A_{av} and B_{av} are the average absorbance of the first and second spectrum respectively. n is the number of data points. The method provides a measure of similarity ranging from identical (SIMILARITY=1000) to different (SIMILARITY=0)[64].

In Publication III, the experimental part in third article was performed on portable CE instrument with capacitively-coupled contactless conductivity detector (C^4D) and the digital microfluidic sampler (DMFS) for the droplets actuation. The portable CE instrument used in this work has been described by Seiman *et al.*[65]. The original portable instrument with cross sampler and C^4D was modified. The cross-sampler was removed, and the electrode array holder was placed there instead. The CE system requires power supply (Spellmann, Hauppauge, NY) capable of delivering voltages up to +25 kV. A schematic diagram of the instrument is shown in Figure 3. The C^4D was constructed according to the ideas outlined by da Silva *et al.*[66] and Zemann *et al.*[67,68]. In all experiments the default frequency was 200 kHz. .

2.3.3 Fabrication of the DMF Sampler for CE

DMF Sampler implements an electrowetting–on-dielectric phenomenon (EWOD), whereas the discrete droplets of conductive aqueous solutions could be manipulated by electrostatic forces on an array of electrodes coated with an insulating dielectric[69-73]. For the fabrication of the DMF platform, the procedure proposed by Abdelgawad and Wheeler[74] was adapted. With the use of the technology described in Publication III, a set of electrode arrays was fabricated. A simple customized clamp with spring-loaded contact pins was used to connect a control box to the electrode array. It was made from copper cladding and had a rectangular opening for exposing the electrode array (Figure 3, parts A and B). On the clamp, a piece of gold-painted capillary was fixed over the electrode array. This capillary served as a ground electrode during the droplet

actuation and CE run. The capillary dimensions were 150 µm o.d. and 8 cm length long. First, the whole capillary was painted in gold and dried at 600 °C for 3 min, then cooled at 50 °C for 30 min. Electrodes had an area of 2 mm × 2 mm, and the gaps between electrodes were estimated as 95 µm. Silicone oil was then dispensed onto the surface of the array and heated on a hot plate at 70 °C for 30 min. The food wrap was placed onto the oil-coated device using tweezers. Finally, the sampler was heated on a hot plate at 70 °C for 2 min, forming a seal between the wrap and the substrate.

Interfacing the DMF sampler into the portable CE analyzer was implemented in the following manner. The separation capillary was directed through the grounded piece of the syringe needle located vertically to the center of the array of electrodes (at a height of 2 mm above the array (Figure3, part 4). The inlet part of a the thin separation capillary (50 cm long, 150 µm o.d., and 50 µm i.d.) was first covered by gold paint and second by silver paint, leaving the end of the capillary covered only with gold to create an electrical contact with the ground (Figure 3).

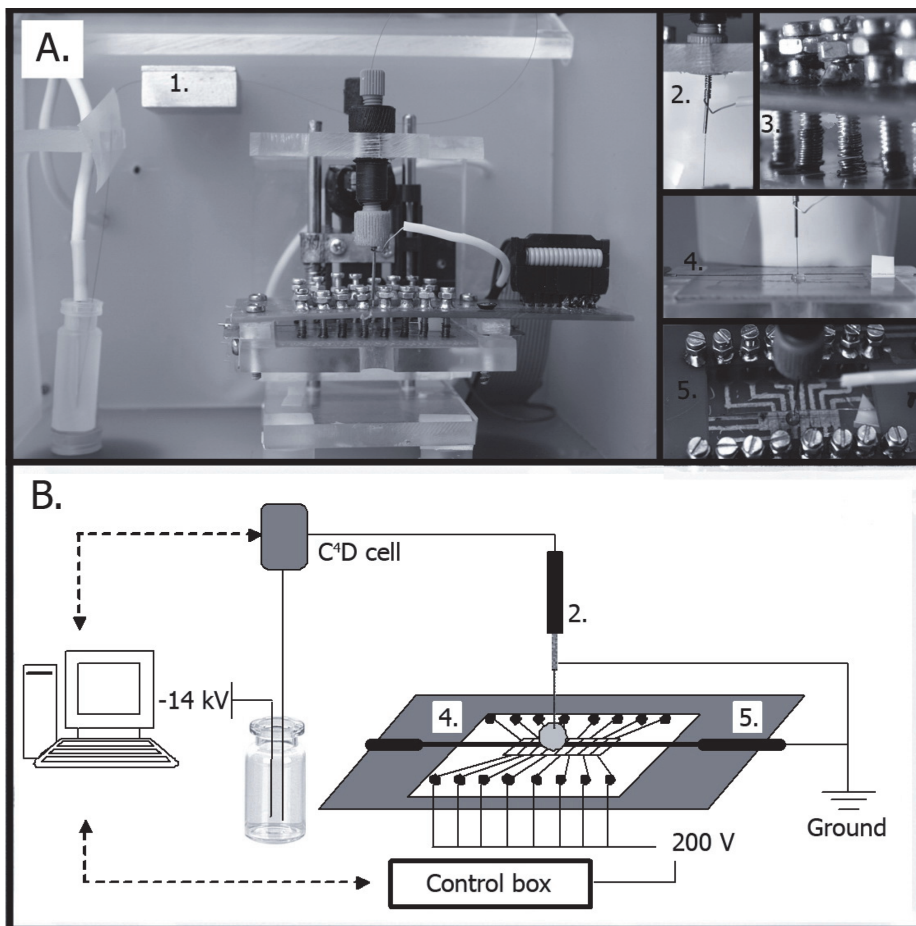


Figure 3. Interfacing the DMF sampler into the portable CE analyzer.
(A) Photograph od the portable CE analyzer with DMF sampler. (B) Instrumentation scheme: 1) capacitively coupled contactless conductivity detector (C^4D); 2) grounded piece of syringe needle with the inlet end of separation capillary; 3) spring-loaded contact pins; 4) ground electrode during droplet actuation; 5) rectangular opening for exposing the electrode array.

2.3.4 Device operation

First step - 10 µL droplets of sample and separation buffer were manually dispensed by pipette on the opposite borders of electrode array. Separation capillary was fixed in the middle of electrode array (Figure 4A). Second step - sample droplet was actuated till the separation capillary inlet end (Figure 4B). Third step - electrokinetic sampling was performed by applying -14 kV for 3 s (Figure 4C). Fourth step – sample was moved back to its initial position and buffer placed under capillary (Figure 4D). Fifth step - buffer droplet stayed under the separation capillary during the whole CE run (Figure 4E). Sixth step – after CE run was completed buffer droplet was returned to its original position (Figure 4F).

2.3.5 Liquid chromatography

For analysis of vitamin C the following protocol was used. Analysis was performed using a Gilson 321 HPLC pumps equipped with a Gilson UV/Vis-151 detector. RP separation was attained using a C-18 column Inertsil ODS-2 5 mm (250 mm id, 4.6 mm) and precolumn Inertsil ODS-2 5 mm (20 mm id, 4.6 mm). The mobile phase was 0.0475 mol/L KH_2PO_4 buffer pH 3.0 with 3% methanol and 2% ACN. The flow rate was 1.0 mL/min, and the detection wavelength was 245 nm.

2.3.6 Humidity test

For humidity tests drying with the help of a Sanyo sterilizer, MOV-112S (Sanyo Electric Biomedical, Japan) with 50 °C temperature berry weighting each 10 min till constant mass was used.

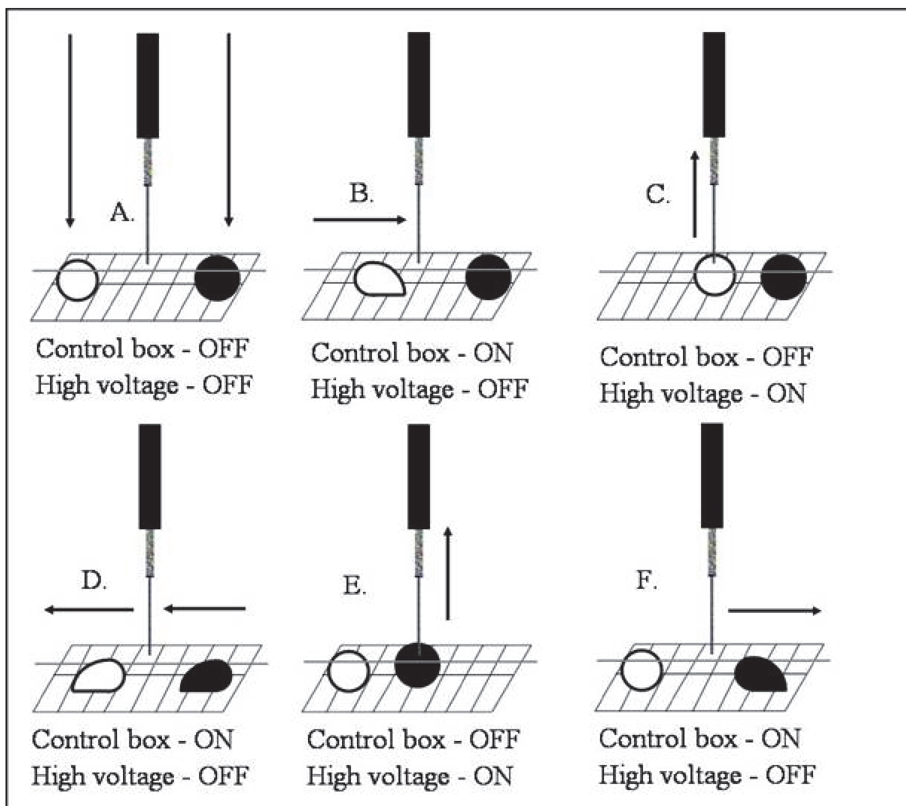


Figure 4. Droplet actuation. The directions of the liquid movements are shown by arrows, sample droplet is colored in white and buffer in black correspondingly. (A) - 10 µL droplets of sample and separation buffer were manually dispensed by pipette on the opposite borders of electrode array. (B) - Sample droplet was actuated till the separation capillary inlet end. (C) - Electrokinetic sampling was performed by applying negative 14 kV for 3 s. (D) - Sample droplet was moved back to its initial position and buffer droplet placed under capillary. (E) – Run of separation. (F) - After CE ran was completed buffer droplet was returned to its original position.

3 Results and Discussion

3.1 Optimization of the analysis of vitamin C and phenolic compounds found in sea buckthorn berries *(Publication I)*

Of special interest in sea buckthorn (*Hippophae rhamnoides*) extracts are ascorbic acid and phenolic metabolites[75], due to their potent antioxidant activity and wide range of pharmacological properties[76] including anticancer[77] and platelet aggregation inhibition activity. Plant phenolics could be extracted from such complex matrixes as berries with the help of supercritical fluid (SFE)[78], pressurised-fluid, microwave-assisted (MAE)[79] and Soxhlet extractions[80], but those techniques require high temperature and pressure conditions that easily destroy ascorbic acid. To avoid this degradation a simple solvent leaching was used. In Publication I, a description for simultaneous extraction of both phenolic compounds and ascorbic acid the methanol solution extraction for CE analysis is presented. Moreover, since methanol is not a common solvent for extracting vitamin C, the results of the latter procedure were verified against a more classical approach, an aqueous solvent extraction. Through extensive experimental trials it became obvious that 70:30 methanol/water solvent provides a good results (in terms of recovery) for both ascorbic acid and phenolic compounds.

For CE analysis the sodium tetraborate as a BGE was used at varying concentrations, from 10 to 40 mmol/L.

It follows from Figure 5 that the sea buckthorn extract is a complex mixture with tens of components present. For identification a spiking experimental technique was used (the addition of various compounds of interest to the sample solutions which results in an increase of the suspected peaks without the appearance of shoulders or split peaks). The identification by spiking was confirmed by comparison of the corresponding electropherograms of extracts and the solution of standards recorded at different wavelengths (Figure 5) and by a visual comparison of the UV spectra of peaks in electropherogram of standards with those of candidate peaks in the electropherogram of the extracts. As a result of identification ascorbic acid, myricetin, and quercetin were found in sea buckthorn extract, however, no *p*-coumaric, caffeic, or gallic acids peaks were observed. Situation with *trans*-resveratrol and catechin remained more uncertain because of the overlapping of the peaks with the peaks of the matrix and their small intensity, therefore presence/absence of *trans*-resveratrol and catechin in the sea buckthorn extract might be detected only after more careful study. Further, the data obtained were compared using the paired *t*-test[81-83]. The results are presented in Table 1.

The concentration of vitamin C was verified using more classical approach – aqueous solvent extraction and HPLC analysis. It follows from

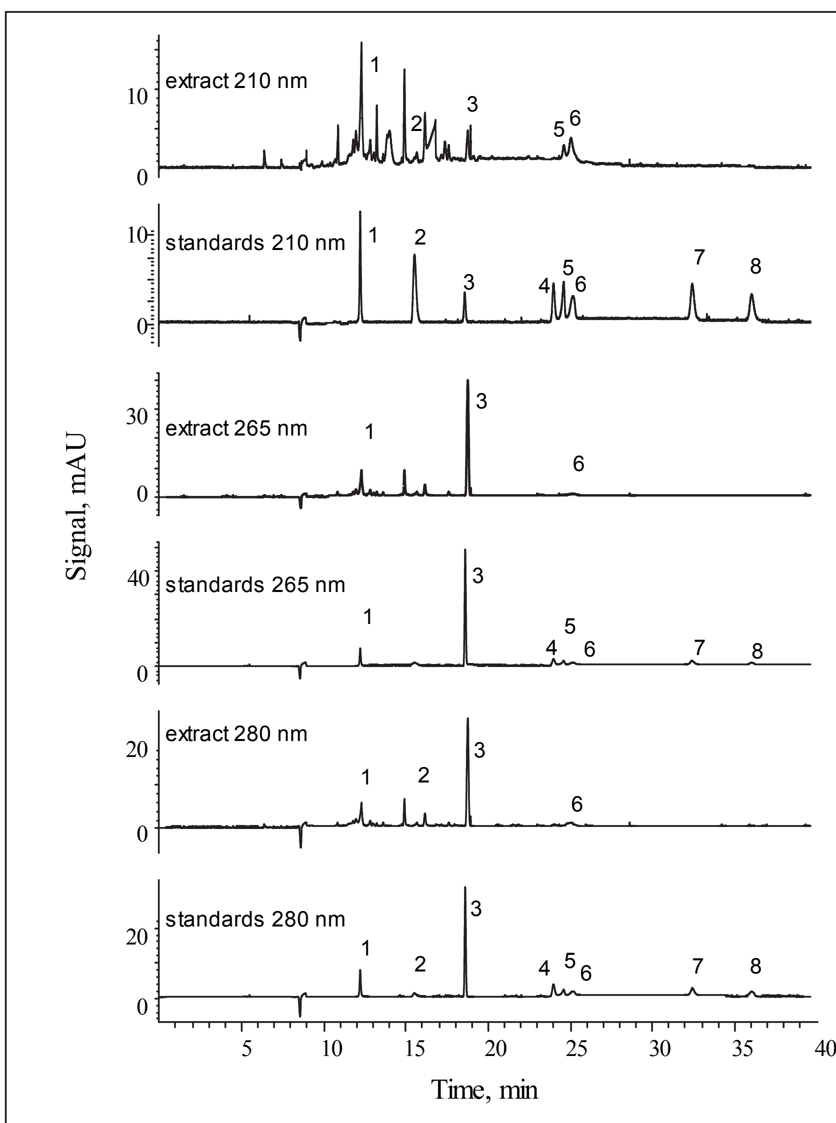


Figure 5. Electropherograms of sea buckthorn extract and mixture of standards at different detector wavelength. Standard mixture consisted of (1) *trans*-resveratrol, (2) catechin, (3) ascorbic acid, (4) *p*-coumaric acid, (5) myricetin, (6) quercetin, and (7) caffeic- and (8) gallic acids with concentrations of 100 µmol/L. Standards were added to sea buckthorn extract one by one and final result is presented in Figure 5.

Table 1 that in general the results of CE and HPLC obtained through the analysis of the same methanol solution extract correlate well. The square of the correlation coefficient is 0.98. However, CE gevies somewhat lower concentration values than HPLC. The calculated paired Student's *t*-test between 5% methanol/HPLC buffer extracts gives t=2.73 which is near to the critical t=2.77 value for the two-sided test at the 95% confidence level. The same also becomes evident from the regression line between *C3* and *C2* where the slope is different from 1 and the intercept from 0 (which can be easily be confirmed by testing the corresponding statistical hypothesis). Surprisingly, for the methanol solution extract the same test gives a lower than critical value of t equal to 0.89, but the correlation between the data is not good which could be expected since the extraction methods were different. The correlation between two different extraction methods is excellent which is confirmed by a lower than critical paired t value of 0.89 and the regression line slope and intercept values do not differ statistically from their values of 1 and 0, respectively.

It follows from this work that CE is a reasonable alternative to HPLC for the analysis of natural compounds found in different varieties of plant extracts. Differences in analyte levels of sea buckthorn varieties can be easily compared. Although the determination of the analyte concentrations by CE correlated well with HPLC determinations, the statistical analysis of the data reveals a small, statistically not very significant bias between the two methods. Origin of this fact needs to be evaluated further.

Table 1. Results of paired *t*-test and regression equations between concentration values of ascorbic acid obtained by CE and HPLC using two different extraction methods.

Extraction / analysis mode		CH_3OH/H_2O (70/30) by CE	5% CH_3OH in HPLC eluent by HPLC
CH_3OH/H_2O (70/30) by CE	Calculated *t* value*	t=0.89	t=0.89
	Regression equation[# $]	C_1 = (1.05± 0,15)C_2-(9.5±0.2)	C_3=(0,66±0.13)C_1+(232.2±0.2)
	Correlation coefficient	$R^2 = 0.922$	$R^2 = 0.866$
5% CH_3OH in HPLC eluent by CE	Calculated *t* value*	X	t=2.73
	Regression equation[# $]		C_3 =(0,77±0.05)C_2+(182.5±0.2)
	Correlation coefficient		$R^2 = 0.980$

* Critical value for two sided at 95% confidence level is $t = 2.77$.
[#$] C_1, concentration determined by CE from the methanol solution extract; C_2, concentration determined by CE from aqueous extract; C_3, concentration determined by HPLC from aqueous extract. The slope and intercept are given with their SDs.

3.2 Vitamins in the extracts of various plants (Publication II)

In Publication II, the simultaneous extraction of plant phenolics and vitamin C, as well as complexes-free forms of thiamin, nicotinamide, pyridoxine and nicotinic acid from the *Solanaceae* family plants was investigated. Since all analytes became negatively charged only at high pH, a BGE contenting 40 mM boric acid with 50 mM SDS was found to be satisfactory in terms of compromise between separation efficiency and analysis time. Peak identification was based on the comparison of the migration times of standard compounds and the extracts of vegetables. The identification was confirmed by spiking (the fortification technique) and the spectra of standard compounds and those of vegetable extract. The quantification was based on an external standard method using calibration curves (Table 2).

For the determination of ascorbic acid only fresh skin extracts of vegetables of the *Solanaceae* family could be used as the acid is easily decomposed. Figure 6 shows the vitamins determined in the skin extracts of tomato, eggplant, and chilli pepper. In the potato extract the vitamins were not detected. L-ascorbic acid was present in the fresh skin extracts of chilli pepper and tomato at a concentrations of 196 ±10 mg and 4 ± 0.2 mg, respectively. But of B-group vitamins, only a free form of pyridoxine was determined in the dried eggplant skin extract at a concentration of 609 ± 10 mg.

Table 2. The analytical parameters of vitamins determination

Analyte	Equation of calibration curve	R^2	LOD*, µmol/L	LOQ**, µmol/L
Nicotinamide	y = 0.0618 x + 5.51	0.995	8	23
Pyridoxine	y = 0.1327 x + 4.85	0.994	4	13
L-ascorbic acid***	y = 0.1037 x + 4.40	0.995	7	21
Nicotinic acid	y = 0.0905 x + 6.96	0.996	6	18
Thiamin	y = 0.1527x + 10.45	0.994	3	10

* Limit of detection (LOD)
** Limit of quantification (LOQ)
*** Measured at 265 nm

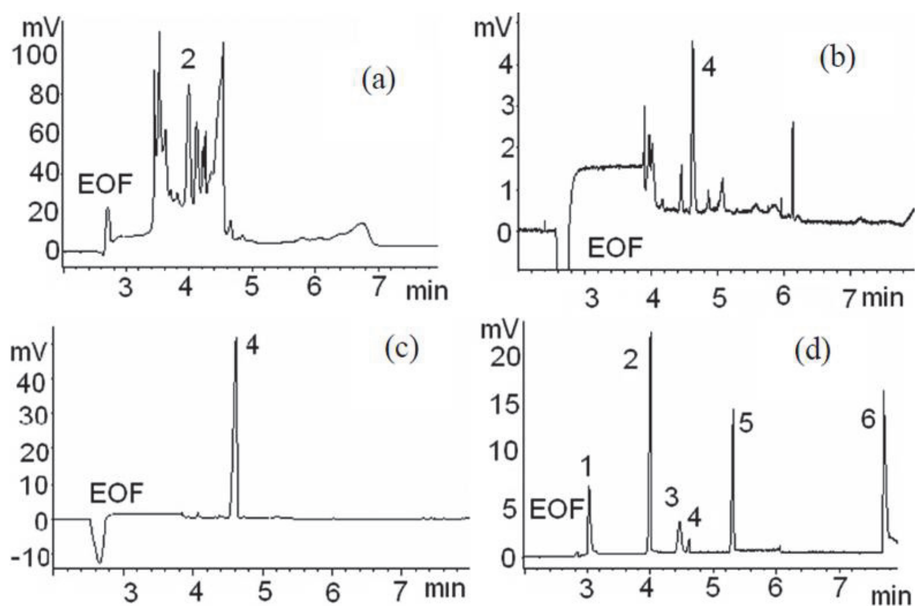


Figure 6. The electropherograms of the vitamins determined in the plant extracts: (a) – dry eggplant skin extract 210 nm; (b) – fresh tomato skin extract 265 nm; (c) – fresh chilli pepper skin extract 265 nm; (d) – a mixture of standard solutions of the vitamins 210 nm (each 250 μM): No. 1 – nicotinamide, 2 – pyridoxine, 3 – D-pantothenic acid, 4 – L-ascorbic acid, 5 – nicotinic acid, 6 – thiamin. The separation conditions: separation buffer 40 mM boric acid with 50 mM SDS (pH = 8.5), the effective length of the capillary 52 cm, applied voltage +16 kV; the injections were performed hydrodynamically 5 s.

