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DEVELOPMENT AND APPLICATION OF VARIOUS ON- AND OFF-LINE ANALYTICAL METHODS FOR THE ANALYSIS OF BIOACTIVE COMPOUNDS

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MITMEMÕÕTMELISTE ANALÜÜSIMEETODITE VÄLJATÖÖTAMINE JA RAKENDAMINE BIOAKTIIVSETE ÜHENDITE ANALÜÜSIL

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II Ehala, S.; Kaljurand, M; Kudrjashova, M.; Vaher, M. Stroboscopic sampling in comprehensive high-performance liquid chromatography-capillary electrophoresis via a pneumatic sampler. *Electophoresis* **2004**, *25*, 980-989.

III Ehala, S; Vaher, M.; Kaljurand, M. Separation of polyphenols and L-ascorbic acid and investigation of their antioxidant activity by capillary electrophoresis. *Proc. Estonian Acad. Sci. Chem.* **2004**, *53*, 21-35.

IV Vaher, M.; Ehala, S.; Kaljurand, M. On-column capillary electrophoretic of rapid reaction kinetics for determination of antioxidative potential of various bioactive phenols. *Electrophoresis* **2005**, *26*, 990-1000.

V Ehala, S; Vaher, M.; Kaljurand, M. Characterization of phenolic profiles of Northern European berries by capillary electrophoresis and determination of their antioxidant activity. *J. Agric. Food Chem.* **2005**, *53*, 6484-6490.

ABBREVIATIONS

AA – L-ascorbic acid
ABTS – 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonate)
AC – antioxidant activity
AEAC – ascorbic acid equivalents
APCI – atmospheric pressure chemical ionization
AP-MALDI MS – atmospheric pressure matrix-assisted laser desorption/ ionisation mass spectrometry
ATT – 6-aza-2-thiothymine
BTT – 1,2,4-butanetriol
CE – capillary electrophoresis
CEC – capillary electrochromatography
CF – continuous flow
CTAB – cetyltrimethylammonium bromide
CZE – capillary zone electrophoresis
DAD – diode array detection
DHB – 2,5-dihydroxybenzoic acid
EMMA – electrophoretically mediated microanalysis
ESI-MS – electrospray ionisation mass spectrometry
FAB-MS – fast atom bombardment mass spectrometry
FIA – flow-injection analysis
FW – frozen weight
GC – gas chromatography
GPC – gel permeation chromatography
HCCA – α-cyano-4-hydroxycinnamic acid
HPLC – high-performance liquid chromatography
IR-MALDI MS – infrared matrix-assisted laser desorption/ionization mass spectrometry
MAE – microwave-assisted extraction
MALDI MS – matrix-assisted laser desorption/ionisation mass spectrometry
MBBA – N-(4-metoxybenzylidene)-4-butylaniline
PAGE – polyacrylamide gel electrophoresis
PD – plasma desorption
PEG – polyethylene glycol
PNA – 4-nitroaniline
RPLC – reversed-phase liquid chromatography
SFE – supercritical fluid extraction
SPE – solid-phase extraction
TAE – tannic acid equivalents
TLC – thin-layer chromatography
TOF-MS – time-of-flight mass spectrometry
TPH – total phenolics

INTRODUCTION

Off- and on-line multidimensional methods of analysis

Due to their complexity, one-dimensional separation methods do not often have sufficient selectivity for the analysis of natural or biological samples. In these cases, multidimensional methods, which may offer higher selectivity, can be used. An increase in the selectivity of a multidimensional system can only be obtained when the dimensions are based on different, but compatible, separation mechanisms. Also, the second dimension of a multidimensional system should not destroy the resolution achieved by the previous one [i]. In addition, adequate interfacing is needed to transport the various fractions from the first to the second dimension.

A coupled system can be composed of various separation and detection methods. Today high-performance liquid chromatography (HPLC) is the most widely employed analytical method in multidimensional separations. Even different HPLC columns are frequently being connected together when dealing with more complicated samples. During the last decade, HPLC combined with electrospray ionization mass spectrometry (ESI-MS) has gained remarkable popularity. On the one hand, ESI-MS has several advantages over the other frequently used mass spectrometric technique, matrix-assisted laser desorption/ionisation mass spectrometry (MALDI MS), including the universality of the ESI method, specifically for efficient ionization of non-peptide low molecular weight compounds, and perhaps the most importantly, ESI's great compatibility for on-line coupling with liquid separations. On the other hand, MALDI MS has also some benefits compared to ESI-MS, such as spectral simplicity due to mostly singly charged molecules (vs. multiply-charged ions for ESI) and high tolerance to contamination and non-volatile buffers (volatile buffers should be used for ESI).

As mentioned above, HPLC has been most widely combined either with its different separation modes (normal-phase, reversed-phase, size exclusion, ion exchange or affinity chromatography), mass spectrometry or capillary electrophoresis (CE). Two electrophoretic separations based on various mechanisms: charge to mass ratio (capillary zone electrophoresis, isotachophoresis), isoelectric point (capillary isoelectric focusing), with size (capillary gel electrophoresis) and differences in hydrophobicity (micellar electrokinetic chromatography) have also been coupled. Besides HPLC, other continuous separation techniques, such as solid-phase extraction (SPE), dialysis or supercritical fluid extraction (SFE), are frequently combined with CE, to concentrate the analytes and remove interfering compounds.

One possible application of multidimensional separation methods would be the studying of bioactive peptides in hydrolysates and phenols in plant extracts, as in both cases, analyzed samples are often very complicated, containing tens or even hundreds of components.

Bioactive compounds

Plant extracts have been used in traditional medicine since ancient times for treatment of various diseases. Several compounds from plant extracts (alkaloids, flavonoids, glycosides/steroids/terpenoids, polysaccharides, proteins, saponins, etc.) have been reported to have different biological functions (i.e. antioxidant, antimicrobial, chemopreventive, anticancer, immunosuppressive, etc.).

The interest in natural products is supported by advances in chromatographic and spectroscopic techniques that have greatly facilitated drug discovery from plants. Nature is recognized as a unique and incomparable source of both novel bioactive molecules and templates for optimization by combinatorial and computational approaches [ii]. Biophenols are the compounds that have attracted increasing attention during the past few years due to their biological activities and natural abundance and are potential targets for the food and pharmaceutical industries.

Besides secondary metabolites, interest towards primary metabolites, such as proteins, has grown over the years, since proteins are sources of many peptides with diverse biological activity. Such peptides are considered as valuable components of foods and are classified as follows: ligands for receptors, enzyme inhibitors, peptides regulating intestinal absorption, anti-microbial peptides, antioxidative peptides and others [iii]. To date, most bioactive peptides are being isolated from milk-based products, though other animal as well as plant proteins, especially soybean, also contain potential bioactive sequences [iv]. Mass spectrometry, more often ESI but also MALDI MS, is frequently used for the analysis of complex peptide mixtures. Commonly, for that purpose, first the fractions are collected after HPLC separation and then analyzed with MALDI.

Objectives of the study

Due to the importance of the bioactive compounds, the present work aims to develop new and improve current analytical methods that could be applied for the analysis of bioactive peptides and phenols (papers I-V). Emphasis was placed on coupling different analytical methods, developing innovative sampling methods and studying antioxidativity of polyphenols.

The main goals of the present work were:

- to investigate the possibility of creating on-line MALDI interface capable of analyzing liquid streams (paper I);
- to develop an interface that allows the hyphenation of HPLC and CE methods and to find a solution to the commonly encountered problem with long analysis times in the second dimension, i.e., to use stroboscopic sampling in the 2-D HPLC-CE system (paper II);
- to use spectrophotometry and capillary electrophoresis either coupled with SPE or separately for the determination of bioactive phenols and their antioxidant activity (papers III-V).

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1 METHODS USED IN DIFFERENT ON- AND OFF-LINE APPLICATIONS

1.1 Matrix-assisted laser desorption/ionization mass spectrometry

Mass spectrometry is a technique which produces gaseous ions in the vacuum and subsequently separates them according to their mass-to-charge (m/z) ratio, using a mass analyzer. The technique was developed in the 1920s. Until the late 1980s, however, most MS ionization methods (e.g., electron impact and chemical ionization) were not applicable for the analysis of complex high molecular weight compounds. Two so-called "soft" desorption/ionization techniques, i.e., characterized by little fragmentation, introduced at the end of the 1980s, greatly expanded the mass range and thus strengthened the role of mass spectrometry in biological research. Matrix-assisted laser desorption/ionization (MALDI) was introduced simultaneously by Tanaka [v] and Karas and Hillenkamp [vi]. Electrospray ionizaton (ESI) was developed by Fenn and co-workers [vii].

1.1.1 General principles of the MALDI method

The mechanism of MALDI is believed to consist of three basic steps. The first step involves the formation of a "solid solution". A low concentration of the analyte is mixed with the matrix onto a probe or metal plate. It is essential for the matrix to be in excess so that the analyte molecules are completely isolated from one another throughout the matrix. This is necessary if the matrix is to form a homogenous "solid solution" (any liquid solvents used in the preparation of the solution are removed when the mixture is dried before analysis), which is required to produce a stable desorption of the analyte.