3.3 The extraction and CE analysis of bioavailable water-soluble vitamins in food products (Publication III)

The are several difficulties that the simultaneous analysis of vitamins of the B complex in foods should cope with and which have not been encountered in the analysis of plant extracts. These include low natural levels of vitamins, presence of multiple metabolic forms, which are sometimes specific to the matrix, substantial differences in physical properties, and a potential of the vitamins to form complexes with macromolecules such as transfer proteins or, less specifically, with carbohydrates. Therefore in Publication III, both the extraction and CE procedure were optimized in order to obtain better results.

Two kinds of samples were used to investigate the possible influence of the sample matrix on the possibility of the analytes determination. Food supplements “Doppelhertz®, (a cardiovascular energy tonic) and a syrup “Floradix Kindervital for Children” were used in order to optimize the CE analysis protocols. These are complex products, considering that Doppelhertz is a mixture of 15 herbal extracts and Floradix of 14 ones. Nevertheless, the supplements are enriched with synthesized forms of vitamins, so no specific exraction procedure is needed and the sample can be directly inserted into the CE capillary. However, most of the analytes of interest in food products require extraction. For optimization of the extraction procedure of natural products that are rich with bioactive forms of vitamins B, yeast “Veski-Mati” (a solid sample) and beer “Saku Kuld” (a liquid sample) were selected. It is obvious that in comparison with supplements, the analytes concentration in yeast (“Veski-Mati”) and beer are lower and they are present in more complex molecular associations. Moreover, during the sample preparation steps proteins degradation occurs causing extract saturation with amino acids and peptides, that interfere with CE analysis.

To optimize the separation of analytes in the sample extracts four different background electrolytes (BGE) were employed in order to perform separation (Table 3). Since thiamin, pyridoxine, nicotinic acid and nicotinamide are zwitterions they have a positive charge at low pH and a negative at high pH. As for ascorbic acids, it becomes negatively charged only at higher pH.

In the case of BGE #1 only for positively charged molecules, like thiamin, pyridoxine, nicotinamide and nicotinic acid, will migrate. As follows from the data analysis only three systems, namely, phosphoric acid (BGE#1), sodium tetraborate (BGE#4) and boric acid with SDS (BGE#3) (Figure 7) were suitable for separation of analytes of interest. With the sodium phosphate (BGE#2) not all analytes were completely separated (Figure 7 pH 7). The resolution between the second and third peaks was only 0.8. Thus, BGE#2 was not used for further analysis.

Table 3 Composition of BGE for CE analysis of food supplements

BGE	Composition	pH	Comment
#1	25 mmol/L phosphoric acid	2.1	No EOF. Successfully was used for analysis of thiamin, nicotinamide, pyridoxine and nicotinic acid in unfortified food samples.
#2	25 mmol/L sodium phosphate	7.0	Poor resolution (0.8) between peaks #2 and #3
#3	40 mmol/L boric acid and 60 mmol/L SDS	8.2	Successfully used for investigation of thiamin, nicotinamide, pyridoxine, nicotinic acid, D-pantothenic acid and ascorbic acid present in fortified food samples. Also was used in Publication II.
#4	25 mmol/L sodium tetraborate	9.2	Successfully used for investigation of thiamin, nicotinamide, pyridoxine, nicotinic acid, D-pantothenic acid and ascorbic acid present in fortified food samples.

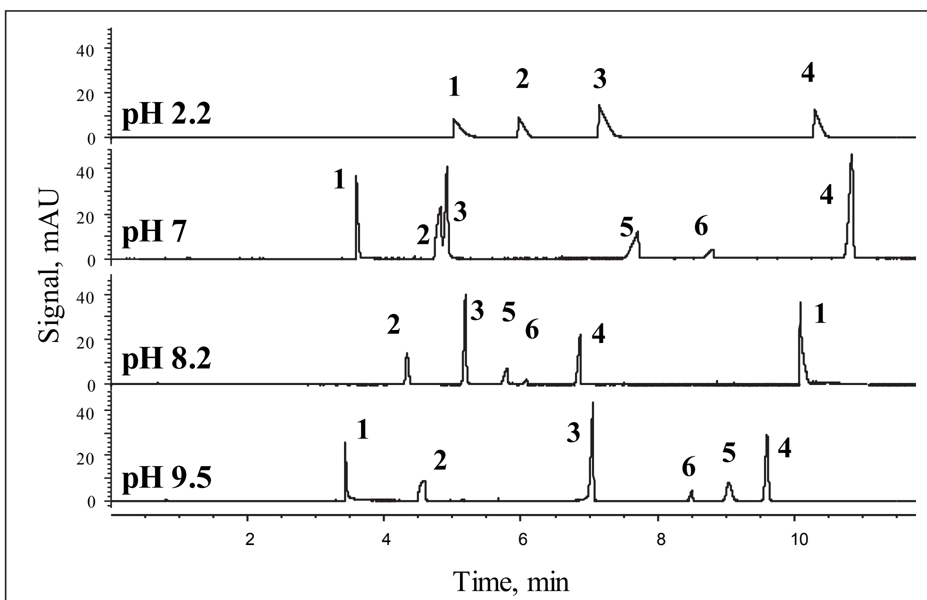


Figure 7. The influence of the background electrolyte on the separation of a standard vitamins mixture at a concentration of 100 µmol/L. Analytes: 1-thiamin, 2-nicotinamide, 3-pyridoxine, 4-nicotinic acid, 5-D-pantothenic acid and 6-ascorbic acid. CE conditions: 25°C, 20 kV, injection 50 mbar, 5 s, capillary effective 50 cm and total 58cm lengths. BGEs: pH 2.1 -25 mmol/L phosphoric acid (BGE#1); pH 7.0 - 25 mmol/L sodium phosphate (BGE#2); pH 8.2 – 40 mmol/L boric acid and 60 mmol/L SDS (BGE#3); and pH 9.5 - 25 mmol/L sodium tetraborate (BGE#4).

For the identification and quantification of vitamins in sample extracts three BGE-s (BGE#1, BGE#3 and BGE#4) and three extraction methods were tested in order elucidate the most suitable one for each of the analytes (in terms of fewer matrix interferences and large recoveries). The peaks of analytes were identified in the electropherograms by calculating the SIMILARITY function (see 2.3.2) between the spectra of the standard peak and candidate peak in the corresponding electropherograms. Figure 8 illustrates the identification procedure. Effect of different extraction procedures is demonstrated in the Figure 9. For a simplified demonstration only the thiamin part of the electropherograms is presented in detail (Figure 8 and 9).

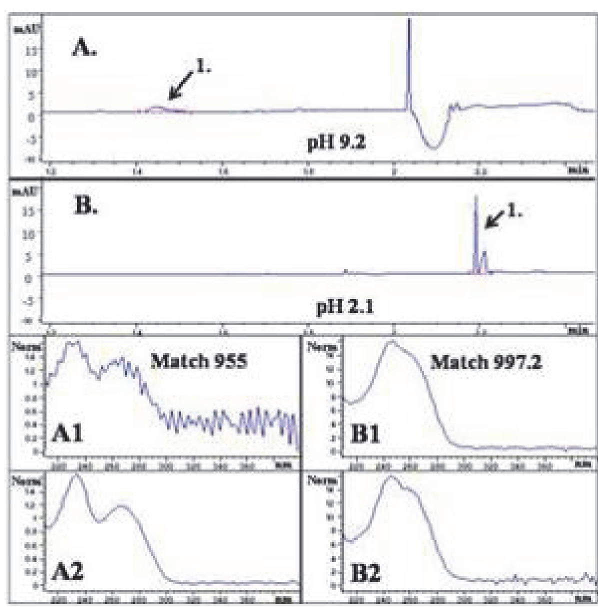


Figure 8. The BGE systems used to determine thiamin (1.) in the yeast extract. CE system: A - 25 mmol/L sodium tetraborate, pH 9.2, A1 - the spectrum of absorbance of thiamin in the sample; A2 – the spectrum of absorbance of thiamin in the standard mixture; B - 25 mmol/L phosphoric acid, pH 2.1, B1 – the spectrum of absorbance of thiamin in the sample, B2 – the spectrum of absorbance of thiamin in the standard mixture. CE conditions: 25 °C, 20 kV, injection 50 mbar, 5 s, capillary effective 50 cm and total lengths 58 cm. Similarities (Match) between the peaks can also be seen.

A comparison of the proposed extraction procedures demonstrated that neither hydrochloric (Figure 9A) nor enzymatic (Figure 9B) hydrolysis gave positive results independently, yet bening combained into the two-step treatment (Figure 9C) produce it.

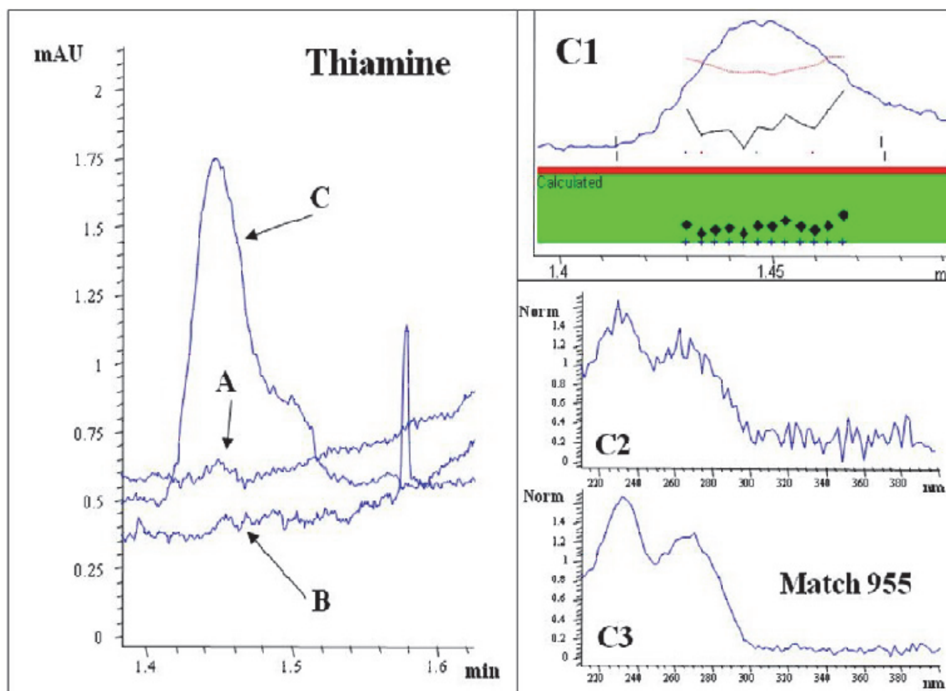


Figure 9. A comparison of the electropherograms of thiamin in the yeast extracts prepared using different sample treatment procedures (for acetic, enzymatic and two-step extraction procedures see 2.2.3). CE conditions: 25 °C, 20 kV, injection 50 mbar, 5 s, capillary effective 50 cm and total 58cm lengths, 232 nm. A – acid hydrolysis, B – enzymatic treatment, C – two-step extraction (the last extraction procedure): C1 – the purity factor of thiamin, C2 – the thiamin absorbance spectra in sample, C3 – the thiamin absorbance spectra in standard mixture. Similarities (Match) between the peaks are also presented.

It follows from Table 4 that for vitamin determination in food supplement samples Floradix and Doppelthertz, the BGE #3 and #4 suit well. Notwithstanding, for analysis of natural food extracts, in which only four vitamins of interest (thiamin, pyridoxine, nicotinamide and nicotinic acid), could be found, since ascorbic acid degrades in high temperatures, the BGE#1 (pH=2.1) was chosen as a separation medium to avoid matrix interferences, shorten the analysis time and use the stacking effect occurring at this pH.

Table 4 The concentration of analytes with 95% uncertainty (n=3)

Vitamin	Yeast	Beer	Doppel-hertz	Floradix
BGE	BGE #1	BGE #1	BGE #3,4	BGE #3,4
Extraction mode*	Two-step extraction	Two-step extraction	Dilution	Dilution
	µg/g	µg/mL	µg/mL	µg/mL
Thiamin	143 ± 0.6	ND	484 ± 37	ND
Pyridoxine	82 ± 4	ND	400 ± 43	410 ± 60
Nicotinamide	115 ± 10	320 ± 40	1400 ± 220	4820 ± 370
Nicotinic acid	ND	ND	96 ± 5	55 ± 6
Ascorbic acid	ND	ND	ND	18900 ± 2700

*Chapter 2.2.3
ND – not detected

3.4 Simultaneous sampling and concentrating of droplets of vitamin solutions using DMF sampler (Publication IV)

When a vitamin sample is extracted from a plant or food product matrix the amount of extract is not usually an issue. Unless the sample has been treated with enzymes, which makes the procedure expensive, then the ability of the CE to analyze small amounts of samples can be advantageously made use of. Nevertheless, it requires the development of proper methods that would allow the introduction and analysis of µL-volume samples. In the fourth publication the proof of the principle of introduction of a limited amount of sample is demonstrated by implementing a DMF sampler mounted on the portable electrophoretic instrument equipped with a C^4D detector. Such an experimental set-up was used to monitor the sample concentration process via evaporation of a µL volume droplet of a vitamin sample on the surface of the digital microfluidic platform. When the concentration process was finished, the droplet of the sample was transported to the inlet of the CE column, using the EWOD actuation of the droplet.

Acetic acid is known as a volatile background electrolyte suitable for use with C^4D [84,85]. The complete volatility of BGE was important for this work because it meant that there was no danger of depositing non-volatile BGE components on the food wrap surface which covers the DMF electrodes array. For experiments a 250 mmol/L acetic acid (pH 2.5) was used as a BGE. Consequently, the analytes mobility in the separation capillary was achieved mostly by electrophoresis since the EOF is either too slow or non-existent at this pH. Typical electropherograms of three analytes: thiamin, pyridoxine and nicotinamide (40 µmol/L of each in Milli-Q water), are shown in Figure 10.

In the initial experiments, the droplet sampler was evaluated for reproducibility. For each new run a new sample and buffer droplets were pipetted to the electrodes array. The performance data of six parallel runs for one of the sample concentrations (40 µmol/L thiamin and 80 µmol/L pyridoxine) are listed in Table 5. When evaluated in replicate trials, the sampler was characterized by good average retention time reproducibility (3% of RSD; 1% RSD for relative migration time) and peak area variation (8% RSD).

For evaporation process monitoring the following experiment was performed. The droplet containing the sample was delivered on one side of the electrode array and the buffer droplet on the opposite side, with the separation capillary located in the middle. During the run, the sample and buffer droplets were alternately transported under the capillary and the electropherograms were recorded for each sample/buffer droplet passage. The detector signal is shown in Figure 5 with zoomed in electropherograms

Table 5. Reproducibility data for DMF sampler

	Thiamin		Pyridoxine		Relative data	
	t_T, s*	S_T**	t_P, s	S_P	t_T/t_P	S_P/S_T
run#1	484	6.04	854	7.93	0.57	1.31
run#2	491	6.80	865	9.22	0.57	1.36
run#3	489	5.87	873	8.54	0.56	1.46
run#4	476	6.58	851	8.54	0.56	1.30
run#5	488	6.19	873	8.58	0.56	1.21
run#6	521	7.09	950	7.48	0.55	1.17
Mean	**490**	**6.43**	**877**	**8.27**	**0.56**	**1.3**
SD	15	0.47	37	0.60	0.01	0.1
%	3.1	7.3	4.2	7.2	1.8	7.7

*t – migration time
**S – peak area

It was then possible to record five electropherograms from the droplet, which was initially 10 µL in size until it evaporated to the size unsuitable for further actuation. The rate of evaporation of a spherical droplet of a pure solvent in still air of constant temperature with an initial radius r_0 and mass m is given by the "d^2-law" derived from the boundary-layer theory of a shrinking droplet . This means that the volume of a droplet, $V(t)$, decreases in time t as a 3/2 power, which in turn indicates that the sample in the droplet concentrates as power 3/2 in time according to the equation below: where $c(t)$ is the concentration at time t, and c_0 is the initial concentration and β accounts for the evaporation process.

$$c(t) = \frac{m}{V(t)} = \frac{3m}{4\pi(r_o\sqrt{(1-\beta t)})^3} = \frac{c_o}{(\sqrt{(1-\beta t)})^3}$$

The least squares fitting of the experimental data to the equation results in an excellent confirmation of this law (Figure 11). Using the Excel Solver, the following values for the parameter β can be calculated: $\beta = (1.04 \pm 0.01)10^{-2}$ min^{-1}, $\beta = (1.14 \pm 0.02)10^{-2}$ min^{-1} and $\beta = (1.19 \pm 0.03)10^{-2}$ min^{-1} for nicotinamide, thiamin and pyridoxine correspondingly. Uncertainties for β were calculated according to the procedure. The β parameters are close but still statistically different. The smallness of the uncertainties (RSD=1÷3%) in β values are another indication of a good performance of the sampler, as well as the precision of the "d^2-law".

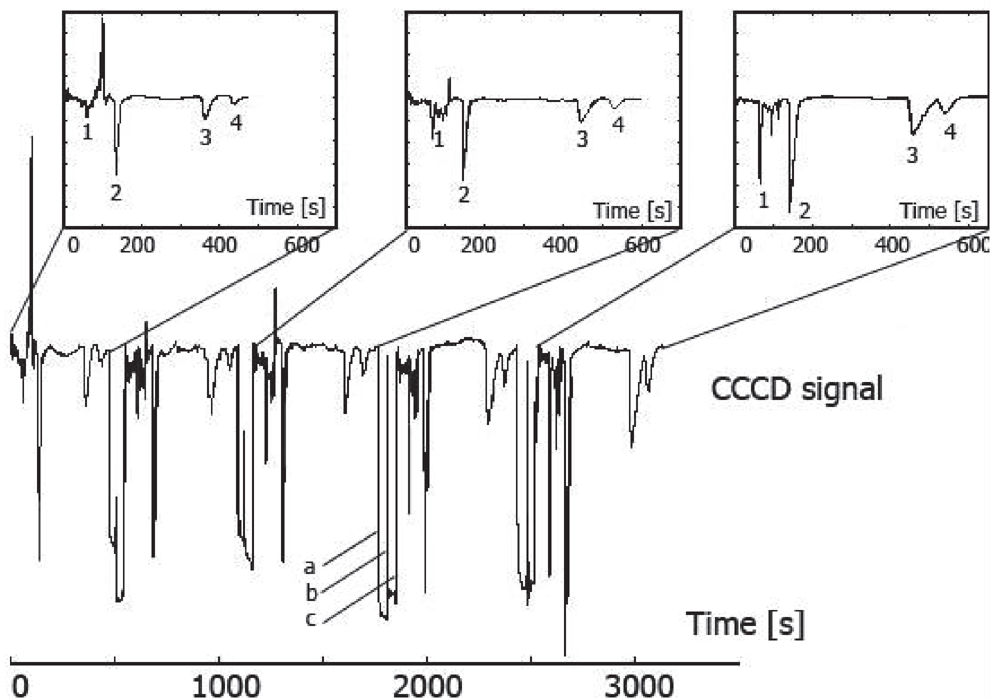


Figure 10. Droplet evaporation monitoring. Peaks 1 – unknown, 2 – thiamin, 3 – pyridoxine and 4 – nicotinamide. Events: a- HV-off, b – HV "on" for sampling, c -– HV "on" for separation. Buffer 250 mmol/L acetic acid. Separation voltage -14 kV.

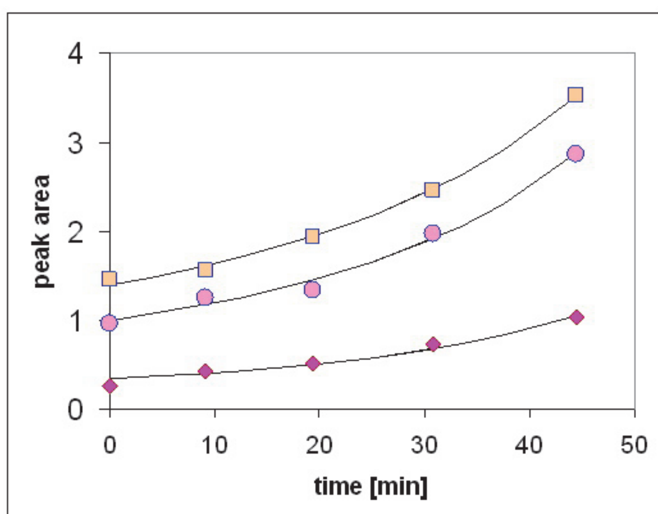


Figure 11. Sample concentration kinetics during evaporation of the droplet. Squares - thiamin, rings - pyrodixine, diamons - nicotinamide.