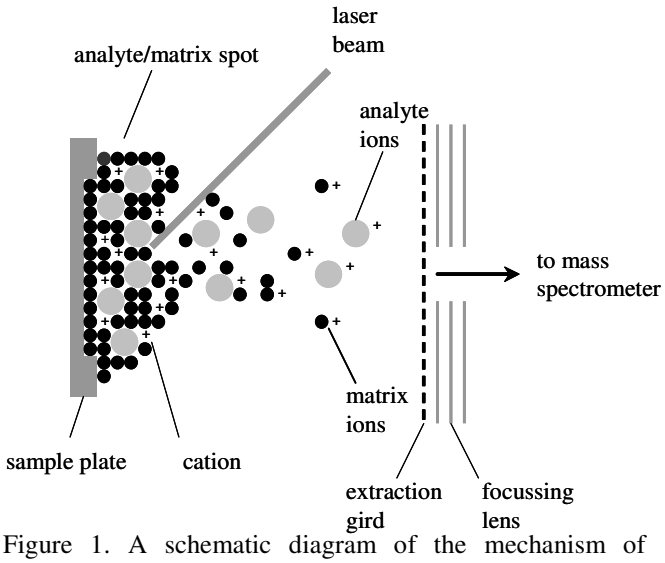


Figure 1. A schematic diagram of the mechanism of MALDI

The second step involves matrix excitation. The laser beam is focussed onto the surface of the matrix-analyte solid solution. Some of the laser energy is absorbed by the matrix, causing rapid vibrational excitation that brings about

disintegration of the solid solution. The clusters ejected from the surface consist of the analyte molecules surrounded by the matrix and salt ions. The matrix molecules evaporate away from the clusters to leave the free analyte in the gas phase.

The third step is the analyte ionization. The photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. In this way the characteristic $[M+X]^+$ (X=H, Li, Na, K etc.) analyte ions are formed. Some multiply charged species, dimers and trimers can also be formed. Negative ions are formed from reactions involving deprotonation of the analyte by the matrix to form $[M-H]^-$ and from interactions with photoelectrons to form the $[M]^{-\bullet}$ radical molecular ions. These ionization reactions take place in the first tens of nanoseconds after irradiance and within the desorbed matrix-analyte cloud just above the surface. The ions are then extracted into the mass spectrometer for analysis.

1.1.2 Description of MALDI matrices

In MALDI, the choice of the matrix material plays a central role for successful experiments. The use of the matrix enhances desorption and ionization processes, thus permitting the analysis of non-volatile and thermally labile molecules. The general utility of any matrix compound is determined by its efficiency in ionizing the biomolecule of interest and by lack of sensitivity to the numerous impurities commonly present in biological solutions. Other properties required of a matrix for MALDI experiments are: it should absorb strongly at the wavelength of the irradiating laser, co-crystallize (solid matrix) or dissolve (liquid matrix) the sample, promote the chemistry that leads to the ionization of the sample, and insulate the sample from thermal degradation during laser desorption.

So far in most MALDI applications solid matrices are employed because of difficulties with using liquid matrices in vacuum. The recent introduction of atmospheric pressure MALDI might enhance interest towards the use of liquid matrices, as with AP-MALDI the ionization takes place at atmospheric pressure and thus makes the use of liquid matrices much easier. When liquid matrices are used, the co-crystallization of the analyte and matrix is not necessary, thus, the analyte and matrix are equally distributed within the droplets, at least if neither of them is a surfactant. This is in contrast to conventional crystallized MALDI samples, where segregation effects of matrix and analyte often occur, leading to the formation of “sweet spots”.

1.2 General principles of capillary electrophoresis

Electrophoresis is defined as the migration of analytes within an electrolyte solution under the influence of an electric field. Capillary electrophoresis (CE)

was developed as a technique in the late 1980s, and since then its popularity has constantly grown in the field of analytical chemistry. The advantages of the method are: very small amounts of sample required, high efficiency due to the non-parabolic fronting, short separation times, low running costs, ease of operation and consumption of limited amount of reagents. Although the basic methodology involves the separation of molecules based on their charge to mass ratio, there are straightforward modifications to the procedure, which allow separations based on the size or isoelectric point, or which permit the separation of non-charged molecules.

Electroosmosis

Capillary electrophoresis is normally carried out in the capillaries made from fused silica. The inside wall of a fused silica capillary is covered with the silanol (Si-OH) groups with a negative charge ($Si\text{-}O^-$) above pH ≈ 2. Negative surface charge results in a distinctive distribution of cationic species in any ionic solution within the capillary. There is a layer of tightly bound cations immediately adjacent to the capillary wall and more loosely associated layer which is also largely cationic in nature. The application of an electric field results in the movement of these more loosely bound cations toward the cathode, and since they are hydrated, the consequence is a bulk flow of liquid in the same direction. The existence of the electroendosmotic flow (EOF) means that neutral molecules and even those with a negative charge and an expected anodic mobility will be swept along towards the cathode.

2 MULTIDIMENSIONAL METHODS

A multidimensional separation involves the coupling of two or more separation mechanisms in order to carry out a single analysis. Although simple in concept, multidimensional separations can be exceedingly powerful in terms of resolution. The need for multidimensional separations arises from the inability of one-dimensional (1D) separation methods to adequately resolve highly complex samples. One field of research that highlights the need for enhanced separation power of multidimensional techniques is proteomics, which involves the characterization of all the proteins expressed by an organism. The most powerful 1D column chromatography techniques typically have the peak capacity of only a few hundred, where peak capacity is defined as the maximum number of peaks that can fit into the accessible separation space side by side with a resolution of 1.0 from each neighbouring peak. Moreover, due to the usually random distribution of peaks and the statistical probability of peak overlap, the actual number of detectable components that can be expected to be fully resolved is substantially smaller than the peak capacity [viii].

Multidimensional separations can be performed either in off-, at- or on-line. In general, the on-line combination of separation systems enables a significantly

faster analysis of a complex matrix in comparison with an at-line or off-line combination. Off-line methods involve collecting fractions from the first dimension, and later subjecting them to separation on the second dimension. In at-line methods, the effluent from the first dimension is transported to the next system by help of a robot. On-line techniques employ switching valves or other instrumentation that allow the fractions from the first dimension to be transferred directly to the second dimension, while usually the first-dimensional separation continues simultaneously.

2.1 Interfacing liquid separation techniques with MALDI MS in off- and on-line mode

MALDI coupled with time-of-flight mass spectrometry (TOF-MS) is, besides electrospray ionization (ESI), one of the most important tools in the investigation of proteins, peptides, nucleic acids, organometallic compounds, and synthetic polymers. While ESI and MALDI MS alone can easily be used for the direct analysis of simple mixtures, additional fractionation of complex mixtures by liquid separation techniques is required prior to mass analysis. The benefits of such a coupling are the reduction of sample preparation time, the on-line monitoring of reactions, and the high information content, i.e., molecular weight and structural information. Due to their low flow rate, CE, capillary electrochromatography (CEC) and μ-HPLC are well suited for introducing a liquid flow into the mass spectrometer.

ESI MS, along with fast atom bombardment mass spectrometry (FAB MS), has been most successful for the direct coupling because the effluent from the liquid separation technique can directly be introduced into the mass analyzer. However, despite the widespread use of ESI with liquid separations, there are several drawbacks of electrospray ionization. First, an ESI mass spectrum depicts multiply charged ions. On the one hand, their presence allows the detection of large ions at relatively low m/z, on the other hand, it complicates the interpretation of the spectra of complex mixtures. Second, the sensitivity is affected by the presence of salts, impurities and organic buffers which are often required in chromatographic separations. Third, ESI solvents are generally incompatible with gradient elution common in chromatography. Finally, ESI sources are generally combined with relatively slow quadrupole analyzers which have to scan the whole mass range. MALDI MS overcomes these disadvantages. Since it is a pulsed technique, it can be combined with a TOF analyzer, resulting in a complete mass spectrum from every single laser shot. MALDI TOF MS therefore allows a high analysis speed which is especially important in a continuous-flow interface for rapidly eluting peaks. MALDI TOF is rather tolerant towards the presence of salts and buffer. However, there is one major drawback of MALDI TOF MS. Typically, a sample co-crystallized with the matrix is analyzed. The sample introduction mode is therefore highly incompatible with a liquid separation technique. Therefore, such interfaces are

not very common and many of them are quite limited for practical use. Hence, mainly only off-line combinations of liquid separation techniques with this method are reported.

Mass spectrometers (MS) have been extensively used as detectors for various separation methods. The off-line and on-line couplings of MALDI MS to liquid separations have been comprehensively summarized in literature [ix,x,xi]. The off-line approach involves subsequent but separate MALDI analysis of fractions collected from HPLC, gel permeation chromatography (GPC) and CE or spots scraped and extracted from thin-layer chromatography (TLC) [xii] and polyacrylamide gel electrophoresis (PAGE). The column fractions can be collected in vials or deposited directly onto MALDI target plates. The matrix can also be mixed with the analyte directly in the vial or onto the MALDI target plate.

With off-line separations, it is important to maintain the separation efficiency and, with small-scale separations, avoiding an excessive dilution of the analyte when the matrix is added. Although the off-line approach simplifies the coupling, it only provides the mass spectral data for several discrete times (HPLC, GPC, and CE) or spots (TLC and PAGE). For continuous monitoring of the entire separation, MALDI can be coupled on-line (real-time detection) with column separations or directly to planar separations. On-line coupling of liquid column separations to MALDI requires that the separation effluent be delivered directly to the mass spectrometer. There have been several approaches to couple MALDI directly to liquid introduction, such as continuous-flow, aerosol introduction, continuous vacuum deposition interface, rotating ball inlet, and atmospheric pressure MALDI.

Various continuous flow (CF) interfaces have been developed for MALDI MS. In one of those interfaces, proposed by Nagra *et al.*, a mixture of analyte and liquid MALDI matrix was delivered through the CF probe and the mixture of analyte and matrix was desorbed directly from the probe tip [xiii]. In this design the probe was positioned orthogonally to the ion acceleration direction. The ion optics were later configured to parallel ion extraction [xiv] to improve the mass resolution. Besides UV MALDI, IR-MALDI has also been combined with continuous flow interfaces [xv]. The advantage of IR-MALDI over AP-MALDI is the wider range of available liquid matrices.

A semi-continuous interface, where the matrix and analyte are allowed to co-crystallize on a porous frit connected to the high vacuum end of a CF-capillary [xvi], has also been demonstrated. The liquid solution containing the analyte and the matrix flows through the frit into the vacuum of the mass spectrometer. The volatile solvent of the sample evaporates rapidly, leaving a crystalline of the matrix and the analyte on the vacuum side of the frit. Regeneration of the interface is achieved by a combination of flushing the frit with a pure solvent and laser ablation.

In another modification of a continuous flow interface two-phase MALDI matrices were applied [xvii]. These matrices consist of solid particles that absorb the laser energy and assist in analyte desorption, and a liquid for

dissolving and ionizing the analyte. Solid particulates were compressed or sintered into a porous frit that was placed at or near the MALDI probe tip, and the effluent from the liquid separation continuously delivered the sample to such a "particle frit". In on-line experiments, the sample was delivered to the outer side of the frit with a fused silica capillary (50 μm i.d.). The sample penetrated through the frit to the vacuum side facing the ion source of the mass spectrometer. The ionization of the analyte was promoted by the liquid matrix. The problem encountered with this interface was that the mechanical stability of the frits was not satisfactory.

In the aerosol MALDI method, the solution containing the matrix and the analyte is sprayed into the mass spectrometer where the solvent evaporates. The dried aerosol particles are ionized with a pulsed laser and analyzed by time-of-flight MS [xviii].

Preisler *et al.* [xix] demonstrated that a device for continuous vacuum deposition of the matrix and the analyte from a solution onto a moving surface inside the mass spectrometer can be used for generating MALDI spectra of a flowing liquid sample. The device makes use of a rotating quartz wheel onto which the liquid is deposited through a narrow fused silica capillary that is kept in contact with the wheel. When the wheel is rotating, the deposited sample is transported into the ion source region where MALDI takes place.

Two different designs have been proposed for the rotating ball interface [xx,xxi]. In the later version, the major part of the ball was outside a vacuum chamber. The analyte and matrix solutions were delivered onto the rotating ball through a narrow fused silica capillary. With this design very good vacuum pressure was maintained, as the solvent evaporated outside the vacuum, leaving a thin crystalline deposit of the analyte and the matrix on the surface of the ball, which was desorbed and ionized by laser irradiation in the vacuum of the mass spectrometer.

Conventional MALDI is a vacuum ionization technique in which sample ionization occurs under vacuum conditions inside a mass spectrometer. Sample ionization in the atmospheric-pressure matrix-assisted laser desorpion/ionization mass spectrometry (AP-MALDI MS) takes place at atmospheric pressure outside the mass spectrometer. The ions generated by the laser irradiation on the sample are transported into the vacuum of the mass spectrometer by means of carrier gas or electric fields. The initial AP-MALDI source was described by Laiko *et al.* [xxii]. A device proposed in this study couples a liquid flow system with AP-MALDI MS.

2.2 Coupling continuous separation techniques to CE

The analysis of real samples by capillary electrophoresis (CE) often requires efficient sample preparation procedures to remove interfering solutes, (in)organic salts and particulate matter. Sample preparation systems reported in the literature are based on chromatographic, electrophoretic, membrane-based

procedures, solid-phase extraction (SPE), and supercritical fluid extraction (SFE) among other techniques.

2.2.1 HPLC coupled to CE

CE and LC operate under fundamentally different separation mechanisms, therefore LC-CE coupling represents a true orthogonal system [viii,xxiii]. CE is a relatively fast separation method compared to LC, which makes it well suited as the second dimension in a 2D system because of its ability to sample the first dimension at relatively high frequency. The first automated comprehensive LC-CE system was developed by Bushey and Jorgenson in 1990 [xxiv]. It was based on the coupling of a commercial microbore RPLC column with a fused-silica capillary using an electrically actuated six port valve with an external sample loop. This system was used to separate mixtures of peptide standards and tryptic digests of ovalbumin with a fluorescence detector.

During the 1990s, LC-CE instrumentation was significantly improved. Enhanced separation efficiency was achieved by using packed fused-silica capillaries for the LC dimension, as opposed to conventional LC columns [xxv]. This also facilitated coupling the two dimensions, because flow rates in capillary LC are more similar to those typically used in CE. Other improvements in instrumentation focused on the interface used to couple LC columns and CE capillaries. The coupling of separation techniques that operate with low flow rates requires the use of an interface design capable of transferring small volumes from LC to CE, while minimizing extra peak broadening. Therefore, an interface was designed, allowing micro-LC and CE to be combined in a comprehensive 2D system. The transverse flow-gated interface first reported in 1993 [xxvi] and improved in 1997 [xxvii] eliminated the need for sample loops that contribute extra-column broadening. In a LC-CE system using this interface, the LC column outlet and the CE capillary inlet are positioned directly opposite to each other, separated by a narrow channel. During most of the run, the CE buffer is continuously flushed through the channel, which carries the LC column effluent to waste. To inject a portion of the LC column effluent into the CE capillary, the flow of flush buffer is stopped and some of the LC effluent is drawn into the CE capillary by electromigration. Fast, reproducible injections can be performed by using an air-actuated switching valve to control the flow of the flush buffer.

An even faster method of performing injections from a LC column onto a CE capillary is provided by optical gating. In this “inverse” injection technique, a LC capillary column and a CE capillary are coupled using a simple interface tee, so that some of the effluent of the first dimension is always being pulled into the CE capillary by electromigration [xxviii]. An intense laser beam is focused on the CE capillary at some point beyond the interface tee; this beam photodegrades the fluorecentely labelled sample passing through the capillary. To make an injection, the gating beam is momentarily blocked with a computer-

controlled shutter. This allows a small amount of undegraded material to be introduced onto the capillary, where its components are separated and detected. The described method is limited to fluorescently labelled samples, but offers the fastest injection method of any LC-CE interface.

The two modes of LC that have been coupled most often with CE are reversed-phase chromatography [xxiv,xxvii,xxviii,xxix,xxx,xxxi] and size-exclusion chromatography [xxv,xxvi,xxx]. Some variants of CE have also been used in 2D LC-CE separations, such as pressurized capillary electrochromatography (pCEC) [xxxii]. LC-CE is expected to have a high separation power, due to the speed and high resolution of CE. Compared to LC-LC, the LC-CE system often seems to be more selective and orthogonal. However, there are several problems associated with coupling LC and CE. Firstly, the elution volumes of conventional LC columns are much greater than sample volumes of CE capillaries, thus in many applications only a small fraction of the LC effluent is transferred to the CE dimension. This, in turn, reduces the sensitivity of the method. Thirdly, the mismatching of the HPLC effluent and CE buffer compositions in the comprehensive 2-D separations causes the appearance of system peaks during the CE separation. The problem becomes especially acute when reversed phase chromatography (RPLC) is used as the first dimension in the HPLC-CE system, because then the separation medias in two dimensions are very different. Another problem encountered in LC-CE systems is the way the electrode is used at the coupling side of the CE and the voltage is applied.

In spite of the difficulties, on-line LC-CE has been shown to be a powerful multidimensional separation method. Today's LC-CE devices have mainly been developed for the field of proteomics, however, many other types of samples can be analyzed using similar methods.

2.2.2 SPE coupled to CE

SPE can be used to simultaneously enrich the trace analytes and remove potentially interfering compounds. It can be combined with CE in several ways using either off-, at- or on-line approach. Solid-phase extraction is frequently required to remove the sample matrix prior to the chromatographic step when plant materials are being analyzed.

3 ROLE OF VARIOUS SEPARATION AND DETECTION METHODS IN THE ANALYSIS OF BIOPHENOLS

The need to identify individual phenolic compounds has led to replacing the traditional methods, such as paper, packed column and thin-layer chromatographic methods, by high-performance chromatographic analyses. Despite the high resolution and sensitivity of GC, the limited volatility of many

phenols has restricted the application of gas chromatography (GC) to their separation. However, with suitable derivatization (e.g., trimethylsilylation) they are amenable to GC and GC-mass spectrometry. Nevertheless, high-performance liquid chromatography (HPLC) currently represents the most popular and reliable technique for the analysis of phenols [xxxiii]. The typical system involves reversed phase liquid chromatography, comprising a C_{18} stationary phase or other alkyl chemistry. Routine detection in HPLC is typically based on the measurement of UV absorption or, less commonly, visible radiation in the case of anthocyanines. Identification of the eluted phenols in GC and HPLC is usually based on the correspondence of retention data with an appropriate standard. Recently MS combined either on-line or off-line with chromatographic or electrophoretic techniques has been successfully used for the separation and determination of phenols. Among the numerous separation systems, only a few procedures have been developed to specifically measure polyphenolic concentrations in several commonly consumed foods. Most of these methods have been developed to measure different groups of polyphenolics in a single plant, or a single or a few groups in multiple plant sources. Obtaining a good resolution is considered to be the main difficulty for a method that is targeted to the separation of multiple polyphenolic groups [xxxiv].

Although HPLC is the most dominating separation technique for polyphenols, capillary electrophoresis (CE) is gaining popularity. CE has several advantages over HPLC: (1) very small sample size requirement, (2) high efficiency due to non-parabolic fronting, (3) shorter analysis time, (4) low cost, and (5) use of no or only a small amount of organic solvent [xxxiii]. In addition to the more commonly used capillary zone electrophoresis (CZE) [xxxv,xxxvi], micellar electrokinetic chromatography [xxxvii] and nonaqueous capillary electrophoresis [xxxviii,xxxix] have also been used for the analysis of polyphenols.

The above discussed separation modes (HPLC, CE) often share the same detection techniques, particularly UV-vis-DAD and MS detectors. Most of the biophenols have highly conjugated double bond or aromatic systems that absorb light in the ultraviolet and visible region. The combination of DAD and HPLC has played an important role in the identification of biophenols. However, for complete structural identification, other techniques such as MS and NMR are often necessary.