On the one hand, the evaporation of the buffer droplet during the CE run can then be considered as a sort of programming of the buffer concentration during the analysis, which may be beneficial in some cases. On the other hand, if monitoring is of interest then reasonable results can be obtained if a new buffer droplets would be dispensed each time in those cases when the interpretation of the electropherograms is difficult. An advantage of the evaporation phenomenon in open DMF systems is the concentrating of the sample in time. As demonstrated in this work, a threefold concentration of the sample occurs during a one-hour experiment. The appearance of small amounts of new products might become possible, which could otherwise remain unnoticed other than in stationary systems.

The method of interfacing the DMF sampler to the separation channel reported in this paper overlaps somewhat with what was reported by Abdelgawad et al[86]. They implemented a microchip with a “cross” layout of sample and buffer channels and the sampling from the droplet to the sample channel of the microchip was preformed. In such design a only very small fraction of sample is used for separation. In our design the sampling from the droplet is done directly to the separation capillary. This allows using much large (in term of micro- and nanofluidic) amounts of sample for separation. Although not demonstrated here the approach used in Publication IV might easily allow the use of concentration methods well known in CE like field amplified sample stacking which reduces the demands to the detector sensitivity even more. Moreover, such experimental arrangement allows easy monitoring of various processes occurring in the droplet as demonstrated here for the case of droplet evaporation. On the other hand, an important advantage of DMF samplers could be computer controlled preprocessing of analytes. Not set as aim in this work the robust DMF sampler design proposed in this paper is rather flexible and replacement of customized (for sample preprocessing) electrodes plate and software should be simple and rapid.

4 Conclusions

The main goal of the present thesis was to elaborate suitable and rapid analytical methods for the isolation, extraction, separation and determination of plant phenolics and water-soluble vitamins in complex matricies like nutritional supplements and unfortified food samples by using CE-based methods. It was demonstrated that in many cases CE is a reasonable alternative to HPLC for the analysis of compounds found in different varieties of plant extracts. The results of the study can be summarized as follows:

- A simple and rapid CE method for a simultaneous analysis of ascorbic acid and plant phenolics contents in sea buckthorn extracts was developed for a further possible routine control of their quantitative level. The developed method was successfully tested on six varieties of sea buckthorn plants obtained from local (Estonian) plantations.
- To confirm and demonstrate that CE is a cheap and convenient alternative to more popular HPLC, the results of ascorbic acid content measurements (in sea buckthorn extracts) were compared and validated against HPLC determinations. The analyte concentrations established by CE correlated well with those of HPLC determinations. However, the statistical analysis of the data revealed a small, yet minor discrepancy between the two methods. Origin of this fact needs to be evaluated further.
- The results of analysis revealed that much to the disadvantage of local producers and processers Estonian sea buckthorn varieties lack many important antioxidants. Nevertheless, ascorbic acid was found which level varied significantly from variety to variety as due to different ripening conditions in different years as well as different farming practices used by different producers.
- It was demonstrated that the composition of plant phenolics (phenolic acids, flavonols, flavones) and vitamins of the skin extracts of the plants of the *Solanaceae* family (tomato, eggplant, chilli pepper and potato skin) can easily be analysed by CE. The procedure for a simultaneous extraction of plant phenolics and vitamins found in free form in plants was developed. The conditions of the separation of water-soluble B complex vitamins, such as thiamin (B_1), nicotinamide and nicotinic acid (B_3), and pyridoxine (B_6), and vitamin C were optimized. The presence of several phenolic compounds in the extracts was established. Of vitamins, pyridoxine was present only in the dried eggplant skin extract, but L-ascorbic acid was present in fresh tomato and chilli pepper skin extracts.
- It was demonstrated in the determination of the vitamins the B complex (thiamin (B_1), nicotinamide and nicotinic acid (B_3), and pyridoxine (B_6)) found in fortified (“Doppelhertz®, (a cardiovascular energy tonic) and syrup

"Floradix Kindervital for Children") and unfortied (yeast "Veski-Mati" and beer "Saku Kuld") food samples that sample preparation is an key step in the analysis of vitamins. The ability of a two-step extraction procedure (acid hydrolysis followed by enzymatic treatment) to elucidate water - soluble vitamins from the vitamin rich samples was demonstrated. Problems with matrix interferences were eliminated by a proper choice of separation BGEs.

- A digital microfluidic technology-based sampler, for processing samples containing very low quantities of vitamins was developed and tested. It follows from this thesis that DMF platforms can easily interfaced to a portable CE instrument which uses contactless conductivity detection. The device shows reasonable performance in terms of reproducibility. The ability of such a system for computer controlled sampling from droplets was demonstrated. To pursue "the spirit of low cost digital microfluidics" it was also demonstrated also that the droplets actuation of can be achieved using an electrode system prepared from the copper substrate of a common printed circuit, coated only with food wrap.
- The preparation of open DMF devices from common consumer products is a simple and robust procedure. This means that the concept of low-cost, rapid prototyping of DMF devices opens interesting opportunities for bioanalysis. In this dissertation the DMF sampler was demonstrated to be capable of monitoring analytes (thiamin, nicotinamide and pyridoxine) concentration in the droplet during its evaporation. Thus, it follows that the combination of DMF sampling with a portable CE analyzer could be an important step toward fully integrating in-line sample processing and separation in bioanalytical chemistry.
- To be addressed in further studies some encountered problems in the performance of the DMF sampler can be listed: droplet evaporation, the need for volatile buffers, and resulting possible low efficiencies.

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ABSTRACT

Nowadays, the qualitative and quantitative analysis of the compounds naturally occurring in foods, including plant phenolics and vitamins has aroused keen interest among the food scientists and nutritionists as well as food producers and pharmaceutical manufacturers, and consumers due to the possible beneficial effects on human health. Consequently, the development of simultaneous multianalyte analysis that is rapid, accurate and sensitive has become important. However, the content investigations in natural products is a challenge-in-itself due to the difficulties the analytical methods must overcome, namely, low natural levels and presence of multiple metabolic forms of analytes, high variability of sample composition and number of interfering compounds.

This thesis describes the development of methods for a capillary electrophoretic analysis of bioactive compounds - plant phenolic compounds and water-soluble vitamins. Special attention has been devoted to the optimization of following procedures, sample treatment and concentration as well as analytes separation and data analysis.

In this study, the possibility of a simultaneous CE analysis of plant phenolics and water-soluble vitamins found in representatives of *Hippophae*, *Solanum* and *Capsicum* families in free form was explored. The work also included the analysis of B_1, B_3 and B_6 vitamins in fortified and unfortified foods, where analytes are present in multiple metabolic forms or in the form of complexes with proteins or carbohydrates.

In the last section of the paper, with a view to investigating the sample concentration process of μL volume droplets, a Digital MicroFluidic sampler mounted on the portable electrophoretic instrument equipped with C^4D detector was developed. The sample and BGE droplets were placed on the surface of the digital microfluidic platform and transported using the Electro-Wetting-On-Dielectric phenomenon in succession under the CE capillary inlet end allowing the capillary to be immersed into the sample/buffer droplet The CE separation was performed by applying a high voltage between the (grounded) buffer droplet and CE outlet reservoir.

KOKKUVÕTE

Tänapäeval pakub toiduainetes leiduvate komponentide, sealhulgas taimsete fenoolide ja vitamiinide, kvalitatiivne ning kvantitatiivne analüüs üha suuremat huvi toitumisspetsialistidele ja –teadlastele, ravimitootjatele ning ka toidutootjatele ja tarbijatele just nende ainete võimaliku mõju tõttu inimese tervisele.

Tervisliku toitumise trendidest tulenevalt on tekkinud vajadus kiirete, täpsete ja tundlike analüüside väljatöötamiseks, mis võimaldaks mitme analüüdi samaaegset uurimist.

Loodustoodete koostisosade uurimine on omaette väljakutse, mis tähendab olemasolevate analüütiliste meetodite tõsist proovilepanekut ja mitmete takistavate asjaolude ületamist. Põhilisteks proovikivideks on töötamine madala kontsentratsiooniga analüütidega, analüüdi mitme metaboolse vormi olemasolu, segu koostisosade suur varieeruvus looduslikus proovis ning lisaks mitmete komponentide võimalikud omavahelised interaktsioonid.

Antud töö kirjeldab meetodite väljatöötamist bioaktiivsete ainete-taimsete fenoolide ja vees lahustuvate vitamiinide elektroforeetiliseks analüüsiks. Erilist tähelepanu on pööratud protseduuride optimiseerimisele, proovi käsitlemisele, analüütide eraldamisele ja tulemuste (andmete) analüüsile.

Teostatud töö eesmärgiks oli uurida perekondade *Hippophae*, *Solanum*, *Capsicum* esindajate leiduvate taimsete fenoolide ja vees lahustuvate vitamiinide (vabas vormis) üheaegse analüüsimise võimalusi, kasutades kapillaarelektroforeesi (KE) meetodit. Uurimus jätkus B_1, B_3 ja B_6 vitamiinidega rikastatud ja rikastamata toitude analüüsiga, kus analüüdid esinevad mitmes metaboolses vormis või on kompleksis valkude või süsivesikutega.

Viimases osas töötati välja digitaalsel mikrofluidikal baseeruv kontaktivaba juhtivusdetektoriga KE sisendseade, mis on ette nähtud väikeste proovi koguste (µL suurusjärgus tilkade) analüüsimiseks. Sellised proovi kogused on aktuaalsed bioanalüütilistes uuringutes. Digitaalsele mikrofluidika plaadile paigutatud proovi ja taustelektrolüüdi tilkade liikumine saavutati dielektrikuelektromärgamise fenomeniga, mille abil suunati tilgad järjestikku kapillaari sisend-otsa vahetusse lähedusse, võimaldades paigutada kapillaari ots otse tilga sisse ja sisestada kordamööda analüüt ja puhver. Järgnev KE lahutamine saavutati kõrgpinge rakendamisel (maandatud) taustelektrolüüdi tilga ja väljund-anuma vahele.

Original publications

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Jelena Gorbatsova[1]
Tiina Lõugas[2]
Raivo Vokk[2]
Mihkel Kaljurand[1]

[1]Department of Science, Tallinn University of Technology, Tallinn, Estonia
[2]Department of Food Processing, Tallinn University of Technology, Tallinn, Estonia

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Research Article

Comparison of the contents of various antioxidants of sea buckthorn berries using CE

The increased interest in sea buckthorn (*Hippophae rhamnoides* L.) made it possible to investigate the antioxidant content in it. To address this issue, the presence of following antioxidant compounds were analyzed: *trans*-resveratrol, catechin, myricetin, quercetin, *p*-coumaric acid, caffeic acid, L-ascorbic acid (AA), and gallic acid (linear range of 50–150 μmol/L) in six different varieties of sea buckthorn berries extracts (sea buckthorn varieties: "Trofimovskaja (TR)," "Podarok Sadu (PS)," and "Avgustinka (AV),") received from two local Estonian companies. *Trans*-Resveratrol, catechin, AA, myricetin, and quercetin were found in extracts of sea buckthorn. Moreover, AA, myricetin, and quercetin contents were quantified. The biggest average AA content was found in TR (740 mg/100 g of dried berries, respectively). Furthermore, the same varieties gave the biggest quercetin content 116 mg/100 g of dried berries, respectively. For analysis, CZE was used and the results were partly validated by HPLC. Statistically no big differences in levels of antioxidants were consistently found in different varieties of sea buckthorn extracts investigated in this work.

Keywords:
Antioxidants / CE / HPLC / Phenolics / Sea buckthorn DOI 10.1002/elps.200700362

1 Introduction

Sea buckthorn (*Hippophae rhamnoides* L.) is a hardy bush, which belongs to the Elaeagnaceae family and naturally distributed over Asia and Europe [1, 2]. Various parts of sea buckthorn, especially berries, were used in traditional medicines, mainly in Far East and Middle Asia [2, 3]. The berries have been used as raw materials for foods and medicines for centuries in China and Russia. Recently, the nutritional importance of the berries has been increased in North America and Europe. Sea buckthorn berry products are among popular foods in the United States, Canada, Finland, Germany, and some other European countries. Especially after restructuring of agriculture in post-Soviet countries sea buckthorn is considered as a prospective alternative to cultivate in those countries.

Industrial utilizations and having different biological activities of the berries and other parts of sea buckthorn caused to increase the necessity of compositional information for selecting the best raw materials for food and drug industries. Sea buckthorn comprises of a series of chemical compounds including carotenoids, tocopherols, sterols, flavonoids, lipids, L-ascorbic acid (AA), tannins, *etc*. These compounds are of interest not only from the chemical point of view, but also because many of them possess biological and therapeutic activity including antitumoral, hepatoprotective, radioprotective, and immunomodulatory properties [4, 5]. Of special interest in sea buckthorn extracts are AA and phenolic metabolites due to their potent antioxidant activity and wide range of pharmacological properties including anticancer and platelet aggregation inhibition activity [6]. Phenolic metabolites are common constituents of fruits and vegetables [7] that function in the defense against herbivorous insects [5, 8].

When the composition of sea buckthorn is widely studied by different methods, however, there is very little information on the impact various cultural practices have on the production of bioactive metabolites in plants and the effect of postharvesting processing procedures on their levels. Also variation of the bioactive compounds between different sea buckthorn varieties is of interest to select appropriate variants to be introduced into a local agricultural practice. Given that increasing evidence indicates a role for plant phenolics in human health, efforts need to be directed in understanding the relationships between cultivating and postharvesting processing practices, and phenolics levels in crops.

Correspondence: Jelena Gorbatsova, Department of Science, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia
E-mail: jgorbatsova@yahoo.com
Fax: +372-620-4325

Abbreviations: AA, L-ascorbic acid; **AV,** Avgustinka; **PS,** Podarok sadu; **TR,** Trofimovskaja

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This in turn has necessitated the development of new methods for the analysis and quantitative measurement of bioactive components of sea buckthorn. The methods for the determination of chemical compounds of sea buckthorn include thin layer chromatographic and gas chromatographic separation [9], HPLC [10, 11], and HPLC-MS [12]. Chromatographic techniques used for the quantization of chemical compounds in plant material have been exhaustively reviewed in the recent paper by Guliyev *et al*. [13]. Compared to chromatography CE is more efficient in terms of analysis speed with sample volumes in the nanoliter range. There is an extensive literature concerning the detection and quantification of bioactive compounds like flavonoids by CE (see recent publications [14–22]). Recently CE was proposed for the analysis of sea buckthorn extracts [23–30]. Despite that, to date a few studies have investigated the variations of different varieties of sea buckthorn on the levels of antioxidative metabolites.

In the present study, we describe the development of a simple and rapid capillary electrophoretic method for the simultaneous analysis of AA and polyphenols in sea buckthorn extracts for the further possible routine control of their quantitative level. The developed method was applied to six varieties of sea buckthorn plants obtained from local (Estonian) plantations. The aim of this study was to optimize the CZE method for the analysis of flavonoids in sea buckthorn extracts and to investigate the effects of extract preparation, its storage conditions, and raw material collection on the analyte concentrations in the extracts. To address this issue, the concentration of AA and various polyphenols were measured in six different varieties of sea buckthorn using CE. The study aims to demonstrate that CE is a cheap and convenient alternative to more popular HPLC. To confirm this, the results of AA measurements were compared and validated against HPLC determinations. Fruit selection was based upon the availability of similarly matched and controlled fields that were harvested at the same time.

2 Materials and methods

2.1 Sea buckthorn berries and postharvesting treatment conditions

Raw material was divided first by variety, second by harvest year and producer. Three different varieties of sea buckthorn were investigated, namely "Trofimovskaja" (TR), "Podarok Sadu" (PS), and "Avgustinka" (AV). Berries were collected in years 1998, September by A-Mari, Southern Estonia (TR1, PS1, and AV1), and in 2005, September by Polli Horticultural Research Centre, Middle Estonia (TR2, PS2, and AV2).

2.2 Chemicals

Trans-Resveratrol, *p*-coumaric acid, quercetin dihydrate, (hereinafter quercetin), *trans*-3,4-dihydroxycinnamic acid (hereinafter caffeic acid), and gallic acid were purchased from Sigma–Aldrich Chemie (Germany), myricetin from Sigma–Aldrich Chemie (USA), (±)-catechin hydrate (hereinafter catechin) from Fluka Chemie (Switzerland), and L-(+)-AA, from Riedel-de Haën (Germany). Methanol was of HPLC grade from Fluka Chemie (Switzerland). ACN was of HPLC grade from Rathburn Chemicals, Walkerburn (Scotland). Deionized water was prepared by a Milli-Q Water Purification system (Millipore, MA, USA). All chemicals were used without any further purification.

The stock solutions of *trans*-resveratrol, catechin, *p*-coumaric acid, myricetin, quercetin, caffeic acid, and gallic acid were prepared by dissolving in a pure methanol at concentration 10 mmol/L. AA stock solution was diluted in Milli-Q water at concentration 100 mmol/L. Working standard solutions were obtained by diluting the corresponding stock solutions in CH_3OH/H_2O (70:30) to achieve concentrations 100 μmol/L. In preparation of all solutes, deionized water (Milli-Q, Millipore S.A., Molsheim, France) was used. All the samples, including standard solutions, were filtered through a 0.45 μM PTFE filter before analysis.

2.3 Sample preparation

Two extraction types were used, first classical for determination of AA (acid and water) and second for analyzing both phenolic compounds and AA (methanol/water mixture). For the first type extraction berries (5 g) were homogenized with mortar and pestle with extraction solution (0.0095 mol/L KH_2PO_4 buffer pH 3.0 with 5% methanol), and transferred to a 50 mL volumetric flask, and then 10 mL of extracts were centrifuged at 27 000 × *g* for 5 min at 20°C. The supernatant was filtered through a Whatman no. 1. This extract was used for both CE and HPLC analysis. For the second extraction, a weighed portion (10 g) of frozen berries was squeezed and extracted one time during 1 h with 20 mL of a CH_3OH/H_2O (70:30) mixture and stored in refrigerator overnight. After extraction, all berry samples were filtered through a 0.45 μm PTFE filter and kept at +4°C until analyzed. This extract was used only for CE analysis. From the same berry variety, three extracts were prepared for statistical evaluation of the results.

2.4 CE analysis

CE analysis was performed by using two instruments. First, an in-house built CE system was used which was equipped with a fused-silica capillary (Polymicro Technologies, Phoenix, AZ), 70 cm (effective length 35 cm), 50 μm id, high-voltage power supply (Spellmann, Hauppauge, NY), and UV detector (Linear 1000, Prince Technologies). The UV detector was coupled to a personal computer. Data acquisition was done by the software written in-house, using a LabView program (National Instruments, Austin, TX). The software recorded the detector signal *via* an ADAM 4018/4060 interface (Advantech, Taipei, Taiwan). The second instrument was an Agilent CE System (Agilent Technologies, Waldbronn, Germany) with DAD using an applied voltage of 16 kV at

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25°C. Recording of electropherograms was performed at various wavelengths depending on the UV absorption spectra of the analytes. Total length of the capillary (360 μm od, 50 μm id, Polymicro Technologies, Phoenix, AZ) was 60 cm and length to the detector was 52 cm.

To compensate for migration time variations, facilitate comparison of electropherograms, and help identification of peaks all electropherograms were subjected to the simple linear migration axis compression/stretching procedure which preserves the peak area value. The procedure implements a pair of peaks (first and last) on two electropherograms to be aligned. The chosen first peak on both electropherograms was identified as corresponding to the same first compound. Similarly, the chosen last peak on both electropherograms was identified as corresponding to the same second compound. The procedure (written in-house in Matlab, Mathworks, MA, USA) assigned the same migration time value to the same first compound peak in all electropherograms, and similarly the same migration time value to the same last compound peak and aligned rest of the electropherograms between first and last peaks.

2.4.1 CE methodology

The capillary was conditioned prior to use with 1 mol/L NaOH for 20 min and with H_2O for 30 min. After each run, the capillary surface was regenerated by sequential washing with 0.1 mol/L NaOH, H_2O, and the separation buffer for 3 min each. The standards and samples were injected into the capillary gravitationally at a fixed time of 20 s from 12 cm (homemade system) or by 50 mbar for 5 s (Agilent CE System).

Several research groups have successfully used the borate buffer for the separation of antioxidant compounds by CE [24, 29, 31] which suggested to use the same approach. The separation of phenols in CE with borate buffer is based on the differences in charge-to-mass ratios of these compounds and on their complex formation with tetraborate molecules when the phenolic compound has *o*-hydroxy groups. In this work, the buffer concentration was varied from 10 mM borate to 40 mM (with 10 mM increments), and the latter value was found satisfactory in terms of compromise between separation and the analysis time.