Mass spectrometry (MS) can be carried out on-line, coupled with chromatographic or electrophoretic techniques or off-line as a stand-alone instrument. However, it is the former that provides unsurpassed opportunities in the identification and structure elucidation of biophenols. There are two main types of ionization techniques for biophenols, the ion-spray techniques, such as electrospray ionization (ESI), thermospray and atmospheric pressure chemical ionization (APCI), and the ion desorption techniques which include fast atom bombardment (FAB), plasma desorption (PD), and matrix-assisted laser desorption ionization (MALDI). Sensitivity and selectivity of detection can be

increased using tandem mass spectrometry, i.e., two (MS-MS) or more (MS^n) mass analyzers coupled in series. MS-MS and MS^n produce more fragmentation of the precursor and daughter ions, therefore, provide additional structural information for the identification of biophenols.

3.1 Determination of antioxidant activity, the most studied bioactivity of the phenols

The importance of oxidation in the body and in foodstuffs has been widely recognized. Oxidative metabolism is essential for cell survival. A side-effect of this dependence is the production of free radicals and other reactive oxygen species, such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) that cause oxidative changes. Different studies have shown that the free radicals present in humans cause oxidative damage to different molecules, such as lipids, proteins, and nucleic acids and are thus involved in the initiation phase of some degenerative illnesses. Antioxidant compounds that are capable of neutralizing free radicals are believed to play an important role in the prevention of certain diseases, such as cancer and cardiovascular diseases.

An antioxidant may be defined as "any substance that when present at low concentrations, compared with those of the oxidizable substrate, significantly delays or inhibits oxidation of that substrate" [xl]. The anitioxidant activity is, by definition, the capability of a compound to inhibit oxidative degradation, e.g., lipid peroxidation. Phenolics are the main antioxidant components of food. While in plant oils and fats these are basically monophenolics, first of all, tocopherols (vitamin E), water-soluble polyphenols are more typical in water-soluble products (fruits, vegetables, tea, coffee, wine). Although antioxidant activity (AC) of polyphenols is associated with various mechanisms, the elevated reactivity of phenolics towards active free radicals is considered as the most principle mechanism [xli].

Polyhphenols act as free radical scavengers due to the presence of one or several phenolic groups. These groups can react according to [xlii]

$$R^{\bullet} + XOH \rightarrow RH + XO^{\bullet} \qquad (1)$$

The antioxidant activity of the phenols is then promoted by the lability of the O–H bond in phenols and the low reactivity of the phenoxyl radical, which favours termination reactions, thereby reducing the length of free radical chains. Most polyphenols bear more than one potentially reactive group, and their antioxidant activity mainly functions on the basis of an efficient hydrogen transfer capacity.

Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. The features of an oxidation are a substrate, an oxidant and an initiator, intermediates and final

products and the measurement of any one of these can be used to assess antioxidant activity [xl].

Various strategies have been developed for measuring the antioxidant activity as the ability to scavenge free radicals generated in aqueous and lipophilic phases. One approach involves the generation of a free radical species and direct measurement of its inhibition due to the addition of antioxidant(s). This approach is employed in commonly used ABTS assay, which is based on the determination of the relative ability of antioxidants to scavenge a radical cation 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$). This can be measured spectrophotometrically, by measurement in the near-infrared region at 734 nm.

In the second approach employed in this study, the antioxidant activities of L-ascorbic acid and polyphenols were characterized by monitoring their oxidation by H_2O_2 by CE. The decomposition of H_2O_2 in an aqueous solution occurs by dissociation (2) and the homolytic cleavage of O-H or O-O bonds (3, 4) with the formation of highly reactive products: perhydroxyl anion (HOO^-), and perhydroxyl (*OOH) and hydroxyl (*OH) radicals.

$$H_2O_2 \rightarrow H^+ + HOO^- \quad k=1.55 \times 10^{-12} \quad (s^{-1}) \tag{2}$$

$$HOOH \rightarrow *OOH + *H \tag{3}$$

$$HOOH \rightarrow 2*OH \tag{4}$$

Thus, the degradation of biophenols by H_2O_2 is primarily affected by the amount of free radicals and HOO^- anion formed by decomposition and dissociation, respectively.

3.1.1 Reaction kinetics studies with CE

The most well-known method proposed for studying reaction kinetics by capillary electrophoresis is electrophoretically mediated microanalysis (EMMA). Following the pioneering paper by the Reginer's group [xliii], EMMA was developed by the efforts of several groups, such as Schepdael *et al.* [xliv], Glatz *et al.* [xlv], and Gomez *et al.* [xlvi]. EMMA uses different electrophoretic mobilities of enzyme and substrate(s) to initiate the reaction inside a capillary and to separate the components of the reaction mixture from each other for the final on-capillary quantification.

There are basically two ways to mix the reaction components in a capillary under electrophoretic conditions. In the continuous mode of EMMA (long contact mode), the capillary is initially completely filled with one of the reactants while the second reactant is introduced. In contrast, the plug-plug mode of EMMA (short contact mode) is based on a plug-plug interaction of reactants in the capillary (Figure 2). Using the latter mode, the reactants are

introduced into the capillary as separate bands. Upon the application of an electric field, the two bands merge due to the differences of their electrophoretic mobilities. The reaction takes place and the resultant products migrate away from the reaction zone and separate. Thus, the detector can individually determine the amount of the non-reacted substrate and products.

In all the above described EMMA modes, the mixing of the reactants is accomplished by exploiting the variability in transport velocity among the chemical species in the chosen electrophoretic medium. Following the injection of the reactants into the capillary, electrophoretic mixing is initiated by the application of an electric field. To monitor reaction kinetics it is important to control the reaction start- and endpoint. Control over reaction endpoint in EMMA can be achieved by turning off the voltage when the two zones are completely mixed, which allows the reaction to continue in the absence of an electric field. This technique is called "zero potential EMMA" [xlvii]. The reaction is stopped by turning on the voltage. However, in the case of electrophoretic mixing it is difficult to determine the exact moment when the two reactant zones are mixed. Therefore, the above described different modes of EMMA do not suit well for studying reaction kinetics.

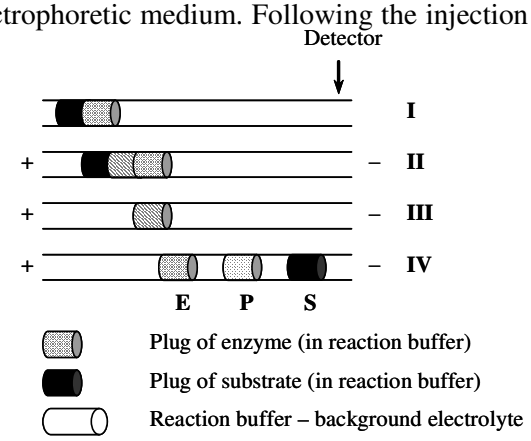


Figure 2. Schematic of the classical plug-plug mode of EMMA (E: enzyme; S: substrate; P: product of enzymatic reaction). (I) A plug of enzyme and substrate are introduced consecutively in the capillary. (II and III) Upon the application of an electric field, the two zones interpenetrate due to differences in their electrophoretic mobilities, and enzymatic reaction takes place. (IV) The reaction product and the unreacted substrate are electrophoretically transported to the detector

However, among different plug-plug modes there is one technique, at-inlet technique, where the reactants are not mixed electrophoretically but by diffusion [xlviii,xlix]. Using sandwich mode injection, the injection of enzyme is followed by the substrate and the enzyme. Then the overlaid plugs of the enzyme and substrate(s) are allowed to react at the capillary inlet. Subsequently, voltage is applied and reaction compounds are separated and quantified. However, neither does this method make it easy to evaluate the exact moment when the zones are completely mixed. In this study another method is proposed, where the reagents are mixed just before introduction to the CE capillary and the reaction is terminated and reactants and reaction products are separated when high voltage is applied.

4 CHEMICALS AND REAGENTS

4.1 Chemicals and reagents used in HPLC-MALDI application

The MALDI matrices α-cyano-4-hydroxycinnamic acid (HCCA, M=189.2 Da), 2,5-dihydroxybenzoic acid (DHB, M=154.1 Da), protoporphyrin IX (M=562.7 Da), 6-aza-2-thiothymine (ATT, M=143.2 Da), 4-nitroaniline (PNA, M=138.1 Da), 3,5-dimetoxy-4-hydroxycinnamic acid (sinapic acid, M=224.2 Da), 3,4-dihydroxycinnamic acid (caffeic acid, M=180.2 Da), and 1,2,4-butanetriol were purchased from Fluka (Switzerland). Harmane (M=182.2 Da) was obtained from Acros (Belgium). The liquid matrix N-(4-metoxybenzylidene)-4-butylaniline (MBBA, M=267.4 Da) was purchased from Aldrich (USA). The peptide analytes angiotensin II (M=1046 Da), [Ile^7]-angiotensin III (M=897 Da), α-casein(90-96), $P_{14}R$ (M=1532.8 Da), Bradykinin fragment 1-7 (M=756.8 Da), and cytochrome C were purchased from Sigma (USA), and polyethylene glycols (PEG1000, M=800-1200 Da; PEG1500, M=1400-1600 Da) were obtained from Fluka (Switzerland). All the solvents, matrices and analytes were obtained at the highest purity available and used as received.

4.2 Chemicals and reagents in CE applications

2,2'-Azinobis-(3-ethyl-benzothiazoline-6-sulphonate) (ABTS), potassium persulfate, ferric chloride, potassium ferricyanide ($K_3[Fe(CN)_6]$), hydrochloric acid, quercetin (3,3',4',5,7-pentahydroxyflavone), rutin (3,3',4',5,7,-pentahydroxyflavone-3-rutinoside), morin (2',3,4',5,7-pentahydroxyflavone), catechin (trans-3,3',4',5,7-pentahydroxyflavane), cinnamic acid (3-phenyl-2-propenoic acid), ferulic acid (*trans*-4-hydroxy-3-metoxycinnamic acid), *p*-coumaric acid (*trans*-4-hydroxycinnamic acid), caffeic acid (3,4-dihydroxycinnamic acid), gallic acid (3,4,5-trihydroxybenzoic acid), chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), *trans*-resveratrol (3,4',5-trihydroxy-trans-stilbene) and L-ascorbic acid, as well as sodium tetraborate, disodium hydrogen phosphate, sodium hydroxide, sulfuric acid, and cetyltrimethylammonium bromide (CTAB) were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Potassium dihydrogen phosphate, methylsuccinic acid and 2-hydroxyisovaleric acid were from Reakhim (Moscow, Russia). Phenol, resorcinol, 2,5-dimethylphenol, 3-methylphenol, 5-methylresorcinol, hippuric acid, benzoic acid, 3-aminobenzoic acid, 2-aminobenzoic acid, 3-nitrobenzoic acid, salicylic acid, and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Tannic acid and 2,6-dimethylphenol were obtained from Sigma (St. Louis, MO, USA). Methanol was purchased from Rathburn Chemicals Ltd (Walkerburn, Scotland) and acetonitrile form Sigma-Aldrich (Steinheim, Germany) and were of a chromatographic grade. All chemicals were used without any further purification. For the preparation of buffers and solutions of analytes, ultra pure Milli Q water (Millipore S.A. Molsheim, France) was used

throughout the experiments. All the samples, including standard solutions, were filtered through a 0.45 µM syringe filter before analysis.

In different applications, sample preparation conditions varied. The stock solutions of polyphenols were prepared by dissolving these compounds in CH_3OH/H_2O or CH_3CN/H_2O mixtures at different concentrations, L-ascorbic acid was dissolved in H_2O; working standard solutions were obtained by diluting the corresponding stock solutions with the same mixture. The buffer used in all applications was sodium tetraborate with slightly different concentrations (20 and 35 mM, pH ~9.3).

5 COUPLING OF AP-MALDI MS WITH LIQUID DELIVERY SYSTEMS

5.1 Sample preparation

Solid samples were prepared by the dried droplet method, where a drop of aqueous matrix solution (about 100 times more concentrated) is mixed with analyte solution and dried under vacuum. Liquid matrices were prepared from solid matrices (in low concentration) dissolved in different solvents or by adding a liquid mediator, 1,2,4-butanetriol (BTT) to the solution of solid matrix to avoid the crystallization. Also, a neat liquid matrix N-(4-metoxybezylidene)-4-butylaniline (MBBA) was used.

5.1.1 AP-MALDI with solid samples - an initial design

To develop an on-line coupling between liquid separations and AP-MALDI, firstly, the AP-MALDI source was constructed and its characteristics were investigated with solid samples.

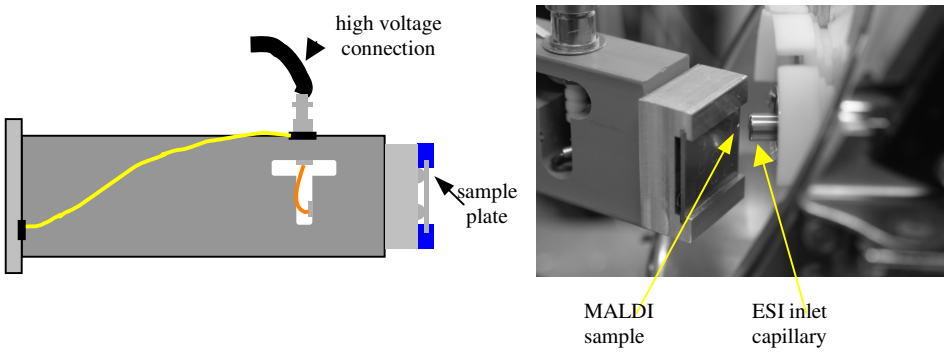


Figure 3. Schematic of the AP-MALDI sampling device used for solid samples

The initial AP MALDI source was composed of a sample holder and a sample plate (24.5×25.5 mm in size), which was connected to high voltage power

supply. Ultraviolet radiation at 337 nm from a pulsed nitrogen laser (20 Hz, 200 μJ pulse $^{-1}$, Laser Science Inc., USA) was focused by a lens with a focal length of 300 mm onto this small replaceable stainless steel target plate. The sample plate was placed approximately 5 mm from the inlet of the mass spectrometer and was kept on the ground potential while the voltage of -2 to -4 kV was applied to the ESI inlet capillary. AP-MALDI mass spectra were recorded using the program "Tof aquire". The AP-MALDI device was combined with the ESI-MS instrument by simply replacing the ESI ion source with the AP-MALDI source. More detailed schematics of sample plate placement and the laser spot is shown in Figure 3.

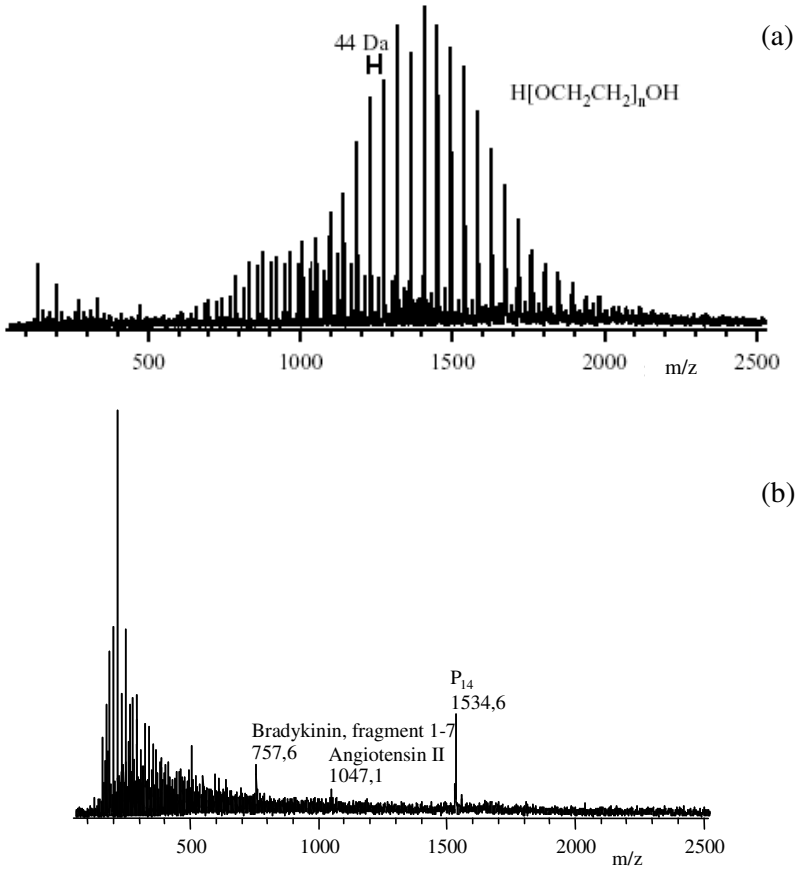


Figure 4. Positive ion mode AP MALDI mass spectra of different analytes and matrices. (a) Equal mixture of PEG 1500 (1 mM in H_2O), matrix (100 mM DHB in C_2H_5OH:H_2O, 1:1) and NaCl (10 mM in H_2O). (b) Equal mixture of peptides (33 μM of each peptide in H_2O) and matrix (100 mM PNA in CH_3CN:H_2O, 1:1)

The ESI-MS instrument used was a prototype, orthogonal injection time-of-flight mass spectrometer from Aglient (USA), consisting of a differentially pumped interface to the time-of-flight analyzer consisting of a heatable transfer capillary, two skimmers, and an octapol ion guide for collisional cooling and

focusing. Ions were orthogonally extracted by a pulsed extraction field at a repetition rate of 4 kHz, and guided *via* a single stage reflectron to the detector (Burle, USA). The detector signal was amplified and digitized with a computer (Hewlett Packard, USA) at a sampling frequency of 250 MHz. 10 000 to 50 000 spectral transients were summed and transferred to PC for further processing.

To test the constructed AP-MALDI source, standard solutions of polymers or peptides were anayzed, using classical MALDI matrices, such as 2,5-dihydroxybenzoic acid (DHB) and *para*-nitroanilin (PNA) (Figure 4).

5.1.2 Flow injection AP MALDI

5.1.2.1 Experiments with old set-up

The initial experiments with liquid samples were performed either with the same set-up by applying the sample droplet on the sample plate or with a continuous flow mode. In the latter case, the analyte/matrix mixture was connected to the exit capillary by HPLC coupling. To obtain the analyte/matrix mixture, the analyte solution was either premixed with the matrix solution, or the analyte and matrix were mixed online by mixing T. The mixture was delivered through the exit capillary by a syringe pump (Harvard Apparatus, USA) or a micro flow pump (ISCO, Germany) at flow rates between 1-10 µl min^{-1}. A small droplet was formed at the end of the exit capillary (static fused silica capillary, 50 µm i.d., 360 µm o.d., USA). A pulsed laser beam was focused onto the surface of the droplet, desorbing and ionizing the matrix and the analyte at atmospheric pressure. The ions produced were guided by electric fields to the inlet of mass spectrometer. Figure 5 shows a mass spectrum of PEG 1500 obtained with the flow injection mode.