2.5 HPLC analysis

Analysis was performed using a Gilson 321 HPLC pumps equipped with a Gilson UV/Vis-151 detector. RP separation was attained using a C-18 column Inertsil ODS-2 5 μm (250 mm id, 4.6 mm) and precolumn Inertsil ODS-2 5 μm (20 mm id, 4.6 mm). The mobile phase was 0.0475 mol/L KH_2PO_4 buffer pH 3.0 with 3% methanol and 2% ACN. The flow rate was 1.0 mL/min, and the detection wavelength was 245 nm. For analysis the first type extraction was used (for details see [32]).

2.6 Humidity analyzes

For humidity analyzes the drying by Sanyo sterilizer, MOV-112S (Sanyo Electric Biomedical, Japan) with 50°C temperature and berry weighting each 10 min till constant mass was used [33].

3 Results and discussion

3.1 Separation and identification of antioxidants in a standard mixture and sea buckthorn extract by CE

Several antioxidants, frequently encountered in many berries extracts (for possible list see *e.g.*, publications [12, 13, 34, 35]) were looked for in sea buckthorn extract electropherograms. For this analysis the second type of extraction was used. Typical electropherograms of one of the extracts, namely TR1, is presented in Fig. 1. The patterns of the electropherograms of the other varieties looked similar (although different in intensity of the particular peaks) and are not presented here. Figure 1 presents electropherograms of sea buckthorn extract and standard mixture at three different wavelengths (210, 265, and 280 nm). Standard mixture consisted of *trans*-resveratrol, catechin, AA, *p*-coumaric acid, myricetin, quercetin, caffeic acid, and gallic acid with concentration of 100 μmol/L. All this standards have strong absorption around 200 nm and various absorption bands between 200 and 300 nm which were taken into account by choosing detection wavelengths. Moreover, analytical parameters of proposed standards are found in Table 1 (with calibration curve correlation coefficient square root, R^2, LOD, and LOQ).

It follows from Fig. 1 that sea buckthorn extract is a complicated mixture with tens of components presented. For identification, spiking experimental technique was used (the addition of standards to the sample solutions resulted in an increase of the analyte peak without the appearance of shoulders or split peaks). The identification by spiking was confirmed by comparison of corresponding electropherograms of extracts and standards recorded at different wavelengths (Fig. 1) and by visual comparison of UV spectra between peaks on the electropherogram of the standards and candidate peaks on the electropherogram of the extracts (an opportunity provided by Agilent ChemStation software, results shown in the Fig. 2). As a result of identification AA, myricetin, and quercetin peaks were found in sea buckthorn extract, however, no *p*-coumaric, caffeic, or gallic acids peaks were found. Situation with *trans*-resveratrol and catechin is more uncertain because of the overlapping of the peaks with peaks of the matrix and their small intensity, therefore presence/absence of *trans*-resveratrol and catechin in the sea buckthorn extract might be detected only after more careful study.

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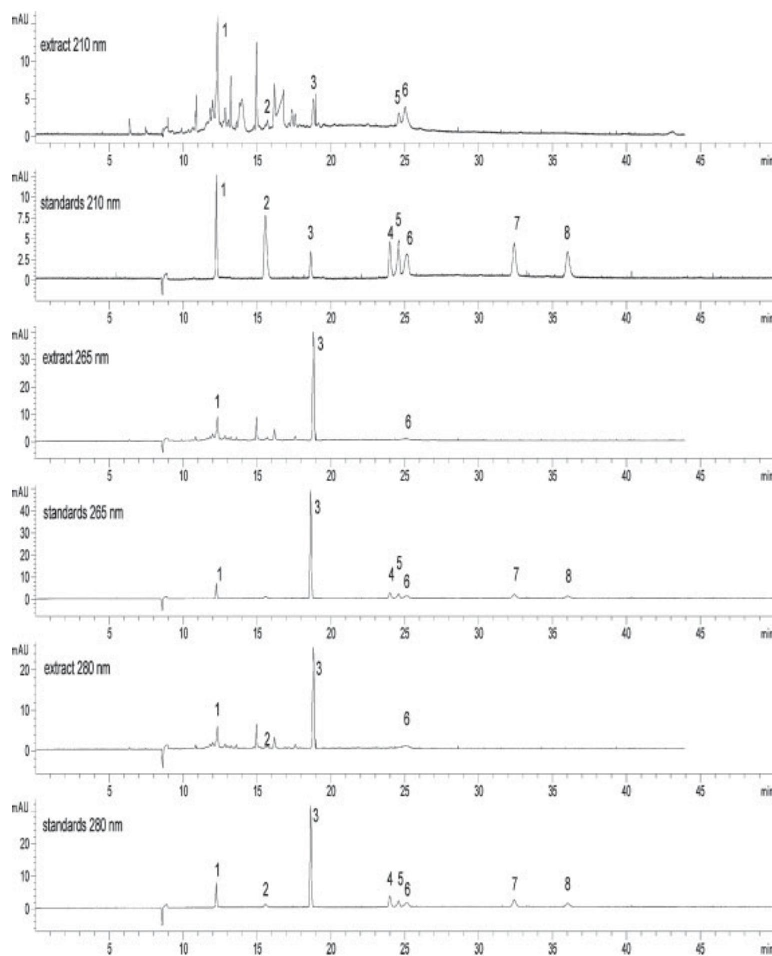

Figure 1. Electropherograms of sea buckthorn extract and mixture of standards at different detector wavelength. Standard mixture consisted of (1) *trans*-resveratrol, (2) catechin, (3) AA, (4) *p*-coumaric acid, (5) myricetin, (6) quercetin, and (7) caffeic and (8) gallic acids with concentrations of 100 μmol/L. Standards were added to sea buckthorn extract one by one and final result is presented in Fig. 1.

Table 1. Analytical parameters of polyphenols and AA[a)]

Analyte	Linear range (μmol/L)	10^3 ($a \pm S_a$)[b)]	10^3 ($b \pm S_b$)[b)]	R^2	LOD (μmol/L)	LOQ (μmol/L)
Trans-resveratrol	50–150	4.5 ± 0.4	−51 ± 51	0.9913	6	19
Catechin	50–150	21.0 ± 0.6	81 ± 73	0.9992	3	9
AA	50–150	4.5 ± 0.2	−43 ± 21	0.9986	2	6
p-coumaric acid	50–150	13.0 ± 1.2	60 ± 152	0.9910	5	17
Myricetin	50–150	14.1 ± 1.4	37 ± 168	0.9905	4	15
Quercetin	50–150	14.7 ± 1.5	−115 ± 180	0.9901	4	12
Caffeic acid	50–150	8.5 ± 1.1	−36 ± 130	0.9846	5	17
Gallic acid	50–150	15.8 ± 1.1	−33 ± 136	0.9951	4	14

a) Measurements were made by CE, with 50 μm inner diameter capillary, 70 cm (effective length 35 cm), 16 kV, 210 nm, 20 mmol/L sodium tetraborate (pH 9.3) buffer, and gravitational injection from 12 cm for 20 s.

b) *a* and *b*, calibration equation $y = aC + b$ coefficients. S_a and S_b correspond to SDs; *C*, concentration; *y*, UV detector response.

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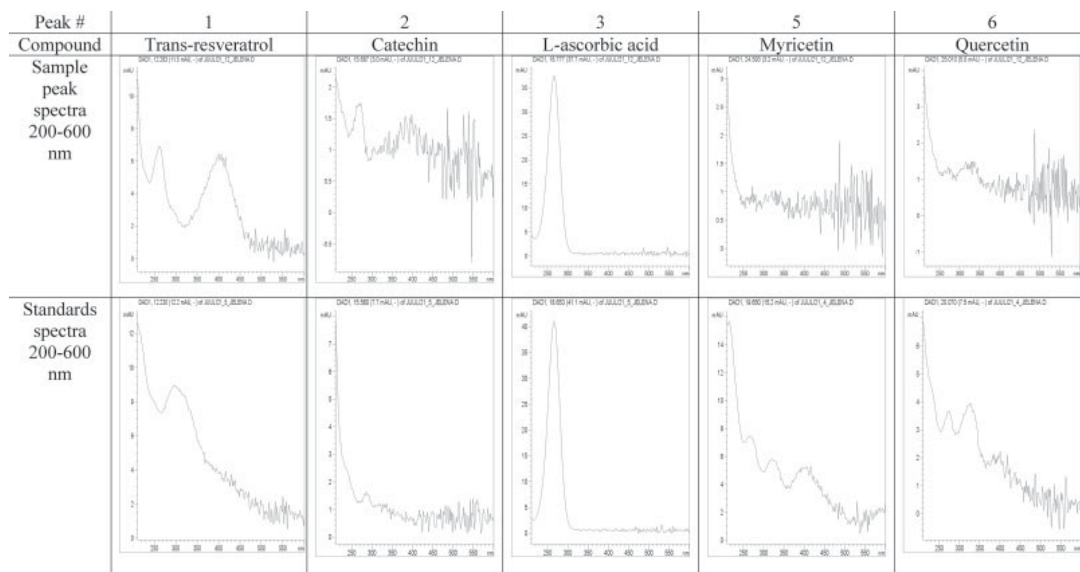

Figure 2. Comparison of UV spectra of the standards with the spectra of the candidate peaks in sea buckthorn extract.

3.2 Quantitative determination of quercetin and AA in berry extracts

Although *trans*-resveratrol and catechin were identified in all extracts, it appeared to be difficult to quantify them. The other compounds of interest were quantified using calibration standards, and the concentrations obtained were verified by the standard addition method for each extract separately. The analytical results for quantity of the substances studied by CE are reported in Table 2, where humidity level was taken to account, so results expressed as mg *per* 100 g of dried berries. As follows from Table 2, all studied varieties contained AA at levels of 300–800 mg/100 g of dried berries and quercetin at levels of 70–120 mg/100 g of dried berries. Table 2 confirms that differences in concentration are statistically significant if judged by analyte concentration means together with the corresponding SDs. There is also an interesting fact that berries collected in the year 1998 by A-Mari contain a higher amount of AA than berries collected in 2005 by Polli Horticultural Research Centre. This might be explained by different ripening conditions in different years (rain, temperature, and soil) and also by different agriculture practice used by different manufactures.

The concentration of AA was measured separately by HPLC to compare the data obtained by the CE method using the paired *t*-test [36]. In this test, the difference between the measurements is calculated for each pair of varieties, and the mean, *d*, and SD of these differences, *S*, is calculated. Dividing the mean by the SD of the mean yields a test statistic, *t*, as follows:

$$t = \sqrt{n}\frac{d}{S} \tag{1}$$

which has Student's *t*-distribution with degrees of freedom equal to 1 less than the number of pairs, *n*. If the calculated *t* is larger than its critical value then the two methods give different results. The results are reported in Table 3. In addition to the *t*-test, the results of correlation analysis between HPLC and CE results are given in Table 3. It follows from Table 3 that in general the results of CE and HPLC obtained in the analysis of the same 5% methanol/ HPLC buffer extract correlate well. The square of the correlation coefficient is 0.98. However, CE measurements give a bit lower concentration values than HPLC. The calculated paired Student's *t*-test between 5% methanol/HPLC buffer extracts gives $t = 2.73$ which is near to the critical $t = 2.77$ value for the two-sided test at the 95% confidence level. The same also becomes evident from the regression line between C_3 and C_2 where the slope is different from 1 and the intercept from 0 (which can be easily confirmed by testing the corresponding statistical hypothesis). Surprisingly, for the methanol/water extract the same test gives a lower than critical value of *t* equal to 0.89, but the correlation between data is not good which could be expected since the extraction methods were different. The correlation between two different extraction methods is excellent which is confirmed by a lower than critical paired *t* value of 0.89 and the regression line slope and intercept values do not differ statistically from their values of 1 and 0, respectively.

When the reason for the slight disagreement between CE and HPLC data is not clear one of the possible reasons could be the narrowness of CE peaks. Our present data registration

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Table 2. Concentrations of AA and various polyphenols in sea buckthorn extract in mg/100 g of dried berries (triplicate analyses for each treatment)

Extraction solution	CH_3OH/KH_2PO_4	CH_3OH/KH_2PO_4	CH_3OH/H_2O			Humidity (%)
Analytical method	HPLC	CE	CE			
Compound	AA	AA	AA	Myricetin	Quercetin	
AV1	554 ± 25	480 ± 12	571 ± 62	34.4 ± 0.8	75.0 ± 2.3	87
AV2	415 ± 13	304 ± 42	299 ± 62	35.0 ± 0.9	89.2 ± 4.3	88
TR1	714 ± 44	714 ± 41	740 ± 66	35.5 ± 0.7	85.9 ± 3.3	85
TR2	681 ± 30	677 ± 47	743 ± 38	44.2 ± 0.8	116.2 ± 9.8	88
PS1	809 ± 5	777 ± 21	783 ± 70	35.1 ± 0.8	69.1 ± 3.9	87
PS2	592 ± 19	512 ± 37	448 ± 35	38.9 ± 0.7	75.4 ± 4.3	86

Table 3. Results of paired *t*-test and regression equations between concentration values obtained by CE and HPLC using two different extraction methods

Extraction analysis mode		5% Methanol in HPLC eluent by CE	5% Methanol in HPLC eluent by HPLC
Methanol/water (70:30) by CE	Calculated *t* value[a)]	$t = 0.89$	$t = 0.89$
	Regression equation[b),c)]	$C_1 = (1.05 \pm 0.15)C_2 - (9.5 \pm 0.2)$	$C_3 = (0{,}66 \pm 0.13)C_1 + (232.2 \pm 0.2)$
	Correlation coefficient	$R^2 = 0.922$	$R^2 = 0.866$
5% methanol in HPLC eluent by CE	Calculated *t* value[a)]	*X*	$t = 2.73$
	Regression equation[b),c)]		$C_3 = (0{,}77 \pm 0.05)C_2 + (182.5 \pm 0.2)$
	Correlation coefficient		$R^2 = 0.980$

a) Critical value for two sided at 95% confidence level is $t = 2.77$.
b) C_1, concentration determined by CE from the methanol/water extract; C_2, concentration determined by CE from 5% methanol in HPLC buffer; C_3, concentration determined by HPLC from 5% methanol in HPLC buffer.
c) The slope and intercept are given with their SDs.

equipment (an ADAM 4018/4060 interface, speed 5 points/s) might not be always satisfactory for a complete description of the peak shape (and the baseline) that in turn might give a lower estimation of peak areas and thus, the corresponding concentration values. The fact that CE sometimes does not perform well in peak area quantization is well known. Nevertheless, this drawback is frequently overweighed by the efficiency of CE as compared to HPLC that is the case with this work as well.

In general, as follows from Fig. 1, the sea buckthorn extract is very rich in different compounds whose identification by spiking of standards – a trivial and cheap technique – is laborious and time-consuming. ESI-CE-MS could be a good alternative to analysis of complicated mixtures of natural extracts [30] that, however, is presently unavailable everywhere. Thus, the spiking remains the way how the identification of analytes in plant extracts has to be done. On the other hand, although the API has revolutionized the application of LC-MS, some problems remain, the major limitation being the strong dependency of the response on the nature of the analyte plus the mobile phase. Thus, the generation of mass spectral libraries is difficult. On rare occasions, LC-MS can provide data sufficient for full structure analysis but more generally it is used to determine molecular mass and to establish the distribution of substituents on the phenolic ring(s). Thus, despite its power CE-MS requires the collecting of the library of mass spectra of individual plant compound standards, too. This is a task that has not been completed yet.

4 Concluding remarks

It follows from this work that CE is a reasonable alternative to HPLC for the analysis of natural compounds found in different varieties of plant extracts. Differences in analyte levels of sea buckthorn varieties can be easily compared. Although the determination of the analyte concentrations by CE correlated well with HPLC determinations, the statistical analysis of the data reveals a small, statistically not very sig-

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nificant bias between the two methods. Origin of this fact needs to be evaluated further.

The results revealed that many important antioxidants are missing in Estonian varieties of the sea buckthorn, namely TR, PS, and AV. Still a reasonable amount of AA was found. The level of AA varied significantly from variety to variety. In addition, the level of AA is higher for berries collected in the year 1998 by A-Mari than for berries collected in 2005 by Polli Horticultural Research Centre. This effect can be explained as due to different ripening conditions in different years as well as different agriculture practice used by different manufactures.

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Publication II

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Characterization of bioactive compounds contained in vegetables of the Solanaceae family by capillary electrophoresis

Kati Helmja*, Merike Vaher, Jelena Gorbatšova, and Mihkel Kaljurand

Department of Chemistry, Faculty of Science, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

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Abstract. Because of their antioxidative capability polyphenols and vitamins are the most important naturally occurring compounds. Several widely consumed vegetables are rich in various phenolic compounds and vitamins. In this study, such vegetables as tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), chilli pepper (*Capsicum annuum*), and potato (*Solanum tuberosum*) of the Solanaceae family were investigated. The phenolic compounds and vitamins were separated and their composition was determined by capillary electrophoresis (CE). The total phenolic content was measured according to the Price and Butler method. In addition, the antioxidative capability of phenolic compounds was monitored and evaluated by CE using a coloured free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Key words: antioxidativity, capillary electrophoresis, polyphenols, vitamins.

INTRODUCTION

The increasing interest in naturally occurring antioxidants (polyphenols, vitamins) is attributed to their capability of scavenging free radicals that are formed in various biochemical processes. The reactive oxygen species like superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) cause an extensive oxidative damage to biomolecules such as nucleic acids, proteins, and lipids. These highly unstable radicals have been found to be related to oxidative stress-related diseases like cardiovascular diseases, cancer, inflammatory disorders, neurological degeneration (Parkinson's and Alzheimer's diseases), premature ageing, etc. [1–6].

* Corresponding author, helmja@staff.ttu.ee

Polyphenols (cinnamic acid derivatives, flavonols, anthocyanins) and vitamins are present in vegetables, fruits, berries, and herbs, which are the main source of natural antioxidants in our daily diet. The basic structure of polyphenols is composed of one or more phenolic rings that are substituted with several hydroxyl groups and these are highly correlated with their strong antioxidant activity [3, 4, 7–9]. Vitamins are structurally a heterogeneous group of compounds, which are essential in the diet for the maintenance of healthy growth and development. In general, vitamins are divided into two main categories, fat and water-soluble ones [10–12].

Among vegetables, tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), chilli pepper (*Capsicum annuum*), and potato (*Solanum tuberosum*), which belong to the Solanaceae family, are important for their richness in healthy components due to which they are also widely consumed. Tomato is rich in phenolic compounds (flavonoids, flavones, cinnamic acid derivatives), phytoalexins, protease inhibitors, glycoalkaloids, and carotenoids, but especially in lycopene and β-carotene. In addition, vitamins C, E, and A have been determined in tomato [5, 7, 9, 13–16]. The main polyphenols found in eggplant are phenolic acids (chlorogenic acid, caffeic acid, *p*-coumaric acid), but this vegetable is poor in provitamin A and vitamin E. However, the presence of vitamins C and B in eggplant has been established [17–20]. It is also rich in anthocyanins like nasunin and delphinidin conjugates [21]. Chilli pepper has been reported to contain flavones (luteolin, quercetin), flavonols (myricetin, quercetin), and capsaicinoids [22–24]. Of phenolic compounds, chlorogenic and caffeic acid, catechin, and also glycoalkaloids have been reported to be the main compounds present in potato [25, 26]. Vitamin C has been also determined in potato [26].

As plant matrices have a complex composition, development of methods of their separation is of crucial importance. Therefore several methods like thin-layer chromatography (TLC), gas chromatography (GC), high-pressure liquid chromatography (HPLC), and mass-spectrometry (MS) have been used to separate polyphenols and vitamins. Nowadays hyphenated techniques like HPLC–MS have been developed, which enable the characterization/determination of the structure of a compound [1, 2, 5, 6, 15, 27–31]. Also microbiological assays have been developed for the determination of vitamins [10]. Due to its relatively short analysis time, ease of operation, minimum sample and reagent consumption, non-use or use of a very low amount of organic solvents capillary electrophoresis (CE) has been found to be a powerful tool allowing the separation of bioactive compounds in biological matrixes. Moreover, CE permits simultaneous analysis of different kinds of analytes in a single run [3, 27, 28, 32–34].

The antioxidative capability of the compounds under study has been evaluated by using different assays like a ferric reducing/antioxidant power assay (FRAP), an oxygen radical adsorption capacity method (ORAC), colourization assays using stable coloured free radicals, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), a 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

radical cation or a reactive oxygen species, due to their intensive absorbance in the visible region [2, 35–38].