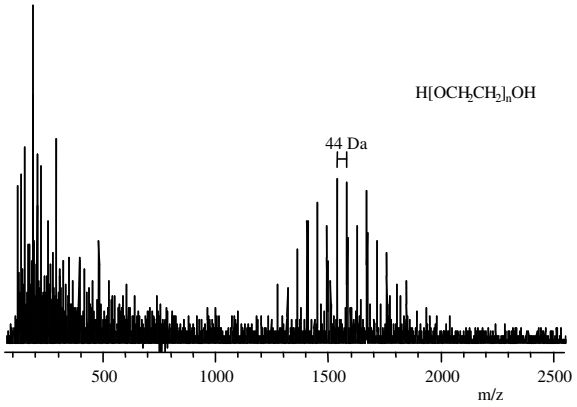


Figure 5. Positive ion mode flow injection AP MALDI mass spectra of equal mixture of PEG 1500 (2 mM in H_2O), 1,2,4-butanetriol (BTT) and matrix (200 mM HCCA in CH_3OH) with a flow rate of 3 µL min^{-1}

5.1.2.2 Improved design

The initial interface was further improved by changing the laser position in relation to the droplet (Figure 6). The pulsed laser beam was then focused from below onto the surface of the droplet.

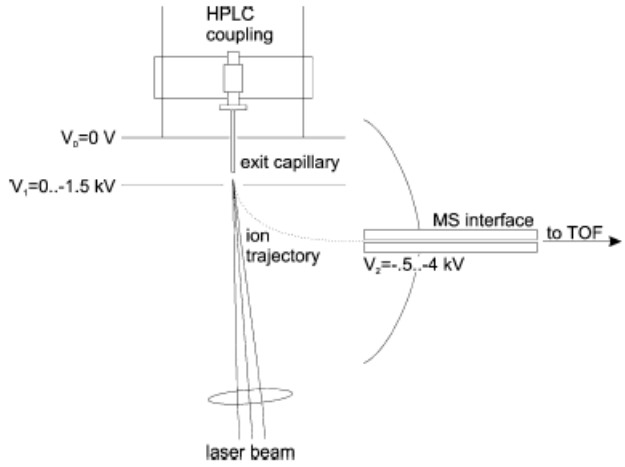


Figure 6. Schematic of the final set-up. The liquid is connected to the ionization source by HPLC coupling, the laser is focused to the end of the exit capillary and the liquid analyte/matrix mixture is desorbed/ionized. The ions are guided by electric fields to the interface of the mass spectrometer

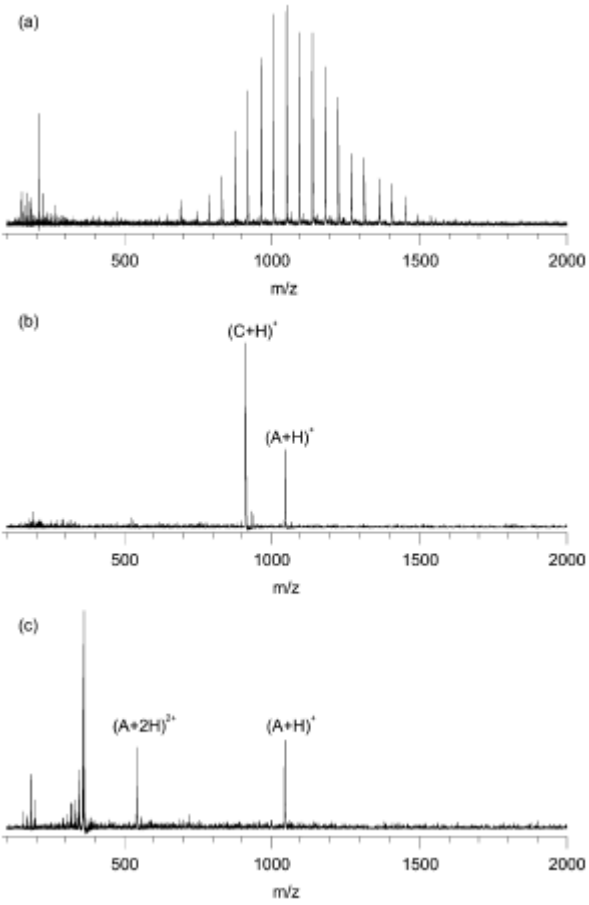


Figure 7. Positive ion mode flow injection AP MALDI mass spectra of (a) 1mM PEG 1000 in MBBA:CH_3OH 1:1 with a flow rate of 5µl min^{-1}, (b) 20 µM α-casein(90-96) (C), 15 µM angiotensin II (A) in 25 mM methanolic HCCA with a flow rate of 1 µl min^{-1}, and (c) 50µM angiotensin II (A) in 80 µM harmane in H_2O with a flow rate of 10 µl min^{-1}

The improved version of flow injection atmospheric pressure MALDI device was applied for the analysis of peptides and polyethylene glycols. For that purpose, various liquid as well as solid matrices dissolved in different solvents were used. Examples of the obtained results are shown in Figure 7, where the spectra of polyethylene glycol 1000, α-casein(90-96), and angiotensin II in different liquid and solid matrices dissolved in water or methanol are presented.

For the analysis of PEG 1000, a known system with MBBA [l] as a liquid matrix was used. Figure 7a shows the intact Na^+ cationized oligomer distribution of 1 mM PEG1000 in an equivalent mixture of MBBA and methanol. No sodium was added to the sample. For the analysis of peptides, solid matrices dissolved in different solvents were used. Figure 7b shows a spectrum of 20 μM α-casein(90-96) and 15 μM angiotensin II obtained when HCCA (25 mM) dissolved in methanol was used as a matrix. Figure 7c presents a mass spectrum of 50 μM angiotensin II with 80 μM harmane dissolved in water as a matrix.

Figure 8 shows three sequential injections of 500 nl of 4μM angiotensin II in 25 mM methanolic HCCA. In this system, 25 mM HCCA dissolved in methanol was used as an effluent. The obtained result clearly shows that the proposed method is applicable for coupling liquid chromatography to AP-MALDI.

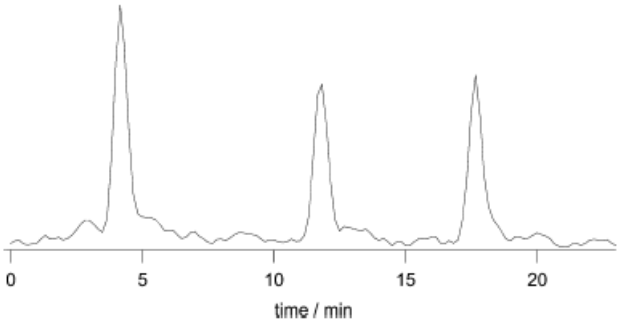


Figure 8. Three flow injections of 500 nl of 4 μM angiotensin II in 25 mM methanolic HCCA; 25 mM methanolic HCCA was used as mobile phase and the applied flow rate was 1 μl min^{-1}. The loss of sensitivity was observed during the measurements

Besides HCCA and harmane, other common solid matrices were investigated to ascertain if they could be used as liquid matrices when dissolved either in water or methanol (Table 1). The solubility of UV MALDI matrices often poses problems. While many matrices show a fairly good solubility in methanol, the solubility in water is often poor, leading to low matrix concentrations in the final analyte/matrix mixtures. Nevertheless, it was possible to measure angiotensin II with all matrices in water and in methanol, except with protoporphyrin IX in methanol. Although angiotensin II could not be detected in the latter experiment, a strong matrix ion signal was detected, indicating that desorption/ionization occurs but

Table 1. MALDI mass signal response of angiotensin II with different matrices in methanol and water: ++, good signal response; +, detectable signal; –, no analyte ion detected

matrix	MeOH	H_2O
HCCA	++	+
DHB	+	++
protoporphyrin IX	––	+
ATT	++	+
harmane	+	++
PNA	++	++
sinapic acid	++	+
caffeic acid	++	+

that the analyte signal might be suppressed by the matrix. The overall signal-to-noise ratio obtained with water samples was worse than the one achieved with methanol, nevertheless, both solvents can be successfully employed for measurements. To improve the signal-to-noise ratio, the matrix concentration in water samples should be enhanced. For that, it is necessary to look for new matrices with better solubility in water.

The above presented experiments were carried out with a nitrogen laser (337 nm) at a repetition rate of only 20 Hz. To use the fast data acquisition of time-of-flight mass spectrometer more efficiently (4000 transients per second for our instrument), a "Power Chip" laser (Nd:YAG, 355 nm, 15 μJ pulse^{-1}, JDS Uniphase, USA) with a repetition rate of 1 kHz was employed instead of a nitrogen laser. As a result the signal-to-noise ratio was improved by a factor >2 (Figure 9b). Although the pulse energy of the high repetition rate laser is lower, a better beam profile allowed us to achieve comparable laser fluence, which is a relevant parameter for successful MALDI experiments. Besides changing the laser, the interface was improved by replacing the static exit capillary with a piezo driven micro pump (Gesim mbH, Germany), where the droplets can be ejected up to a frequency of 1 kHz, and which is usually employed for pipetting picoliter volumes. This was done because a laser must be focused exactly onto the surface of the droplet, but in the case of the fused silica capillary, the droplets had dimensions of millimetres and did not have a constant size. This change improved the signal-to-noise ratio by a factor of three in comparison with the fused silica capillary (Figure 9c).

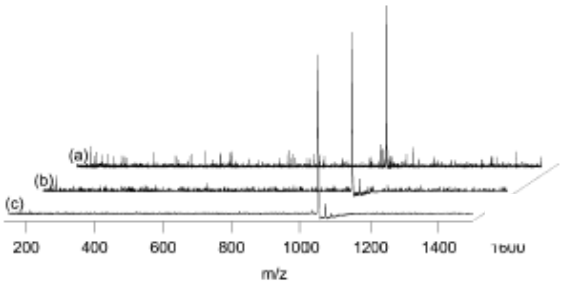


Figure 9. Positive ion mode flow injection AP MALDI mass spectra with different lasers and exit capillaries. (a) 50 μM angiotensin II and 25 mM HCCA in MeOH, flow rate 10 μl min^{-1}, nitrogen laser (20 Hz, 337 nm). (b) 50 μM angiotensin II and 25 mM HCCA in MeOH, flow rate 5 μl min^{-1}, Power Chip Laser (1 kHz, 355 nm). (c) 50 μM angiotensin II and 25 mM HCCA in MeOH, flow rate 5 μl min^{-1}, Power Chip Laser (1 kHz, 355 nm), droplet ejection at 1 kHz

The new set-up also enabled us to perform quantitative measurements, as shown in Figure 10. Angiotensin II was used as an internal standard to account for fluctuations in the total ion current. To estimate the standard deviations and confidence intervals, each concentration was measured for ten times. The noticeably small deviations indicate that the error of preparing the calibration solutions is larger than the error of measurements. For α-casein, the measured concentrations are as low as 500 nM or 83 fmol consumed, for a clearly detectable analyte ion signal. Thus, with the current technique, concentrations

down to 500 nM or 83 fmol are accessible and therefore the sensitivity is comparable to AP-MALDI and to conventional electrospray ionization mass spectrometry: the sensitivity for AP-MALDI for compounds in the mass range of 800 to 1700 Da was estimated by Moyer *et al*. to be 10 to 50 fmol [li].

To determine the upper mass limit for this technique, a solution of 20 μM cytochrome C and 25 mM HCCA in methanol was measured, but neither the singly nor doubly charged species could be detected (data not shown). Sze *et al*. showed the spectra of bovine serum albumin (67 000 Da) recorded from a solution of glycerol, HCCA and 3-amino-quinoline with conventional vacuum MALDI [lii]. They demonstrate that it is possible to measure high mass compounds using co-dissolved matrix / solvent systems. Unfortunately, most atmospheric pressure mass spectrometric interfaces, in particular, those with electrospray interfaces, are optimized for a *m/z* between 1 and 6000 and do not transmit high mass ions effectively, making it difficult or impossible to detect high mass compounds. Thus we believe that it is the instruments interface rather than the desorption/ionization technique that presently hinders the measurement of high mass compounds.

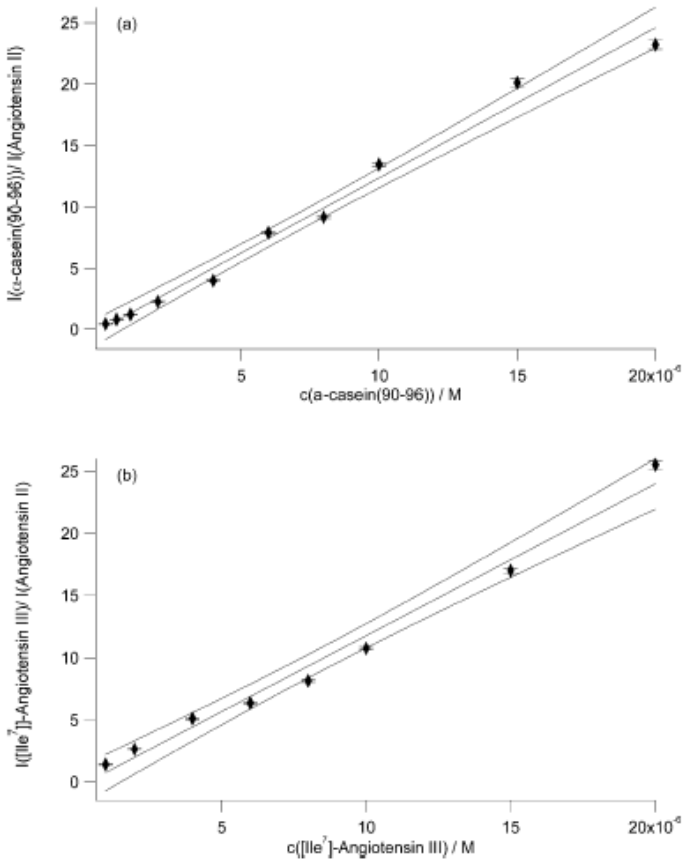


Figure 10. Quantitative measurement with flow injection AP MALDI. (a) 0.1 μM to 20 μM α-casein(90-96) with each 15 μM angiotensin II and 25 mM HCCA in MeOH. (b) 1 μM to 20 μM [Ile^7]-angiotensin III with each 15 μM angiotensin II and 25 mM HCCA in MeOH

- Using the key benefit of atmospheric pressure (AP-MALDI), i.e., that no vacuum has to be maintained a simple, yet an effective way of on-line coupling of liquid sample delivery to AP MALDI mass spectrometry was developed.
- A substantial benefit of this method is that the parameters of the separation, such as solvents and flow rates, do not have to be altered, because there is no need for vacuum compatibility. The matrix is added after the separation, and the overall flow rate can be regulated by the splitting valve prior to desorption.
- The current limit of detection is about 500 nM for methanol as a solvent but may be further improved.
- Due to the homogenous distribution of the matrix and the analytes within the droplets, quantification becomes feasible.
- It should be possible to collect spare sample droplets on a target and to use them for further mass spectrometric analysis or to archive the samples.
- Furthermore, it is well known that water can be employed as a matrix in IR-MALDI experiments. If water can indeed be directly used as a matrix, no additional matrix has to be added to the sample solution. This is especially interesting for measuring biological samples under non-denaturing conditions.
- In contrast to conventional MALDI measurements with liquid matrices in vacuum, the solvents, matrices and analytes do not need to be vacuum stable and therefore a new field of applicable systems opens.

6 APPLICATION OF STROBOSCOPIC SAMPLING IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-CAPILLARY ELECTROPHORESIS

LC and CE are orthogonal methods, and therefore well suited for the 2-D coupled method. Stroboscopic sampling could possibly solve the problem of fast separation necessary for the second dimension of such a system.

6.1 Pneumatic sampler

In LC-CE experiments, an important part of the system was a pneumatic sampler, which was constructed earlier in the Department of Chemistry at Tallinn University of Technology [liii]. The pneumatic sampler was used to introduce sub-samples, obtained after HPLC separation, into the CE part of the 2D system (Figure 11). The sampler device contains one T-shaped (input channel) and two L-shaped channels. Actuators, which consist of 0.4 mm thick Teflon membranes, close the ends of the T- and L-shaped channels. The

actuators were controlled by computer via solenoid valves (Type 6012 Miniature Solenoid Valve; Brükert, Helsinki, Finland). The other ends of L-shaped channels were connected to the buffer and waste vessels. The inlet end of the capillary and one electrode were mounted into the long bath of the input channel of the sampler. The input channel had also an actuator consisting of a membrane at the end of the channel. The actuator was used to close the input channel end to waste. This action enabled the hydrodynamic introduction of the sample into capillary and its flushing by buffer or NaOH. Pressure was applied at two points of the system. One pressure was applied to the actuators so that the membranes closed the ends of the T- and L-shaped channels (P_2-P_4). The other pressure (P_1), somewhat lower, was applied to the waste (effluent) and buffer reservoirs. To fill the long bath of the input channel with a sub-sample from HPLC and/or buffer, the pressure of a particular actuator was released to reach atmospheric pressure.

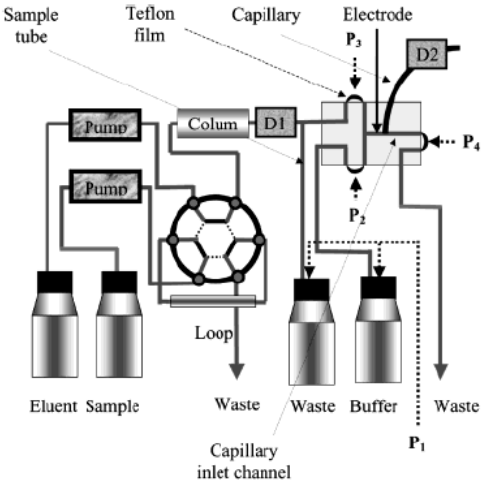


Figure 11. Schematics of HPLC-CE interface. P_1-P_4 pressures; *D1*, HPLC detector; *D2*, CE detector

6.2 Equipment and working principle of LC-CE instrument

The on-line system was build up on the basis of a pneumatic sampler, which is more precisely described in the previous chapter. The main components of the tested HPLC-CE system are presented in Figure 11.

The 2 D HPLC-CE system works with the following principle. Every measuring cycle starts with an injection of the sample into the HPLC column by activating the mechanical valve (Design Bureau of Estonian Academy of Sciences, Tallinn, Estonia). Two HPLC pumps are used in the system, one (Knauer, Germany) for continuous pumping of the sample through a 250 μL loop, and the other (Inchrom, Estonia), for pumping the effluent and injected sample zone at a constant velocity (1.0 mL/min), through the HPLC column. The effluent leaving the column passes the UV detector (Knauer, Germany) and flows to the waste vessel. When the sample is not introduced to the CE system, no pressure is applied to the waste and buffer vessels, but pressure (P_2, P_3) is applied on the actuators closing the sample (sub-sample from HPLC), and buffer channels of the pneumatic sampler (P_2=P_3). During sample introduction, however, pressure P_1 is applied to the waste vessel (P_1<P_2=P_4) and by

simultaneously releasing pressure P_3, a certain amount of effluent leaving the HPLC column is transported to the input channel of the pneumatic sampler. At the same time, pressure P_4 is applied and the input channel of the pneumatic sampler is closed. In the input channel, where a CE capillary and an electrode are inserted, the sampling occurs (combination of hydrodynamic and electrokinetic injection). After sample injection, the input channel of the sampler is filled with the buffer solution by releasing pressures P_2 and P_4 (P_2=P_4=P_{atm}) and by simultaneously applying pressure P_1 to the buffer vessel. When a high voltage is applied, second, electrophoretic separation of the sample takes place in the silica capillary.