The objective of the present study was to determine the composition of polyphenols (phenolic acids, flavonols, flavones) and vitamins of the skin extracts of the plant family of Solanaceae by capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC). The conditions of the separation of water-soluble vitamins such as thiamine (B_1), nicotinamide and nicotinic acid (B_3), D-panthothenic acid (B_5), and pyridoxine (B_6), which are also known as vitamins B complex, as well as vitamin C (L-ascorbic acid) were optimized. Besides, the antioxidative capability of the compounds in question was determined spectrophotometrically, using a free radical like 2,2-diphenyl-1-picrylhydrazyl (DPPH). Additionally, the method for the monitoring of the scavenging capability of a DPPH radical was developed and evaluated by CZE.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade and were used as received. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rutinoside), quercetin (3,3′,4′,5,7-tetrahydroxy-flavonol), naringenin (4′,5,7-trihydroxyflavanone), genistein (4′,5,7-trihydroxy-isoflavone), cinnamic acid ((*E*)-3-phenyl-2-propenoic acid), luteolin (3′,4′,5,7-tetrahydroxuflavone), myricetin (3,3′,4′5,5′,7-hexahydroxyflavone), chlorogenic acid (1,3,4,5-tetrahydroxycyclo-hexanecarboxylic acid 3-(3,4-dihydroxycinna-mate)), *p*-coumaric acid (4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), L-ascorbic acid, thiamine, pyridoxine, sodiumdodecylsulphate (SDS), ferric chloride, potassium ferricyanide, aluminium chloride, sodium nitrite, DPPH, tannic acid, sodium tetraborate, and sodium hydroxide were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Catechin (5,7,3′,4′-tetrahydroxyflavane), nicotinamide, nicotinic acid, and D-pantothenic acid hemicalcium salt were from Fluka Chemie GmbH (Switzerland). Boric acid was from Riedel-de Häen (Germany). The structures of phenolic compounds are given in Fig. 1. Methanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland). Deionized water (MilliQ, Millipore S. A. Molsheim, France) was used for the preparation of all solutions.

Instrumental

All experiments were performed using an Agilent CE System (Agilent Technologies, Waldbronn, Germany) with a diode array detection. A CE Chem-station (Agilent Technologies) was used for instrument control, data acquisition, and data handling. The separation of polyphenols was performed in a fused silica capillary (Polymicro Technology, Phoenix, AZ, USA) with a total length of

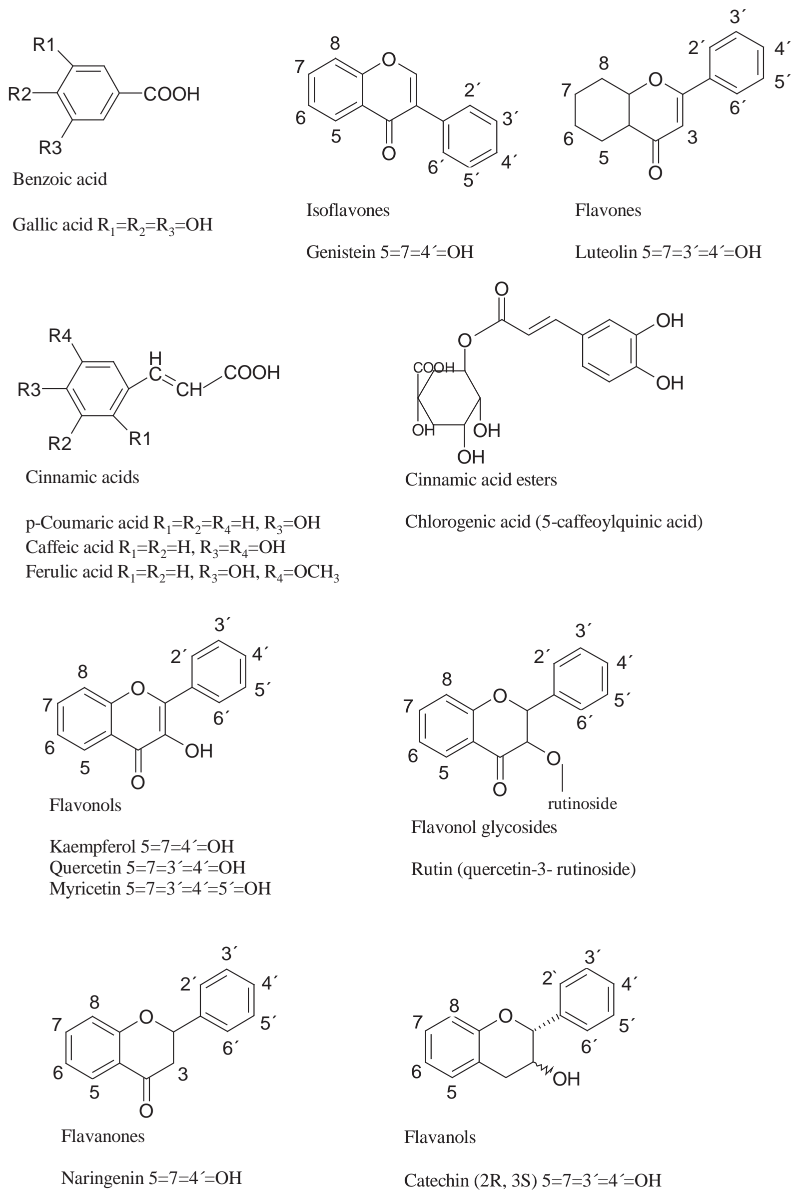

Fig. 1. The structures of polyphenolic compounds found in plants of the Solanaceae family.

75 cm (the effective length 50 cm) and i.d. of 75 μm. A fused silica capillary with a total length of 60 cm (the effective length 52 cm) and i.d. of 50 μm was used to separate vitamins. Prior to use, the capillary was rinsed with a 0.1 M NaOH solution for 5 min and with the separation buffer for 5 min. As a separation buffer 25 mM sodium tetraborate (pH 9.3) was used in the case of polyphenols and 40 mM boric acid with 50 mM SDS (pH 8.5) in the case of vitamins. The voltage applied for the separation of polyphenols and vitamins was +25 and +16 kV, respectively.

Sample preparation

Tomato, eggplant, chilli pepper, and potato were purchased at a local market in the autumn of 2006 and 2007. The vegetable skins were dried at room temperature and for analysis the weighed portions of the dried sample were homogenized into powder. Ultrasonic extraction was performed using a 80 : 20 mixture of methanol and water. For the extraction 0.5 g of the ground skin was weighed and 5 mL of the extraction mixture was added. The sample was left at room temperature for 60 min and in an ultrasonic bath at room temperature for 20 min. The extract was filtered through a 0.45 μm filter and stored at +4 °C in dark. L-ascorbic acid was determined in the extracts of the fresh skin of vegetables under investigation.

Determination of total phenolic content

The total phenolic content of the extracts was determined spectrophotometrically (Jasco V-530, USA) according to the Price and Butler method [39]. Tannic acid was used as a standard (linear range 0.01–2.5 mM). The sample (250 μL) was added to 25 mL of deionized water and mixed. After that 3 mL of $FeCl_3$ was added and, additionally, after 3 min, 3 mL of $K_3[Fe(CN)_6]_3$ was added. The solution was mixed and incubated at room temperature for 18 min. The absorbance was measured at 720 nm spectrophotometrically.

Determination of flavonoid content

The determination of the flavonoid content of plants was performed using the colorimetric assay [40]. At first 50 μL of the skin extract was diluted with 0.5 mL of deionized distilled water and 0.03 mL of 5% $NaNO_2$ was added. Then 0.06 mL of 10% $AlCl_3$ and 0.2 mL of 1 M NaOH were added after 5 min and a further 6 min, respectively. Finally, 0.21 mL of deionized distilled water was added. The absorbance was recorded at 510 nm spectrophotometrically. Rutin was used as a standard.

DPPH radical scavenging capability

The free radical scavenging capability of the compounds under investigation was evaluated using a stable free radical DPPH for the decolorization assay

[41–43]. The assay is based on the reduction of DPPH by phenolic compounds and the adsorbance of DPPH radical at 515 nm. To the cuvette 3.9 mL of a DPPH methanolic solution (6.02×10^{-5} M) was transferred and 0.1 mL of the extract was added. The absorbance at 515 nm was recorded at certain time intervals until a steady state of the reaction was reached. The blank reference cuvette contained a 80 : 20 mixture of methanol and water. The percentage of the DPPH radical remaining at the steady state was determined by the following equation:

$$\%\text{DPPH} = \left(1 - \frac{A_f}{A_0}\right) \times 100,$$

where A_0 and A_f correspond to the absorbance at 515 nm of the radical at the beginning of the reaction and at the steady state, respectively. The time needed to reach the steady state at an EC_{50} concentration of the compound (EC_{50} is the amount of an antioxidant needed to decrease the initial DPPH radical concentration by 50%) was calculated graphically [41–43]. All the determinations were done in triplicate.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents and the antioxidant capability

The total phenolic and flavonoid contents and antioxidant capability of the skin extracts of vegetables of the Solanaceae family are given in Fig. 2. The results indicate eggplant to have the highest total phenolic and flavonoid contents – 1.5 g/L (900 mg/100 g) and 1.1 g/L (660 mg/100 g), respectively, followed by chilli pepper – 0.8 g/L (480 mg/100 g) and 0.4 g/L (240 mg/g), respectively. The

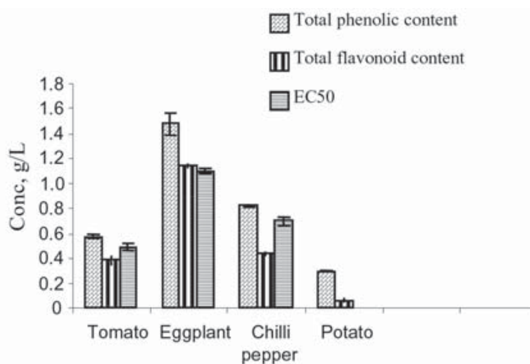

Fig. 2. Comparison of total phenolic and flavonoid content and antioxidant capability of plants of the Solanaceae family.

total phenolic content of the tomato skin extract was 0.6 g/L (360 mg/100 g) and that of flavonoids, 0.4 g/L (240 mg/100 g). Potato had the lowest total phenolic content, 0.3 g/L (180 mg/100 g), and its flavonoid content was 0.06 g/L (36 mg/100 g).

EC_{50} is one of the most frequently measured parameter characterizing the antioxidant capability of plants [41–43]. Its value is inversely related to the antioxidative capability of a compound. Thus, the lower the EC_{50}, the higher the antioxidant power. Figure 2 shows tomato to have the lowest EC_{50}, followed by chilli pepper and eggplant. It is interesting that the decolorization reaction of the potato extract with DPPH was negligible, therefore no data are shown.

Separation of bioactive compounds by CE

Separation of polyphenols

Various phenolic compounds were separated and identified by CZE. Based on the results of our earlier studies, an efficient separation buffer, borate, was used in the case of polyphenols [44, 45]. The electropherogram of the standard mixture of polyphenols was obtained and is shown in Fig. 3. A satisfactory separation was achieved in 12 min.

Polyphenols contained in vegetables of the Solanaceae family were separated and identified. The results are demonstrated in Fig. 4. The phenolic compounds were identified by the spiking of the standard solution to the extract, which resulted in an increase of the analyte peak. As an example, the electropherograms of spiking for the identification of chlorogenic acid in potato extract are

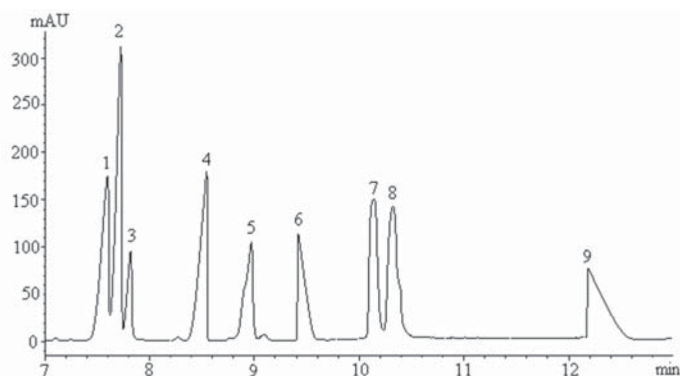

Fig. 3. Electropherogram of the standard mixture of polyphenols (250 mM of each compound): 1 – genistein, 2 – rutin, 3 – naringenin, 4 – cinnamic acid, 5 – chlorogenic acid, 6 – *p*-coumaric acid, 7 – myricetin, 8 – quercetin, 9 – caffeic acid. The separation conditions: separation buffer 25 mM sodium tetraborate (pH 9.3), the effective length of the capillary 50 cm, applied voltage +25 kV, UV detection at 210 nm; injections were performed hydrodynamically 15 s.

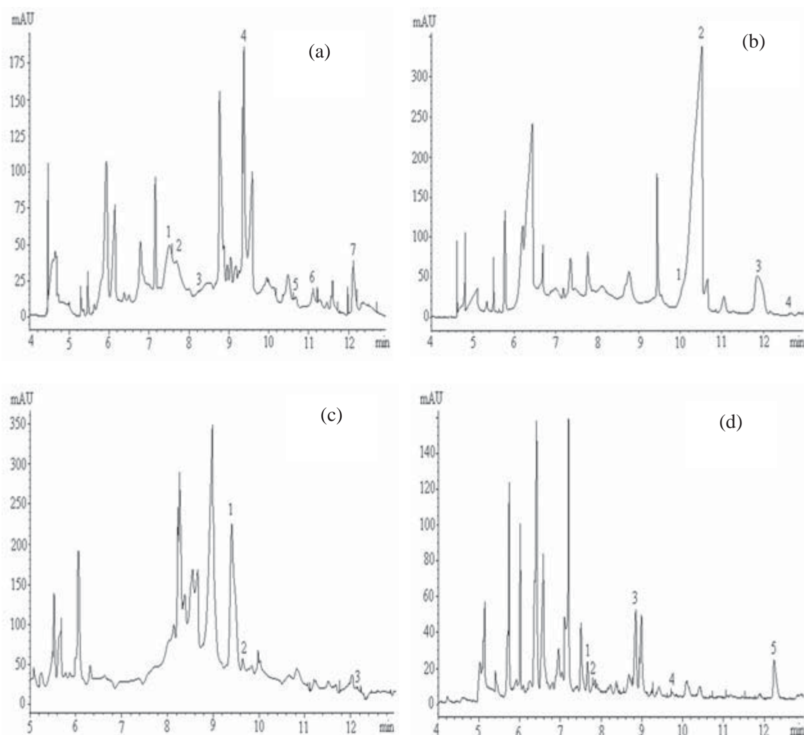

Fig. 4. Electropherograms of skin extracts of plants of the Solanaceae family: (a) skin extract of tomato: 1 – genistein, 2 – rutin, 3 – naringenin, 4 – chlorogenic acid, 5 – myricetin, 6 – quercetin, 7 – caffeic acid; (b) skin extract of eggplant: 1 – cinnamic acid, 2 – chlorogenic acid, 3 – caffeic acid, 4 – ferulic acid; (c) skin extract of chilli pepper: 1 – luteolin, 2 – quercetin, 3 – caffeic acid; (d) skin extract of potato: 1 – catechin, 2 – rutin, 3 – chlorogenic acid, 4 – quercetin, 5 – caffeic acid. The separation conditions were the same as in Fig. 3.

presented in Fig. 5. A certain amount of a standard solution of polyphenols (chlorogenic acid) was added to the extract and a decreased peak was observed. In addition to the spiking procedure, the spectra of the phenolic compounds separated from the skin extracts of vegetables were compared to the spectra of reference compounds using a diode-array detector. Comparison of the electropherograms in Fig. 4 reveals that polyphenols were mainly identified in the tomato skin extract and only a few were identified in the chilli pepper skin extract under the separation conditions applied.

The electropherograms in Fig. 4 demonstrate that the extracts of vegetables are very complex in composition and the determination of individual compounds

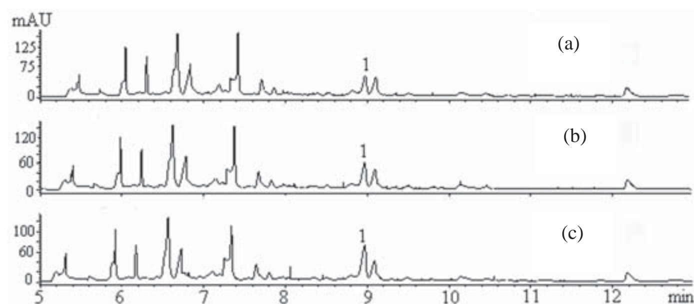

Fig. 5. Identification of a phenolic compound by spiking: (a) – potato skin extract; (b) – 5 μL of 2.5 mM chlorogenic acid (peak 1) was added to the extract; (c) – 5 μL of 250 mM chlorogenic acid added to the extract. The separation conditions were the same as in Fig. 3.

will require the use of several analytical methods. A number of flavonoids may also be present as glycosides and their reference compounds are not commercially available. Thus in the present study most glycosides were not identified, but it could be possible applying MS.

Separation of vitamins

Different techniques of CE like CZE, MEKC, and micellar emulsion electrokinetic chromatography (MEEKC) have been successfully applied to separate vitamins [46–50]. In this study, MEKC was used and as a separation buffer 40 mM boric acid with 50 mM SDS was found to be satisfactory in terms of compromise between the separation efficiency and the analysis time. Peak identification was based on the comparison of the migration times of standard compounds and the extracts of vegetables. It was confirmed by spiking (fortification technique) and the spectra of standard compounds and those in the extracts of vegetables. The quantification was based on an external standard method using calibration curves (Table 1). To assess the linearity of the relationship between the concentration and peak area of analytes, four standard solutions (in the range 250 to 1000 μmol/L) dissolved in milli-Q water were analysed. The limit of detection was evaluated as three times the signal-to-noise ratio.

The composition of vitamins of group B and vitamin C (L-ascorbic acid) was investigated. The fresh skin extracts of vegetables of the Solanaceae family were used for the determination of ascorbic acid as it is easily decomposed. Figure 6 shows the vitamins determined in the skin extracts of tomato, eggplant, and chilli pepper. In the potato extract these vitamins were not detected. L-ascorbic acid was present in the fresh skin extracts of chilli pepper and tomato, but of B-group vitamins, pyridoxine was determined in the dried eggplant skin extract. Table 2 shows quantitative results of vitamin determination in the extracts.

Table 1. The analytical parameters of vitamins

Analyte	Linear range, µmol/L	Equation of calibration curve	R^2	LOD*, µmol/L	LOQ**, µmol/L
Nicotinamide	250–1000	$y = 0.0618x + 5.51$	0.9946	8	23
Pyridoxine	250–1000	$y = 0.1327x + 4.850$	0.9941	4	13
D-Pantothenic acid	250–1000	$y = 0.0405x + 2.95$	0.996	12	36
L-ascorbic acid	250–1000	$y = 0.1037x + 4.4$	0.9948	7	21
Nicotinic acid	250–1000	$y = 0.0905x + 6.96$	0.9963	6	18
Thiamine	250–1000	$y = 0.1527x + 10.45$	0.994	3	10

* Limit of detection (LOD).
** Limit of quantification (LOQ).

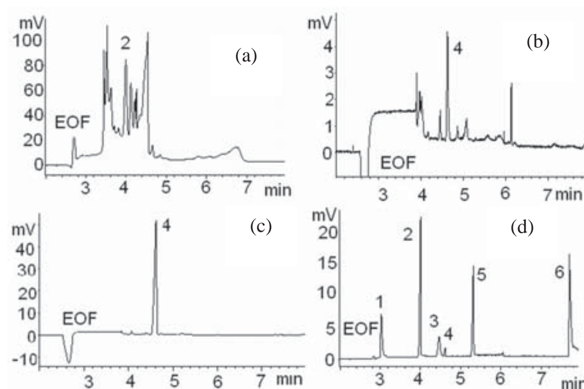

Fig. 6. Electropherograms of the vitamins determined in the plant extracts: (a) – dry eggplant skin extract, peak No. 2 – pyridoxine; (b) – fresh tomato skin extract, peak No. 4 – L-ascorbic acid; (c) – fresh chilli pepper skin extract, peak No. 4 – L-ascorbic acid; (d) – mixture of standard solutions of the vitamins (each 250 µM): 1 – nicotinamide, 2 – pyridoxine, 3 – D-pantothenic acid, 4 – L-ascorbic acid, 5 – nicotinic acid, 6 – thiamine. The separation conditions: separation buffer 40 mM boric acid with 50 mM SDS (pH = 8.5), the effective length of the capillary 52 cm, applied voltage +16 kV; injections were performed hydrodynamically 5 s.

Table 2. Quantification of vitamins found in the skin extracts of vegetables of the Solanaceae family

Fresh tomato	Fresh chilli pepper	Dry eggplant
L-ascorbic acid, 265 nm 4 mg/L ± 0.2	L-ascorbic acid, 265 nm 196 mg/L ± 10	Pyridoxine, 210 nm 609 mg/L ± 10

Monitoring of the free radical scavenging capability by CE

The free radical scavenging capability of phenolic compounds was monitored using CE. Previously, the antioxidative capability had been evaluated mainly spectrophotometrically, but CE allows a simultaneous monitoring of the oxidation of several phenolic compounds over time using a stable free radical like DPPH. Such approach is suitable for qualitative evaluation of the antioxidative capability of phenolic compounds. Due to its richness in polyphenols, the tomato skin extract was used to evaluate the antioxidative capability of compounds.

In Fig. 7 the results of the monitoring of antioxidativity are presented. The electropherogram (Fig. 7b) was taken after 5 min mixing of 10 μL of the tomato skin extract with 90 μL of a 0.5 mM DPPH solution. After 5 min the reaction was completed and therefore no considerable changes were observed. In Fig. 7 the main decreasing peaks are shown. The spectra of the decreased peaks were analysed and compared with the original spectra of the extract. The peaks of rutin, naringenin, chlorogenic acid, and caffeic acid mainly decreased. Besides, Fig. 7 shows four unknown peaks that mostly decreased. Comparing the spectra the unknown peaks X, Y, Z, and Q may be attributed to phenolic acids.

The unoxidized part (%) was determined by the original peak areas (Fig. 7a). Table 3 demonstrates that chlorogenic acid (peak 3) and caffeic acid (peak 4) and

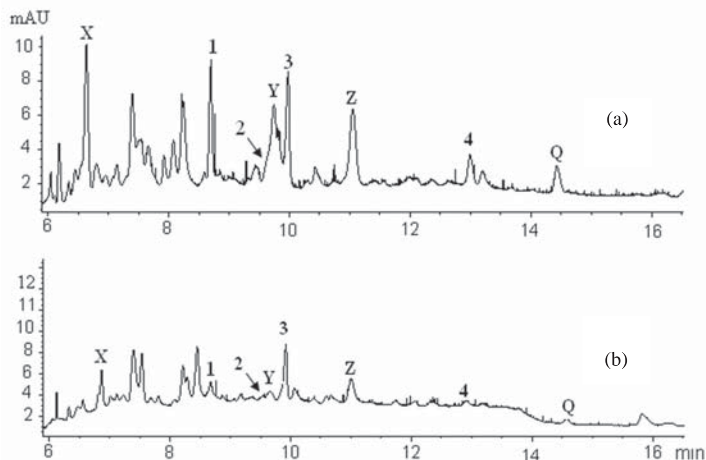

Fig. 7. Electropherograms of the monitoring of the antioxidativity of tomato skin extract using DPPH by CE: (a) 10 μL of tomato skin extract mixed with 90 μL of 80 : 20 methanol/water. Because of dilution the separation should be compared with Fig. 4. Peaks: 1 – rutin, 2 – naringenin, 3 – chlorogenic acid, 4 – caffeic acid; X, Y, Z, Q – unknown compound; (b) the reaction after 5 min. The mixture of the reaction: 10 μL of tomato skin extract mixed with 90 μL of 0.5 mM DPPH. Separation conditions were the same as in Fig. 3.

Table 3. Oxidation of the tomato skin extract by DPPH using CE

Peak No.	Migration time, min	Compound	Unoxidized part*, %
X	6.7	Unknown compound	22.0
1	8.7	Rutin	78.6
2	9.5	Naringenin	81.8
3	9.7	Chlorogenic acid	57.6
Y	9.6	Unknown compound	77.6
Z	11.0	Unknown compound	41.7
4	12.9	Caffeic acid	28.2
Q	14.5	Unknown compound	34.3

* Reaction time 5 min.

also unknown compounds (X, Y, Z, Q) disappeared almost immediately after adding a DPPH solution to the tomato skin extract. It can be said that the antioxidative capability of these compunds is higher compared to the other compounds existing in the extract. The antioxidative capability of phenolic compounds is associated with their structure. Polyphenols, whose hydroxyl group is in the *ortho* or *para* position, undergo the oxidation reaction more easily [28].

CONCLUSIONS

The extracts of vegetables of the Solanaceae family were studied using CE. The presence of several phenolic compounds in tomato, eggplant, chilli pepper and potato skin extract was established. Of vitamins, pyridoxine was present only in the dried eggplant skin extract, but L-ascorbic acid was present in fresh tomato and chilli pepper skin extracts. A traditional method, a decolorization assay, using a stable free radical DPPH, was applied to determine the antioxidative capability of compounds. The monitoring of the oxidation reaction of the extract by CE allows the evaluation of the role of every single phenolic compound separately in this process.

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Bioaktiivsete komponentide määramine Solanaceae perekonna taimedes kapillaarelektroforeesi abil

Kati Helmja, Merike Vaher, Jelena Gorbatšova ja Mihkel Kaljurand

Fenoolsed ühendid (kaneelhappe derivaadid, flavonoolid, flavoonid, antotsüaanid) ja vitamiinid on laialdaselt levinud mitmetes taimsetes materjalides ning nende tähtsaima omaduse – käituda antioksüdandina – tõttu on nende uurimise olulisus pidevalt kasvanud. Antioksüdatiivsus seisneb vabade radikaalide sidumises, mis tekivad pidevalt biokeemiliste protsesside tulemusena. Liiga suures hulgas või sobimatus keskkonnas võivad vabad radikaalid muutuda toksiliseks, kahjustades biomolekule, nagu DNA, lipiidid, valgud, süsivesikud. Tagajärjeks on kudedes tekkivad degeneratiivsed muutused, mis on mitmete haiguste tekkepõhjuseks: põletikud, immuunsüsteemi nõrgenemine, südame- ja veresoonkonnahaigused, vähktõbi, Parkinsoni ning Alzheimeri tõbi ja ka vananemisprotsess.

Käesolevas töös on uuritud maavitsaliste perekonda Solanaceae kuuluvaid taimi, nagu tomat (*Solanum lycopersicum*), baklažaan (*Solanum melongena*), tšillipipar (*Capsicum annuum*) ja kartul (*Solanum tuberosum*), seoses nende bioaktiivsete ühendite rikkaliku sisaldusega (polüfenoolid, vitamiinid). Fenoolsed ühendid ja vitamiinid on ekstraheeritud vastavast taimest ultraheli ekstraktsioonil metanooli-veeseguga (80 : 20). Uuritavate ühendite lahutamisel ja identifitseerimisel on kasutatud kapillaarelektroforeesi (KE). Üldfenoolide sisaldus on määratud Price'i ja Butleri meetodi abil. Lisaks on hinnatud antioksüdatiivsust 2,2-difenüül-1-pikrüülhüdrasüül- (DPPH-)radikaali abil nii spektrofotomeetriliselt kui ka kapillaarelektroforeesiga, mis võimaldab jälgida iga ühendi antioksüdatiivsust eraldi.

Publication III

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Sample preparation for CE-DAD analysis of the water soluble vitamins in food products

Jekaterina Mazina[a] and Jelena Gorbatsova[a]*

[a]*Chair of Analytical Chemistry, Tallinn University of Technology, Akadeemia tee 15, Tallinn, 12618, Estonia*

Abstract

The capillary electrophoretic separation of six water-soluble vitamins (thiamine, nicotinic acid, nicotinamide, d-pantothenic acid, pyridoxine and ascorbic acid) was studied. Four CE backgrounds electrolytes were optimized and the most suitable ones were applied for investigation of analytes in real samples (yeast, beer, syrups). Several extraction procedures were performed in order to extract vitamins from proteins and phosphate groups. The research showed that it is possible to minimize interference from the solution with a complex composition and overcome the problem of peak overlapping by exchanging separation BGEs. Moreover, the second order data generated by CE-DAD instrument and Chemstation software (Agilents Technologies) were used to check of the peak purity.

Keywords: Capillary electrophoresis; Water-soluble vitamins; Extraction procedures; Foodstuff.

1. Introduction

Vitamins are organic substances that are essential to the health and have to be obtained from the diet on a regular basis because, with the exception of vitamin D, they cannot be produced within the body. Although a number of them were termed vitamins between the 1930s and 1950s, the nutritional science now recognizes only 13 substances, or groups of substances, as being true vitamins. The 13 substances are divided into two groups: fat-soluble and water-soluble. The group of water soluble vitamin group contains of eight vitamins collectively known as B-complex vitamins plus vitamin C (ascorbic acid) [1,2,3]. In this article five representatives of the vitamin B complex out of eight are dealt with, thiamine (B1), nicotinamide and nicotinic acid (B3), D-pantothenic acid (B5) and pyridoxine (B6). These vitamins have been chosen to avoid any vitamin-vitamin interactions in a standard mixture [3].

Sample preparation is an important step in the analysis of vitamins. On the one hand, vitamins can form complexes easily. For instance, in phosphorylated forms , e.g. thiamine mono –, di – and triphosphate, nicotinic acid adenine dinucleotide phosphate, nicotinamide adenine dinucleotide phosphate, D-pantothenic acid – 4’ -phosphate and pyridoxine-5’-phosphate. Moreover, free thiamine and nicotinic acid are often bound to proteins. [4,7] On the other hand this step is important for analysis as the major amount of interferents should be excluded. Several procedures have been offered for the extraction of water-soluble vitamins. These are acid hydrolysis,

* Jelena Gorbatsova. Tel.:+372-555-65-089.
E-mail address:jgorbatsova@yahoo.com.

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enzymatic treatment and combination of two previous [5,6,7]. An alternative procedure for extraction has been proposed by H. Okamoto et al., namely, in-capillary enzyme reaction method [8].

of the complex approach should still be considered. In this work, CE systems were optimized in order to find the best conditions for the determination of analytes in the sample.

2. Methods and materials.

2.1. Chemicals and reagents

The purity of chemicals used was either 98% or higher. Nicotinamide ($B3_1$), nicotinic acid ($B3_2$) and calcium D-pantothenate (B5) were purchased from Fluka Chemie Gmbh (Biochemika, Switzerland). Three of the vitamin standards, namely, thiamine hydrochloride (B1) (Sigma-Aldrich, Japan), pyridoxine (B6) (Sigma-Aldrich, Germany) and L-Ascorbic acid (C) (Riedel-de Haën Sigma-Aldrich Laborchemikalien Gmbh, Seelze, Germany) were obtained from Sigma Chemicals. The structure of analytes used in this work is shown in Figure 1.

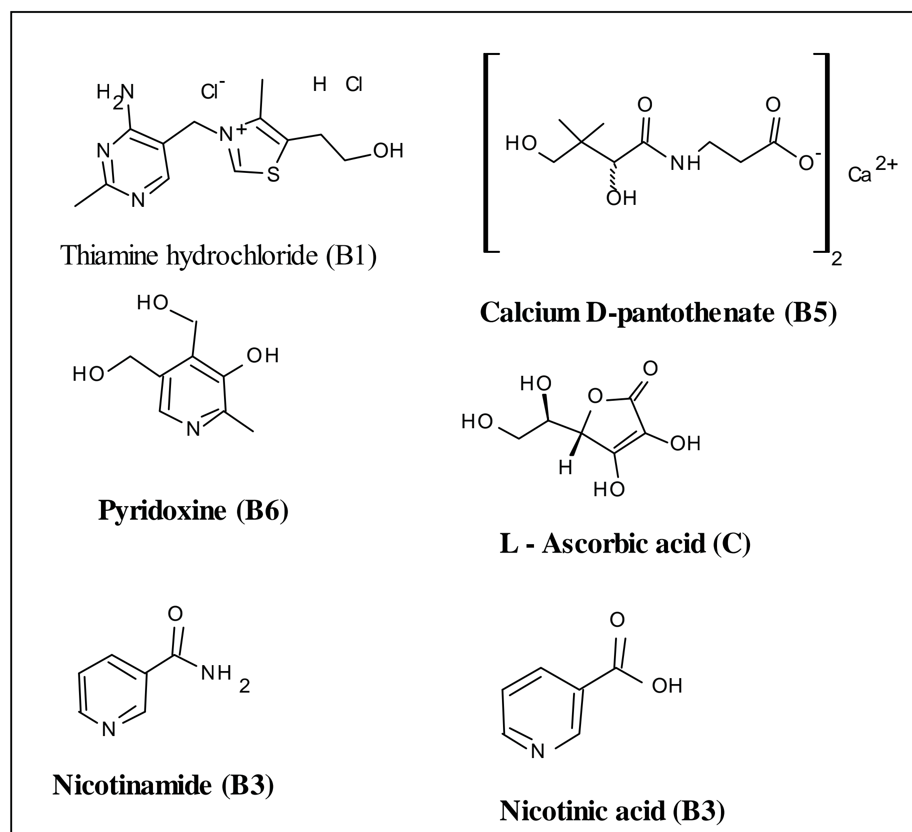

Fig. 1. Structures of the analytes.

Takadiastase from Aspergillus oryzae (No 86250) was obtained from Fluka (Biochemika, Switzerland). The other reagents (phosphoric acid, sodium phosphate, boric acid, sodium dodecyl sulfate (SDS), sodium tetraborate decahydrate, hydrochloric acid, sodium acetate and sodium hydroxide) were purchased from Sigma-Aldrich (Germany). For the preparation of standard solutions the deionised water (pH 7.0), purified with a Milli-Q system (Millipore Corporation, Bedford, USA) was used.

The samples used in the study were following: natural products yeast “Veski-Mati” and beer “Saku Kuld”, which are the rich source of vitamins B, food supplements enriched with synthetic vitamins “Doppelhertz® cardiovascular energy tonic” and syrup “Floradix Kindervital for Children”. All the foodstuffs studied were purchased at local sources.

2.2. Sample preparation

In the present research, four methods of sample preparation were used, viz. three types of extraction, and dilution. Doppelhertz and Floradix were diluted twice and three times with 40 mmol/L boric acid, pH 4.5 in order to decrease viscosity. For extraction 10 mL of a fluid (beer, Doppelhertz or Floradix) or yeast mixture (2 g yeast and 8 ml of water) was weighted in a 15 mL polypropylene (further PP) centrifuge tube.

2.2.1. Hydrochloric acid extraction

100 µL 37% hydrochloric acid was added to the sample in a 15 mL PP centrifuge tube. The solution in the closed tube was placed in an oven at 100°C for 30 min. After having been allowed to cool, it was adjusted to pH 4.5 with a 2.5 mol/L sodium acetate and diluted to 15 mL by Milli-Q water.

The supernatant was separated by centrifugation for 10 min at maximum speed and filtered through a 0.45 µm cellulose acetate filter. The filtrates were used for further investigation.

2.2.2. Enzymatic treatment

The sample mixture was adjusted to pH 4.5 with a 2.5 mol/L sodium acetate. 0.666 g of Takadiastase was added to the sample in a 15 mL PP centrifuge tube. The solution was sonicated at 37°C for 90 min and diluted to 15 mL by Milli-Q water, then thoroughly shaken and cooled.

The supernatant was separated by centrifugation for 10 min at maximum speed and filtered through a 0.45 µm cellulose acetate filter. The filtrates were used for further investigation.

2.2.3. Two-step extraction

100 µL 37% hydrochloric acid were added to sample mixture in a 15 mL PP centrifuge tube. The solution in a closed tube was placed in an oven at 100°C for 30 min. After being allowed to cool, it was adjusted to pH 4.5 with 2.5 mol/L sodium acetate. 0.666 g of Takadiastase was added to the sample in a 15 ml PP centrifuge tube. The solution was then sonicated at 37°C for 90 min and diluted to 15 mL by Milli-Q water. Then thoroughly shaken and cooled.

The supernatant was separated by centrifugation for 10 min at maximum speed and filtered through a 0.45 µm cellulose acetate filter. The filtrates were used for further investigation.[7, 6, 9]

2.3. CE systems

Several research groups have successfully used the all the three types of electrophoresis to separate vitamins [10], namely CZE [11], MEKC [12] and MEEKC[13,14, 15]. In present study following BGEs were used CZE phosphoric acid (pH 2,1), sodium phosphate (pH 7) and sodium tetraborate (pH 9.2), from MEKC boric acid/SDS (pH 8.2) were used. The best separation of vitamin standard mixture was achieved with MEKC, boric acid/SDS. The final BGE concentration was varied from 10 mM boric acid/12.5 mM SDS to 40 mM boric acid/60 mM SDS (with 10 mM increments). The last value was found satisfactory in terms of compromise between separation and the analysis time.

Analysis was performed using a CE apparatus from Agilent Technologies equipped with Chemstation software. An uncoated silica capillary (Agilent Technologies) with a total length of 58 cm (an effective length 50 cm, 50 µm i.d./365µm o.d.) was used for separation. Prior to use the capillary was rinsed with a 0.1M NaOH solution for 3 min and with the separation BGE for 3 min. The injection of the sample was performed hydrodynamically, 50 mbar 5 s. A positive electrical voltage, 20 kV was applied to the inlet reservoir for separation. A DAD detector was used with a spectral range of 200 to 400 nm. The fixed wavelengths for thiamine were at 232 nm (at pH 2.1) and 265 (at higher pH), for nicotinamide and nicotinic acid at 260 nm, for pyridoxine at 290 nm, for D–pantothenic acid at 210 nm and for ascorbic acid at 265 nm.

To optimize the separation of analytes in the sample extracts four different background electrolytes (further BGE) were chosen in order to perform separation based on as different CE mechanisms as possible. Since thiamine, pyridoxine, nicotinic acid and nicotinamide are zwitterions they have a positive charge at low pH and a negative at high pH. As for D-pantothenic and ascorbic acids they could only be negative. The first BGE was 25 mmol/L phosphoric acid with pH 2.1 for CE system with no or minimum EOF influence (only for positively charged molecules like thiamine, pyridoxine, nicotinamide and nicotinic acid). The second BGE was 25 mmol/L sodium phosphate with neutral pH. The third BGE was 25 mmol/L sodium tetraborate with pH 9,2. Finally, the forth MECK BGE was 40 mmol/L boric acid and 60 mmol/L SDS. The systems were evaluated in terms of selectivity, resolution and efficiency. The data is presented in Table 1 and Figure 2. As follows from the analysis of data only three systems, namely, phosphoric acid, sodium tetraborate and boric acid with SDS (Figure 2) were suitable to proceed with. Since, with the sodium phosphate BGE analytes were not completely separated (Figure 2 pH 7). Moreover, the resolution between second and third peaks was only 0.8.

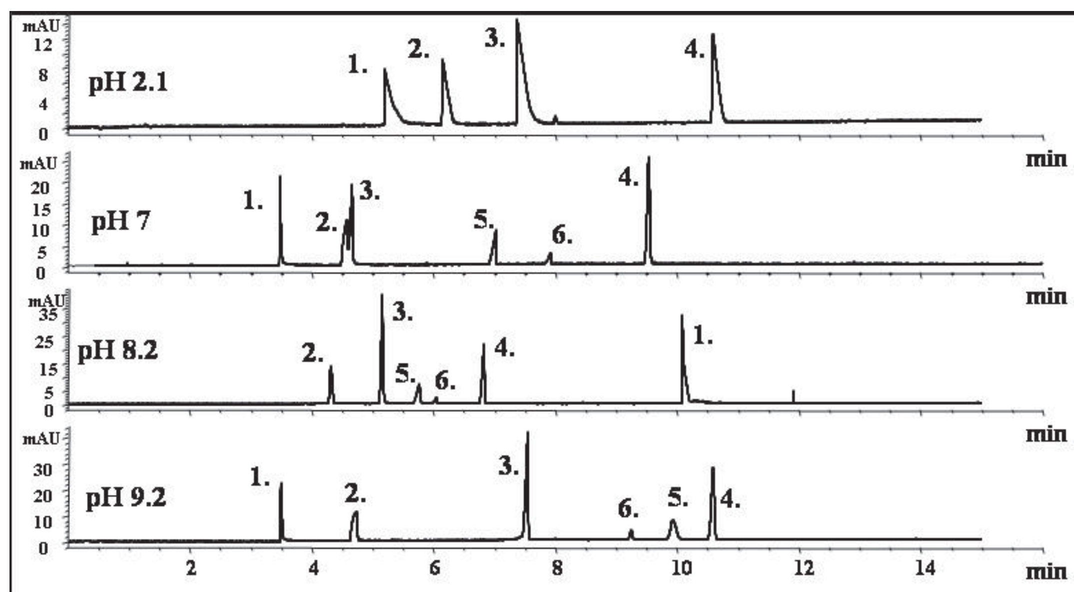

Fig. 2. The influence of background electrolyte influence on the separation of the standard vitamins mixture of the 100 µmol/L. Analytes: 1-thiamine, 2-nicotinamide, 3-pyridoxine, 4-nicotinic acid, 5-D-pantothenic acid and 6-ascorbic acid. CE conditions: 25°C, 20 kV, injection 50 mbar, 5 s, capillary effective 50 cm and total 58cm lengths. BGEs: pH 2,1 -25 mmol/L phosphoric acid; pH 7 - 25 mmol/L sodium phosphate; pH 8,2 – 40 mmol/L boric acid and 60 mmol/L SDS; and pH 9,5 - 25 mmol/L sodium tetraborate.

Table 1. Parameters of CE systems

		Selectivity, α				Resolution, R				Number of theoretical plates, N 10^4			
	pH	2,1	7	9,2	8,2	2,1	7	9,2	8,2	2,1	7	9,2	8,2
1	Thiamine	-	-	-	1.5	-	-	-	60.1	1.3	17.2	15.0	103.6
2	Nicotinamide	1.2	1.3	1.4	-	5.6	12.7	12.2	-	2.4	1.7	1.3	5.0
3	Pyridoxine	1.2	1.0	1.6	1.2	6.6	0.8	23.3	14.7	1.9	7.2	16.2	15.8
4	Nicotinic acid	1.4	1.2	1.1	1.1	17.1	18.6	4.9	10.2	6.7	21.9	19.9	15.8
5	D-pantothenic acid	ND	1.5	1.1	1.1	ND	27.4	5.5	8.5	ND	7.5	5.3	5.7
6	Ascorbic acid	ND	1.1	1.2	1.2	ND	9.3	19.9	14.1	ND	12.4	3.9	9.3

ND – not detected

3. Results and Discussion

The procedure for the identification and quantification of analytes in sample extracts included the following steps.

First, the presence of analytes in sample extracts was also determined by analyzing pherographic data and using spectroscopic techniques, i.e. by analyzing second-order signals or spectral- time second-order data provided by CE-DAD [16, 17, 18, 19]. Also, specific ChemStation programs for CE-DAD signal interpretation were used. A peak purity program was applied to the confirmation of peak selectivity. . To identify analytes in the sample solution a similarity program was used. The latter is based on the match of peak spectra with one another and could be defined by the following equations. [20, 21]:

$$\text{SIMILARITY} = 1000 \times r^2 \quad (1)$$

$$r = \frac{\sum_{i=l}^{i=n} \left[(A_i - A_{av}) \times (B_i - B_{av}) \right]}{\sqrt{\sum_{i=l}^{i=n} (A_i - A_{av})^2 \times \sum_{i=l}^{i=n} (B_i - B_{av})^2}} \quad (2)$$

where A_i and B_i are absorbencies measured at the first and second spectrum at the same wavelength. Moreover, A_{av} and B_{av} are the average absorbance of the first and second spectrum respectively. n is the number of data points. The method provides a measure of similarity ranging from identical (=1000) to different (=0). [22] In an ideal case no difference in the peaks spectra should be found. At the extremes, a similarity factor of 0 indicates no match and 1000 indicates identical spectra. Generally, values which are very close to the ideal similarity factor (greater than 995) indicate that the spectra are very similar, while values which are lower than 990 but higher than 900 are indicative of some similarity in spectra and the underlying data should be considered more carefully.

Second, for the identification and quantification of vitamins in sample extracts three CE BGE-s were tested in order elucidate the most suitable one (in terms of finding fewer overlapping peaks). Through an extensive testing, it was observed that separation with 25 mmol/L phosphoric acid at pH 2.1 was the best for thiamine (Figure 3), pyridoxine, nicotinamide and nicotinic acid. At the same time no D-pantophenic acid was identified in sample extracts. This may be first explained by that the peak of the sample matrix completely overlapped with that of D-pantothenic acid making identification of the compound impossible.. Second, the amount of D-pantothenic acid was too small enable the detection of the compound. Ascorbic acid was found only in Floradix solutions.

For Doppelhertz and Floradix acidic, enzymatic and two-step extractions gave the negative results and no vitamins were identified whatever. However, it appeared that direct injection of the product (diluted three times) could help to reveal the analytes. The data are presented in Table 2 and Figure 5.

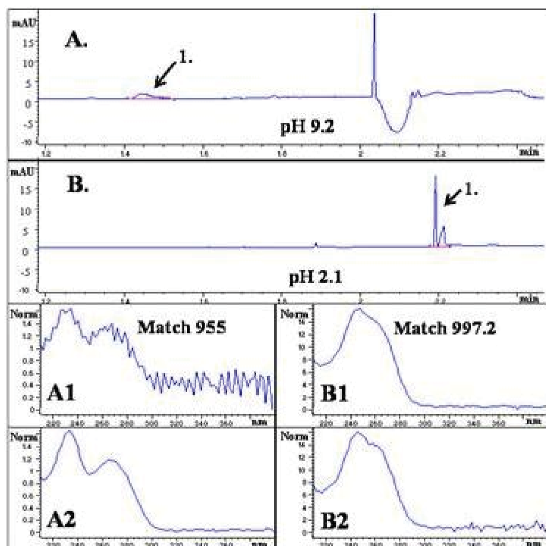

Fig. 3. The BGE systems used to determine thiamine (1.) in the yeast extract. Analytes: 1-thiamine. CE system: A - 25 mmol/L sodium tetraborate, pH 9.2, A1 - the spectrum of absorbance of thiamine in the sample; A2 – the spectrum of absorbance of thiamine in the standard mixture; B - 25 mmol/L phosphoric acid, pH 2.1, B1 – the spectrum of absorbance of thiamine in the sample, B2 – the spectrum of absorbance of thiamine in the standard mixture. CE conditions: 25°C, 20 kV, injection 50 mbar, 5 s, capillary effective 50 cm and total lengths 58cm. Similarities (Match) between the peaks are also presented.

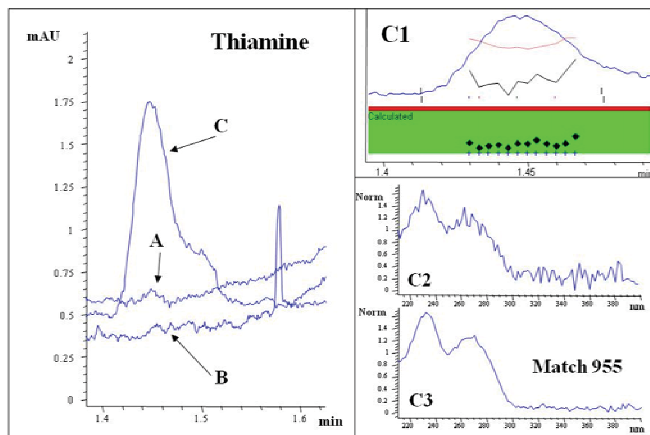

Fig. 4. The comparison between the electropherogramms of thiamine in the yeast extracts prepared using different extraction procedures (A, B and C). CE conditions: 25°C, 20 kV, injection 50 mbar, 5 s, capillary effective 50 cm and total 58cm lengths, 232 nm. A – acid hydrolysis, B – enzymatic treatment, C – two-step extraction (the last extraction procedure): C1 – purity factor of thiamine, C2 – thiamine absorbance spectra in sample, C3 – thiamine absorbance spectra in standard mixture. Similarities (Match) between the peaks are also presented.

Table 2. The amounts of analytes established in sample solutions, with the uncertainty 95% for n=3.

	µg/g	µg/10 ml	µg/10 ml	µg/10 ml
	Yeast	**Beer**	**Doppelhertz**	**Floradix**
Thiamine	143 ± 0.6	ND	484 ± 37	ND
Pyridoxine	82 ± 4	ND	400 ± 43	410 ± 60
Nicotinamide	115 ± 10	320 ± 40	1397 ± 219	4820 ± 370
Nicotinic acid	ND	ND	96 ± 5	55 ± 6
Ascorbic acid	ND	ND	ND	18964 ± 2741
D-Pantothenic acid	ND	ND	ND	ND

ND – not detected

As follows from Table 2, the major water-soluble vitamin found to be present in yeast was thiamine 143 µg/g , while pyridoxine was present in the lowest amount, 82 µg/g. In beer, only nicotinamide was found at a concentration of 320 µg/10 ml. Unfortunately neither ascorbic or D-pantothenic acid were determined in yeast and beer extracts. This may be accounted for the use of extraction conditions like high temperature for ascorbic acid or the complex composition of the solution for D-pantothenic acid (the spectra of the analyte could easily be overlapped by those of the other substances). Pyridoxine, nicotinamide and nicotinic acids were quantified in both Doppelhertz and Floradix. Between the two products there were only slight differences in pyrodixine concentration, 400 and 410 µg/10 ml, respectively. Ascorbic acid was determined only in Floradix at a concentration of 18.96 mg/10 ml.

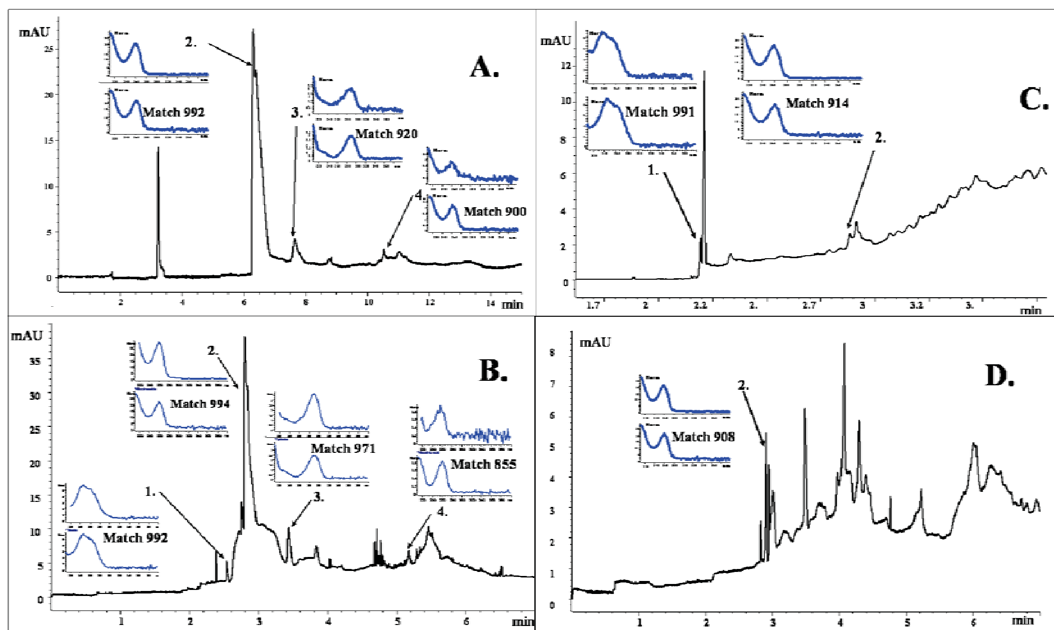

Fig. 5. Electropherograms of sample solutions with the vitamins detected. Analytes: 1-thiamine, 2-nicotinamide, 3-pyridoxine, 4-nicotinic acid, 5-D-pantothenic acid and 6-ascorbic acid. CE system: 25 mmol/L phosphoric acid pH 2,1, 25°C, 20 kV, injection 50 mbar 5 s, capillary effective 50 cm and total 58cm lengths. Samples: A – Floradix, B – Doppelt, C – yeast and D – beer. Similarities between the peaks are also shown: above - the spectrum

of absorbance of the analyte in the sample, below - the spectrum of absorbance of the analyte in the standard mixture.

4. Conclusion

It follows from this work that it is possible to minimize matrix interferences from the solution with complex composition by optimized choice of separation BGEs. As for more, the three extraction procedures were under investigation and only one, two-step extraction (acid hydrolysis followed by enzymatic treatment) was found satisfactory for natural products. Finally, a method was developed for the determination of water-soluble vitamins in various food products.

Acknowledgment

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Publication IV

J. Gorbatšova, M. Jaanus, M. Kaljurand.- Digital Microfluidic Sampler for a Portable Capillary Electropherograph - *Analytical Chemistry* 2009, 81, 8590–8595.

Anal. Chem. **2009,** *81,* 8590–8595

Digital Microfluidic Sampler for a Portable Capillary Electropherograph

Jelena Gorbatsova,† Martin Jaanus,‡ and Mihkel Kaljurand*,†

Department of Chemistry, Faculty of Science, and Department of Computer Control, Faculty of Information Technology, Tallinn University of Technology, Tallinn, Estonia

A new sample introduction/analysis approach was developed by combining a digital microfluidic (DMF) device with a portable capillary electrophoresis (CE) analyzer based on short separation capillary and contactless conductivity detection. The DMF sample injection was performed by transporting sample and buffer droplets in succession under the CE capillary inlet end allowing the capillary to be immersed into the sample/buffer droplet, and CE separation was performed by applying a high voltage between the (grounded) buffer droplet and CE outlet reservoir. Electrowetting on dielectric (EWOD) phenomenon was used for droplets actuation. With the use of the DMF sampler, CE separation of a mixture of vitamins was achieved. A droplet evaporation process with simultaneous concentration of sample in the droplet was monitored. It was found that the concentration process closely followed the theoretically predicted function.

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Ever since the landmark work by Jorgenson and Lukacs in the early 1980s[1,2] capillary electrophoresis (CE) has become a widely used and established analysis technique. Once it was considered a competitive method to separation ones such as gel electrophoresis and high-performance liquid chromatography, but its development stagnated in recent years. CE is widely applied in bioanalytical research, such as separating amino acids, peptides, proteins, DNA and RNA fragments, small molecules, single-cell analysis, and chiral separation.[3] But activity in the instrumental development in the electroseparations field has moved toward development of microfluidic chips.[4] However, the fabrication of microchips usually requires expensive equipment and complicated operations, which may limit its broad application in routine analysis. Even more, the lack of convenient *word-to-chip* interfaces[5] requires otherwise elegant miniature microfluidic designs to be surrounded by bulky supporting devices such as power supplies, pumps (feeding the microchip with necessary solutions), and detectors.[6] Although the field of microfluidics is still looking for its "killer application",[7] there is room for instrumental development in conventional CE systems. The appearance of small capacitively coupled contactless conductivity detectors (C^4D)[8,9] and small-sized high-voltage power supplies makes possible the development of portable CE apparatus. Such instruments could be a solution for in situ and point-of-care analysis. There are several reports of portable CE devices based on C^4D.[10,11] If the miniaturization of supporting equipment is successfully solved such devices could easily accommodate both separation platforms, namely, microchip or capillary. The advantage of the latter approach is the fact that the capillary is a disposable material and significantly cheaper than tailor-made microchips. However, sample introduction remains a bottleneck in both variants. If the sample is available in large quantities, then sampling portable CE analyzers could be solved by exchanging buffer/sample vessels[10] or using sample injection between two pieces of capillary via a syringe.[11] Sampling microvolumes is more difficult. Commercial CE analyzers equipped with an autosampler carousel usually require 50 μL of sample. There has been some research on sampling from flow (i.e., coupling flow injection analysis with the CE). It has proved that it is more flexible and easier to computerize than carousel autosamplers[12,13] but still requires a large amount of sample. As for biological samples they frequently come in small amounts. Sampling small amounts of sample has long been of interest. In 1991, Jorgenson's group had already developed the optical-gating injection technique for CE systems,[14,15] the flow-gating injection technique,[16,17] and Zare's group the spontaneous sample injection.[18] Recently, a picoliter-scale translational spontaneous sample introduction for CE was proposed by Zhang et al.[19] This sampler utilizes slotted sandwich reservoirs for sample and buffer droplets mounted on a computer-

* To whom correspondence should be addressed.
† Department of Chemistry.
‡ Department of Computer Control.

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programmed translational platform. The sample injection was performed by linearly moving the stage between the buffer and sample droplets, allowing the capillary inlet first to enter the sample solution and then move it into the buffer droplet. However, it is difficult to imagine such sampler design being implemented as a portable one.

Small droplets of liquids can be actuated by electrowetting on dielectric (EWOD), a phenomenon discovered by Berge in 1993.[20] In electrowetting experiments, a droplet of conductive fluid is positioned over an electrode coated with a hydrophobic insulator. When a potential is applied across the insulator, it becomes charged, making it attractive for the fluid to wet the surface. In a typical electrowetting device, ~50−100 V is applied across a 1 μm thick insulator, causing the contact angle to decrease from 115° to 75°. EWOD has attracted the interest of many researchers as a means for controlling the movement of microliter-sized (and smaller) droplets of fluids on surfaces. This gives rise to a distinct paradigm of channel microfluidics—digital microfluidics (DMF). The DMF concept has many prospective applications in bioanalysis.[21−23] But its application is still rather limited to a small enthusiastic group of proponents because of the expense and complicated process of making DMF devices (i.e., the fabrication of electrodes and coating with layers of dielectric and hydrophobic materials). Abdelgawad and Wheeler speculate[24] that a fast, accessible, and inexpensive method for forming DMFs devices will have a similar impact on promising DMF technology as the development of soft lithography[25] had on channel microfluidics. To address the need for simplifying the preparation of DMF devices the authors demonstrated that DMF devices could be formed from copper substrates or gold compact disks using rapid marker masking to replace photolithography.[24] The consumer product Saran food wrap (polyethylene film) and commercial water repellents were used as dielectric and hydrophobic coatings, respectively, replacing commonly used and more expensive materials such as Parylene-C and Teflon-AF.

DMF is inherently an array-based technique and is a good match for array-based biochemical applications. At first sight, the DMF platform seems ideally suited to electrokinetics-based separations. It could be seen as a programmable sampler in cases when only microscopic amounts (<10 μL) of sample are available. However, separations have been difficult to implement into the DMF platform. Pessimism expressed by Fair et al.[22] on this matter has been dissolved only by the recent work by Abdelgawad et al.,[26] which described a hybrid microfluidic device where sample was delivered to the separation chip channel by a DMF device. The hybrid microfluidic device was prepared using soft lithography for the separation microchip part, and the glass substrate was coated by multiple thin layers of various materials, i.e., using a technology beyond the reach of the typical mainstream CE laboratory. In this article, we attempt to solve the problem of interfacing DMF platforms to common CE using a portable CE-C^4D instrument for separation and DMF device for computer-controlled sampling from droplets. To pursue "the spirit of low-cost digital microfluidics" advanced by Abdelgawad and Wheeler,[24] we aim to demonstrate that the actuation of droplets can be achieved using an electrode system prepared from the copper substrate of the common printed circuit, coated by only food wrap (without the hydrophobic layer).

EXPERIMENTAL SECTION

Materials and Reagents. The substrate used for fabricating the DMF chips was epoxy single-sided copper-clad laminate (copper layer 17.5 μm; Elfa Elektroonika AS). Other fabrication materials included food wrap (Lindner Haushaltsprodukte GmbH, D-51149, Köln) with an estimated thickness of 10 μm, silicone oil (Valvoline Europe, a division of Ashland Inc., The Netherlands), and car windscreen water repellents Rain Away (Motip Dupli BV, Netherlands) and Nano Vitro (Motip Dulpi GmbH, Germany). An uncoated capillary (Agilent Technologies, Germany) with dimensions of 150 μm o.d. and 50 μm i.d. was used during the studies. Chemicals used in device testing unless otherwise indicated were purchased from Sigma-Aldrich.

Fabrication of the DMF Sampler. For the fabrication of the DMF platform, we adapted the procedure proposed by Abdelgawad and Wheeler.[24] First, an electrode array design (a double row of electrodes with electrical contacts but without gaps between electrodes) was printed by an HP LaserJet 2420 printer to a HP color laserjet transparency film creating a photomask. Second, substrates were exposed (by a UV exposure unit LV 202E, MEGA) through a photomask and developed in liquid photoresist developer (Seno 4006 liquid photoresist developer concentrate, Mega Electronics Ltd., Cambridge). Third, copper was etched on the surface of the substrate in ferric chloride. Fourth, gaps between electrodes were formed using the corner of a razor blade. Fifth, the substrate area—excluding an outline of the electrode array—was covered with nail polish and dried out. Sixth, the plate was replaced in a ferric chloride solution to etch copper between the gaps of the electrodes. It was proved that the last two steps were vital in creating gaps of the necessary size. If only photolithography was used the sizes of the gaps between the electrodes were either unexpectedly big or there were no gaps at all. Moreover, if only scratching was used the short-circuiting between electrodes was detected frequently. So in the end to increase the possibility to receive a "working plate" both techniques were applied. After this step, the plate was washed in acetone and Milli-Q water. Electrodes had an area of 2 mm × 2 mm, and the gaps between electrodes were estimated as 95 μm. Silicone oil was then dispensed onto the surface of the array and heated on a hot plate at 70 °C for 30 min. The food wrap was placed onto the oil-coated device using tweezers. Finally, the sampler was heated on a hot plate at 70 °C for 2 min, forming a seal between the wrap and the substrate. With the use of the technology described above, a set of electrode arrays was fabricated.

As in most cases, treatment of the dielectric layer with hydrophobic material was used as the final stage of fabrication. We tested several consumer products (car windscreen water

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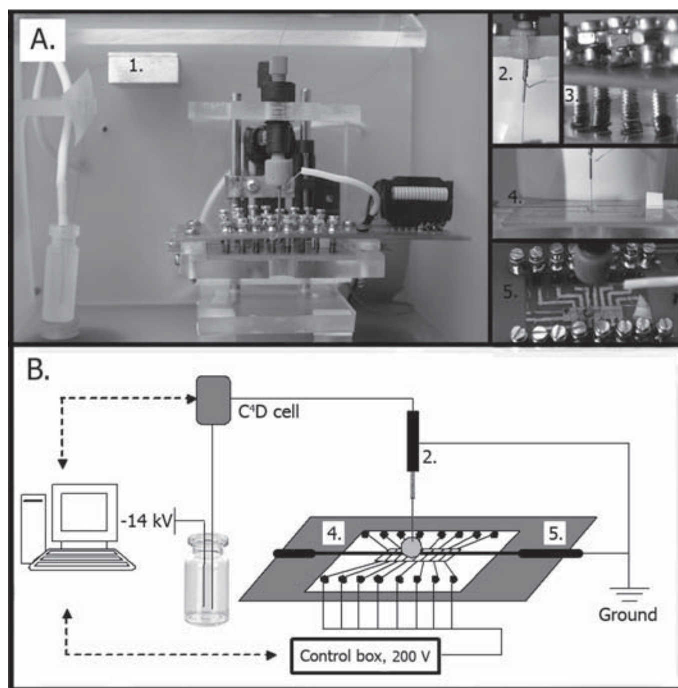

Figure 1. Interfacing the DMF sampler into the portable CE analyzer. (A) Portable CE analyzer with DMF sampler. (B) Instrumentation scheme: 1, capacitively coupled contactless conductivity detector (C^4D); 2, grounded piece of syringe needle with the inlet end of separation capillary; 3, spring-loaded contact pins; 4, ground electrode during droplet actuation; 5, rectangular opening for exposing the electrode array.

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repellents) and Teflon-AF for forming the hydrophobic layer. The water repellents were applied to a piece of food wrap by wiping with a dampened tissue. Also 1%, 2%, and 3% Teflon-AF solutions in Fluorient FC-75 were spin-coated at 2000 rpm for 1 min followed by annealing on a hot plate (75 °C, 2 min).[27] Through extensive testing, we observed no significant improvement of the performance of the DMF (e.g., in terms of the increase of the droplet contact angle) when the food wrap was coated with each of the water repellents. However, during CE runs (when high voltage was applied) and following droplet actuation the Teflon-AF layer tended to form a white deposit, which shows that probably Teflon-AF was coming off from the food wrap. This indicates that the process of coating food wrap with hydrophobic material needs to be studied further. After those experiments it became clear that droplets can be actuated (perhaps less rapidly but satisfactory for our purposes) on uncoated food wrap as well.

A simple customized clamp with spring-loaded contact pins was used to connect a control box to the electrode array. It was made from copper cladding and had a rectangular opening for exposing the electrode array (Figure 1, parts A and B). On the clamp, a piece of gold-painted capillary was fixed over the electrode array. This capillary served as a ground electrode during the droplet actuation and CE run. Capillary dimensions were 150 μm o.d. and 8 cm length. First, the whole capillary was painted in gold (Glanzgold NF 12% 2 g, Schjerning, Denmark) and dried at 600 °C for 3 min, then cooled at 50 °C for 30 min. Second, a silver paint (silver conductive paint, Electrolube, England) was applied to the end of capillary at room temperature (approximately 2 cm above capillary end) and dried. The clamp performed electrical contacts between the electrode array and a custom-built electronic control box. A PC was used to handle the control box, which was capable of independently switching each output between ground and the voltage of a 200 V ac. The signal frequency was 10 kHz. Software was written in Visual Basic. The clamp construction allowed the quick and simple replacement of an electrode array if malfunction occurred.

Interfacing the DMF Sampler into the Portable CE Analyzer. The CE instrument used in this work has been described by Seiman et al.[11] The original portable instrument with cross-sampler and C^4D was modified. The cross-sampler was removed, and the electrode array holder was placed there instead. The separation capillary was directed through the grounded piece of syringe needle located vertically to the center of the array of electrodes (at a height of 2 mm above the array (Figure 1A, part 4). The inlet part of a thin separation capillary (50 cm long, 150 μm o.d., and 50 μm i.d.) was covered first by gold paint and second by silver paint (as described previously), leaving the end of the capillary covered only with gold to create an electrical contact with the ground (Figure 1). Although such contact is unnecessary in the present setup (the grounding is established via a horizontal electrode on the electrode array holder), it provides flexibility if another electrode array configuration ever became of interest. Separation voltage was −14 kV.

Device Operation. In the first step, 10 μL droplets of sample and separation buffer were manually dispensed by pipet on the opposite borders of the electrode array. The separation capillary

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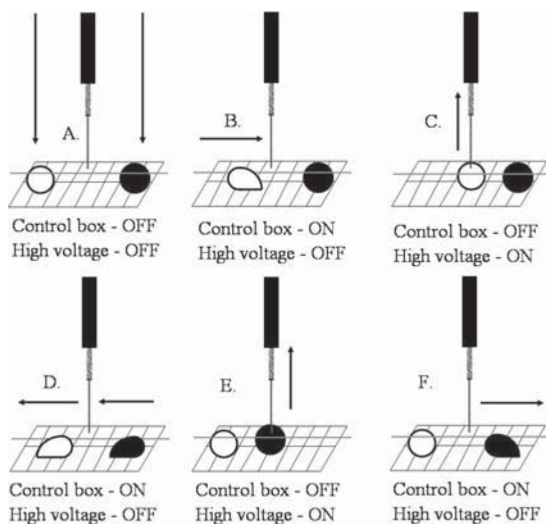

Figure 2. Droplet actuation. The directions of the liquid movements are shown by arrows. Sample droplet is colored in white, and buffer is in black, correspondingly. (A) The 10 μL droplets of sample and separation buffer were manually dispensed by pipet on the opposite borders of the electrode array. (B) The sample droplet was actuated until the separation capillary inlet end. (C) Electrokinetic sampling was performed by applying negative 14 kV for 3 s. (D) The sample droplet was moved back to its initial position, and the buffer droplet was placed under the capillary. (E) Run of separation. (F) After the CE run had been completed the buffer droplet was returned to its original position.

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was fixed in the middle of the electrode array (Figure 2A). In the second step, the sample droplet was actuated until the separation capillary inlet end (Figure 2B). In the third step, electrokinetic sampling was performed by applying −14 kV for 3 s. (Figure 2C). In the fourth step, sample was moved back to its initial position and buffer was placed under the capillary (Figure 2D). In the fifth step, the buffer droplet was left under the separation capillary for the whole CE run (Figure 2E). In the sixth step, after the CE run was completed the buffer droplet was returned to its original position (Figure 2F). For reproducibility studies, new sample and buffer droplets were dispensed onto the electrode array for each electropherogram run. For studying the process of droplet evaporation one buffer droplet and one sample droplet were periodically moved to the capillary until the size of evaporated droplets could no longer be actuated. Droplet actuation was monitored and recorded by a video camera positioned over the top of the device.

RESULTS AND DISCUSSION

Although the described experiments could be achieved with a single row of electrodes, we implemented a double row for droplet actuation. Driving voltage was simultaneously applied to two adjacent electrodes in the array when the upper electrode was grounded. The idea of using such a catenary was proposed by Dubois et al.[28] Indeed as suggested in Figure 1, actuation of the droplet is possible because of the direct electrical contact of

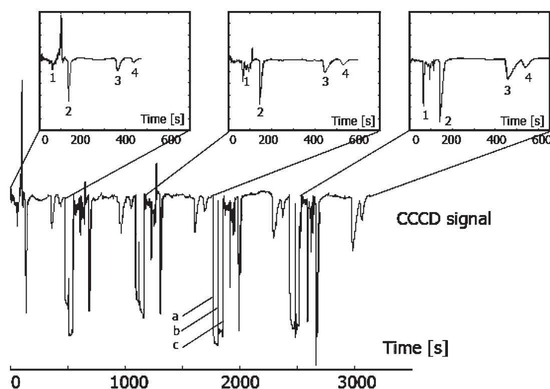

Figure 3. Droplet evaporation monitoring. Peaks: 1, unknown; 2, thiamine; 3, pyridoxine; 4, nicotinamide. Events: a, HV off; b, HV "on" for sampling; c, HV "on" for separation. Buffer was 250 mmol/L acetic acid. Separation voltage was −14 kV.

the droplet with gold-painted capillary over the substrate. This catenary serves both a guide and an electrode, guiding the droplet like a tramway cable. No great positioning precision of the catenary is required. It appeared that the droplet movement was more robust on the double-row array than on a one-row array. The electrode array occasionally malfunctioned either because of damage to the food wrap cover (perhaps by dielectric breakdown) or the short-circuiting of two adjacent electrodes. In such cases, replacing the food wrap sheet was straightforward and rapid. In the case of short-circuiting, the cap between electrodes could be cleaned easily from the conducting deposit under the microscope.

Performance of the DMF Sampler. Acetic acid is known as a volatile background electrolyte (BGE) suitable for use with C^4D.[29,30] The complete volatility of BGE was important for this work because it meant there was no danger of depositing nonvolatile BGE components on the food wrap surface which covers the electrode array. For experiments 250 mmol/L acetic acid at pH 2.5 was used as a BGE. Consequently, analytes movement was achieved mostly by electrophoresis because electroosmotic flow (EOF) is either too slow or does not exist at all at this pH. A typical electropherogram of three analytes, thiamine, pyridoxine, and nicotinamide (40 μmol/L of each in Milli-Q water), is shown in Figure 3. Perhaps as a result of the low pH of the BGE, the analytes experienced severe electrokinetic dispersion, which reduced the efficiency of the peaks as low as $N = 2000$. However, since the baseline resolution of the analyte peaks had been achieved, improving the efficiency by investigating other possible BGEs was not a primary goal of this study.

During the initial experiments, we evaluated the droplet sampler for reproducibility using the procedure described above (see the Device Operation section). For each new run new sample and buffer droplets were pipetted to the electrode array. Performance data of six parallel runs for one of the sample concentrations (40 μmol/L thiamine and 80 μmol/L pyridoxine) are listed in Table 1. When evaluated in replicate trials, the sampler was characterized by good average retention time reproducibility (3%

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Table 1. Reproducibility Data

	thiamine		pyridoxine		relative data	
	t_T [s][a]	S_T[a]	t_P [s]	S_P	t_T/t_P	S_P/S_T
run no. 1	484	6.04	854	7.93	0.57	1.31
run no. 2	491	6.80	865	9.22	0.57	1.36
run no. 3	489	5.87	873	8.54	0.56	1.46
run no. 4	476	6.58	851	8.58	0.56	1.30
run no. 5	488	6.19	873	7.48	0.56	1.21
run no. 6	521	7.09	950	8.27	0.55	1.17
mean	490	6.43	877	8.34	0.56	1.30
SD	15	0.47	37	0.60	0.01	0.10
%	3.1	7.3	4.2	7.2	1.8	7.7

[a] t, migration time; S, peak area.

of RSD; 1% RSD for relative migration time) and peak area variation (8% RSD).

Calibration lines were constructed using seven concentration values for thiamine and pyridoxine and five concentration values for nicotinamide with at least five parallels for each value. Thiamine and pyridoxine had correlation coefficients CC = 0.995 and CC = 0.992, correspondingly (over concentration ranges from 2 to 40 and 4 to 80 μmol/L). However, for nicotinamide the correlation coefficient was CC = 0.97 over a linear range of 20−80 μmol/L. The limit of detection for analytes was determined from the calibration line according to the procedure described by Meier and Zund.[31] It was 0.6 μmol/L for thiamine, 1.7 μmol/L for pyridoxine, and 4.2 μmol/L for nicotinamide. Calibration data are summarized in Table 2.

Abdelgawad et al. speculate that the primary source of the peak area variance is sample dispensing (i.e., pipetting) and that in future experiments with on-chip dispensing from reservoirs (instead of pipetting to the surface), the peak area reproducibility of the on-chip method should be substantially improved.[26] Other sources of the variation (both the peak migration time and area) might be due to the robust construction of our portable CE instrument, e.g., the absence of thermostatting. This conclusion is supported indirectly by good reproducibility data of the relative peak position.

On-Chip Sample Monitoring. The DMF is typically implemented in one of two different configurations: the closed format (also known as the two-plate format), in which droplets are sandwiched between two substrates patterned with electrodes,[32] and the open format (also known as the single-plate format), in which droplets are placed on top of a single substrate, housing both actuation and ground electrodes.[33] In this work, despite the using of the catenary, the design of the DMF device has an open configuration. Obviously, evaporation rates of droplets are higher in open-format devices, which may be advantageous or inconvenient depending on the application. To demonstrate the utility of the sampler for monitoring the droplet evaporation process the following experiment was performed. The droplet containing the sample was delivered on one side of the electrode array and the buffer droplet on the opposite side, with the separation capillary located in the middle. During the run, the sample and buffer droplets were alternately moved under the capillary and electropherograms were recorded for each sample/buffer droplet passage. The detector signal is shown in Figure 3 with zoomed-in electropherograms. It was then possible to record five electropherograms from the droplet, which was initially 10 μL in size until it evaporated to a size unsuitable for further actuation. The rate of evaporation of a spherical droplet of a pure solvent in still air of constant temperature with initial radius r_0 and mass m is given by the "d^2 law" derived from the boundary-layer theory of a shrinking droplet.[34] This means that the volume of a droplet, $V(t)$, decreases in time t as a 3/2 power, which in turn indicates that the sample in the droplet concentrates as power 3/2 in time:

$$c(t) = \frac{m}{V(t)} = \frac{3m}{4\pi(r_0\sqrt{(1-\beta t)})^3} = \frac{c_0}{(\sqrt{1-\beta t})^3} \quad (1)$$

Here $c(t)$ is the concentration at time t, c_0 is the initial concentration, and β accounts for the evaporation process. The least-squares fitting experimental data to eq 1 results in excellent conformation of this law (Figure 4). Using Excel Solver, the following values for the parameter β can be calculated: $\beta = (1.04 \pm 0.01) \times 10^{-2}$ min^{-1}, $\beta = (1.14 \pm 0.02) \times 10^{-2}$ min^{-1}, and $\beta = (1.19 \pm 0.03) \times 10^{-2}$ min^{-1} for nicotinamide, thiamine, and pyridoxine, correspondingly. Uncertainties for β were calculated according to the procedure described by Bevington and Robinson.[35] The β parameters are close but still statistically different. The smallness of the uncertainties (RSD = 1−3%) in β values is another indication of the good performance of the sampler, as well as the precision of the "d^2 law".

Evaporation of sample droplets can be ignored if new droplets are dispensed for each measurement and analysis conditions are reproducible. On one hand, the evaporation of the buffer droplet during the CE run can then be considered as a sort of programming of the buffer concentration during the analysis, which may be beneficial in some cases. This work demonstrates there is no problem carrying on the analysis. On the other hand, if monitoring is of interest then reasonable results can be obtained using a reliable internal standard. New buffer droplets should be dispensed each time in those cases when the interpretation of the electropherograms is difficult. An advantage of the evaporation phenomenon in open DMF systems is the concentrating of the sample in time. As demonstrated in this work, 3-fold concentration of the sample occurs during a 1 h experiment.

The method of interfacing of the DMF sampler to a separation channel reported in this article overlaps somewhat with that which was reported by Abdelgawad et al.[26] They implemented a microchip with "cross" layout of sample and buffer channels. Sampling from the droplet was preformed to the sample channel of the microchip. In such a design only a very small fraction of sample is used for separation. In our design the sampling from the droplet is done directly to the separation capillary. This allows us to use much more larger amounts of sample for separation. Additionally the droplet evaporation process could be used in

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Table 2. Performance Data from the Droplet Sampler

compounds	no. of points	linear range, μmol/L	calibration line equation[a]	CC, R^{2b}	LOD, μmol/L[c]
thiamine	26	2−40	$y = (2720 \pm 40)x + (200 \pm 800)$	0.995	0.6
pyridoxine	25	4−80	$y = (1370 \pm 30)x + (3000 \pm 1000)$	0.992	1.7
nicotinamide	17	20−80	$y = (390 \pm 20)x - (400 \pm 900)$	0.970	4.2

[a] x, concentration; y, C^4D response; line parameters are given with standard deviations. [b] R^2, square of calibration line correlation coefficient CC. [c] LOD, limit of detection estimated from the calibration line according to Meier and Zund (ref 31).

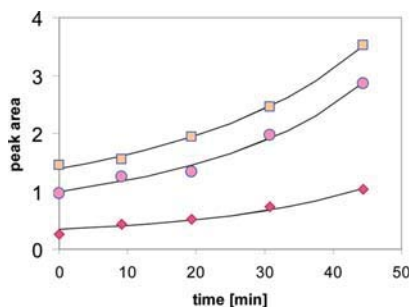

Figure 4. Sample concentration kinetics during evaporation of the droplet: squares, thiamine; rings, pyridoxine; diamonds, nicotinamide.

order to concentrate the sample, which reduces the demands to the detector sensitivity. Although not demonstrated here the approach used in this article might easily allow the use of concentration methods, well-known in CE (like sample stacking), which reduce the demands to the detector sensitivity even more. The design proposed in this work enables us to use one droplet for delivering sample and another one for buffer delivery. Such arrangement allows us to monitor various processes occurring in the droplet easily (e.g., chemical reactions) as demonstrated here in the case of droplet evaporation. But an important advantage of DMF samplers could be computer-controlled preprocessing of analytes as was well-demonstrated in Abdelgawad et al.'s paper. In spite of the fact that it has not been the aim of this research the DMF sampler design proposed in this paper is rather robust and flexible. The replacement of the customized (for sample preprocessing) electrode plate and software should be simple and rapid.

CONCLUSION

We have proposed, fabricated, and tested a device that integrates a DMF sampler with a portable CE analyzer. The device had reasonable performance in terms of reproducibility. It uses an array of electrodes covered by food wrap for transporting sample/buffer droplets to an electrophoresis capillary for analytical separations. The sampler was demonstrated to be capable of monitoring droplet content during its evaporation. However, as the start of the study encountered problems (e.g., droplet evaporation, the need for volatile buffers, and resulting possible low efficiencies), these aspects need to be addressed in further studies. Still, we found that preparing open DMF devices from common consumer products is a simple and robust procedure. This means the concept of low-cost, rapid prototyping of DMF devices, as advanced by Abdelgawad and Wheeler, opens an interesting field of opportunities. We believe that the combination of DMF sampling with a portable CE analyzer is an important step toward fully integrating in-line sample processing and separation in bioanalytical chemistry.

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Curriculum vitae

1. Personal Data

Name	Jelena Gorbatšova
Birth date	03.08.1981, Tallinn
Citizenship	Estonian

2. Contact information

Aadress	Akadeemia tee 15-325, Tallinn, 12618, Estonia
Contact phone	+37255565089, +3726320183
E-mail	jgorbatsova@yahoo.com

3. Education

TUT, 2006, bio –and food technology, M.Sc.
TUT, 2004, bio –and food technology, B.Sc.
Tallinna Laagna Gymnasium, 1999, secondary education

4. Special courses

2009 -	Graduate school "Functional materials and processes"
23.02-27.02.2011	Data analysis for chemistry, Estonia
13.02-14.02.2010	Introduction to chemometrics, Russia
	Chemometrics in Excel, Russia
10.02-12.02.2010	AAS analytical methods, Estonia
2006-2008	Graduate school "New production technologies and processes"

5. Professional employment

2010 – 2011 TUT, Department of Chemistry, project assistant
2007 - 2010 ˇ TUT , Chair of Analytical Chemistry, scientific research in the implementation of projects under the Estonian Ministry of Defense
2006 – 2009 Teaching in the fields of analytical and inorganic chemistry

6. Defended theses

Master's thesis
"Determination of L-ascorbic acid and plant phenolics in three different sorts of sea buckthorn by capillary electrophoresis"
Bachelor's thesis
"Isolation and determination of black, red, green and white tea aromas"

7. Main area of scientific work/Current research topics

Capillary electrophoresis, digital microfluidics, droplet microfluidics, miniaturization, analysis of environmental and biological samples, multidimensional analysis, chemometrics.

8. Theses supervised

Jekaterina Mazina, M. Sc. (TUT, 2010)
Jekaterina Mazina, B. Sc. (TUT, 2008)
Marina Turkina, B. Sc. (TUT, 2006)

9. List of original publications

1) J. Gorbatšova, T. Lõugas, R. Vokk, M. Kaljurand. - Comparison of the contents of various antioxidants of sea buckthorn berries using capillary electrophoresis - *Electrophoresis 2007, 28, 4136-4142.*

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4) J. Gorbatšova, M. Jaanus, M. Kaljurand. - Digital microfluidic sampler for a portable capillary electropherograph - *Analytical Chemistry 2009, 81, 8590–8595.*

5) J. Mazina, J. Gorbatšova. - Sample preparation for CE-DAD analysis of the water soluble vitamins in food products - *Procedia Chemistry 2010, 2, 46-53.*

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7) M. Vaher, J. Gorbatšova, M. Koel, M. Kaljurand. - Separation of ionic liquids in non-aqueous capillary electrophoresis using contactless conductivity detector - *Proceedings 31st International Symposium on High Performance Liquid Phase Separations and Related Techniques, HPLC 2007, Ghent, Belgium, 2007.*

8) J. Gorbatšova, M. Turkina, M. Kaljurand. - Comparison of the contents of various antioxidants of sea buckthorn berries using capillary electrophoresis - *Proceedings 5th Singapore International Chemistry Conference /Asia-Pacific International Symposium on Microscale Separation and Analysis, Singapore, 2007.*

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Elulookirjeldus

1. Isikuandmed

Ees- ja perekonnanimi	Jelena Gorbatšova
Sünniaeg ja - koht	03. august, 1981, Tallinn, Eesti
Kodakondsus	Eesti

2. Kontaktandmed

Aadress	Akadeemia tee 15-325, Tallinn, 12618, Eesti
Telefon	+3726204322, +37255565089
E-posti aadress	jgorbatsova@yahoo.com

3. Hariduskäik

TTÜ, 2006, Bio-ja toiduainete tehnoloogia, magister
TTÜ, 2004, Bio-ja toiduainete tehnoloogia, bakalaureus

4. Täiendusõpe

2009….	Doktorikool „Funktsionaalsete materjalide ja tehnoloogiate“
23.02-27.02.2011	Andmetöötlus keemias, Eesti
13.02-14.02.2010	Sissejuhatus kemomeetriasse, Kemomeetria EXCEL'is, Venemaa
2006-2008	Doktorikool "Uute tootmistehnoloogiate ja – protsesside doktorikool

5. Teenistuskäik

2010 – 2011	TTÜ, Keemiainstituut, projekti assistent
2007 - 2010	TTÜ, Analüütilise keemia õppetool, uurimistöö EV Kaitseministeeriumi projektide täitmise raames
2006 – 2009	Õppetöö läbiviimine analüütilise – ja anorgaanilise keemia alal

6. Kaitstud lõputööd

Bakalaureusetöö
„Musta, punase, rohelise ja valge teede eeterlike õlide koostise uurimine kasutades gaaskromatograafiat"
Magistritöö
„Taimsete fenoolide ja L- askorbiin hape leidmine ja määramine erinevatest astelpaju sortides"

7. Teadustöö põhisuunad

Kapillaarelektroforees, programmeeritavad mikrotilgad, miniaturiseerimine, keskkonna – ja bioloogiliste proovide uurimine, kemomeetria.

DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

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