In the chromatographic part of the system the following LC columns were used: ion-exclusion column BioRad, AMINEX Ion Exclusion HPX-87H, anion-exchange column IonPac AG9-SC, 40 × 4 mm (Dionex, Sunnyvale, CA, USA), and reverse-phase column BioRad Hi-Pore RP-304, 250 × 4.6 mm (Richmond, CA, USA). CE separation was performed in the silica capillary (Polymicro Technologies, Phoenix, AZ, USA), with dimensions of 60 cm (40 cm to detector window) × 50 μm. A high voltage of 20 kV was applied with Spellman 2000 High Voltage Power Supply (Spellman, Hauppauge, NY, USA) throughout the experiments. After CE separation, UV detection (Bischoff Lambda 1000 Spectrophotometer, Leonberg, Germany) was used. The sampling process was controlled and HPLC and CE detector signals were acquired with LabVIEW software (National Instruments, Austin, TX, USA) written in-house. The detector signals were recorded via ADAM 4018/4060 interface blocks (Advantech, Taipei, Taiwan). The acquired detector signal was further processed (baseline reduction, spike removal) with Matlab 5.6 (Mathworks, Natick, MA, USA) software written in-house.

6.3 Sampling procedures

Two different sampling procedures were tested on the constructed HPLC-CE system. The first procedure included a single injection of the sample into a LC column and multiple injections of the effluent into a CE capillary. In this case, the sample injection frequency is limited by the CE analysis time (practically not shorter than 5 minutes). The summed CE detector signal must comprise well readable fragments, which consist only of the peaks of analytes and are not interfered by the peaks from previous or next sample injection. Figure 12 presents an example of a real experiment, where one can see a flat and asymmetrical LC peak (the upper line) and a series of ferograms obtained with multiple injections to the CE system (the lower line). One can clearly see the appearance of methylsuccinate and hydroxyisovalerate peaks. The intensity of the peaks depends on the composition of the sub-sample from the LC column.

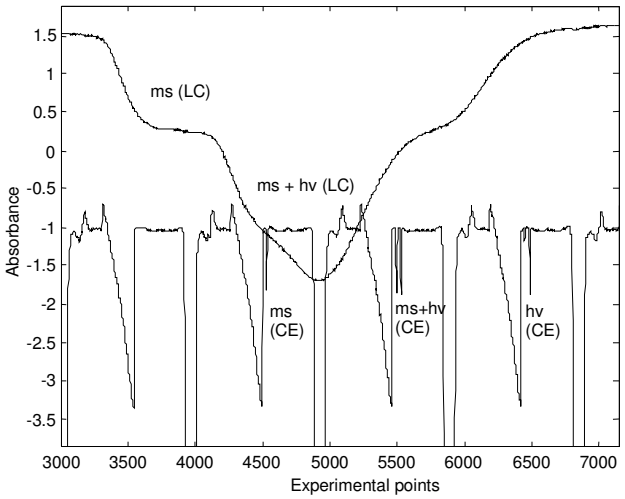


Figure 12. Sample: methylsuccinic acid (ms) and 2-hydroxyisovaleric acid (hv) in H_2O. LC: ion-exclusion column (BioRad, AMINEX Ion Exclusion HPX-87H), 0.008 N H_2SO_4, 0.6 ml/min, 254 nm. CE: 10 mM C_6H_5COOH, NaOH, 1 mM CTAB, pH 5.20, 19 kV, 5 μA, 235 nm

The second method that was used for sample introduction in the on-line HPLC-CE system is called stroboscopic sampling. When using this method, the experiment consisted of multiple measuring cycles. A single measuring cycle embraces single injection of an original sample into a LC column, taking a sub-sample (a "cut") from the LC effluent flow exiting the column and performing a

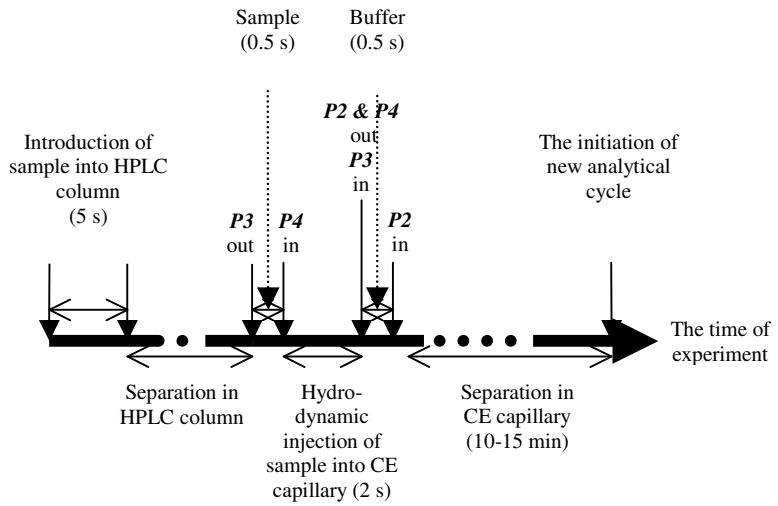


Figure 13. Actions during one stroboscopic sampling cycle

CE analysis of this sub-sample. With this method, the sample is introduced into the chromatographic column for several times. However, the time interval between injecting the sample into the LC column and taking the sub-sample from the LC effluent is prolonged with every single cycle. This action enables us to perform electrophoretic analysis of the LC effluent at a certain moment in

time, which corresponds to a certain area on the chromatogram. With this so-called stroboscopic technique one can scan the whole chromatogram, which is long-term, but a very informative procedure because it enables us to perform a detailed analysis of concrete chromatographic peaks which might consist of several components, not separated in the particular LC column. The latter method, stroboscopic sampling, was found to be the most suitable and optimal method for sampling in the HPLC-CE system. Thus, it was used to perform all the following experiments. A detailed description of the stroboscopic cycle is presented in Figure 13.

6.4 Quasi-flow-injection-analysis, (quasi-FIA)

To evaluate the performance of the HPLC-CE interface, the so-called quasi-FIA experiment was performed. The LC column was replaced by a Teflon tube (100 × 1.5 mm), to where the mixture of three organic acids (10 mM of each in water) was regularly introduced by a mechanical valve. Sample zone widened when passing the tube, and the LC detector registered an unsymmetrical concentration profile.

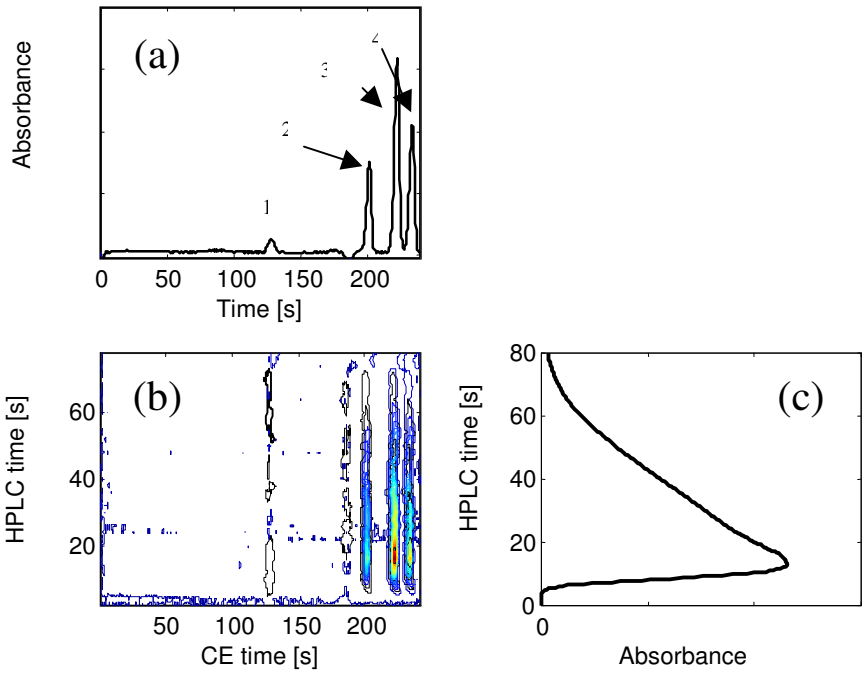


Figure 14. Quasi-FIA experiment. Chromatographic effluent and CE buffer: 20 mM $Na_2B_4O_7$*$10H_2O$. (a), electropherogram at 210 nm; (b), contour plot of the 2-D separation; (c), LC detector signal averaged over 40 experiments. Peaks: 1, effluent; 2, hippuric acid; 3, 3-aminobenzoic acid; 4, benzoic acid

At the first dimension, 40 regularly spaced FIA peaks were registered. An averaged signal of 40 experiments after the first dimension is presented in Figure 14c. An average position of the maximum, the area and half-width values of these peaks were 13 ± 0.4, 1.00 ± 0.06 (normalized), and 30 ± 2 s, respectively (the values for the relative standard deviation are 3.1, 6, and 7%, respectively).

The effluent exiting the Teflon tubing was at a certain time moment directed to the CE capillary, where the mixture was separated into single components. The time interval between injections to the CE system was determined by the time needed for the electrophoretic separation of the analytes, which was 240 seconds. In Figure 14a, an averaged electropherogram of 40 single experiments of the separation of organic acids is presented. The intensity of the corresponding peaks (concentration of analytes) on the individual electropherograms depended on the "place of sampling" from the effluent, because each time the sub-sample ("a cut") was taken from a different part of the sample zone. In real time, the period of time between the pherograms was 240 seconds, but finally, a 80-second chromatogram was scanned for 40 times with a 2-second interval. In Figure 14b, the result of the 2-dimensional separation of organic acids is presented as a contour plot.

It can be seen from the comparison of Figures 14a, 14b and 14c that the system consisting of a HPLC pump, a tube replacing the HPLC column, a pneumatic sampler and a CE instrument worked well when stroboscopic sampling was used. Thus, the used interface suites for the studied method. Also, the obtained contour plot is in accordance with the averaged chromatogram and electroferogram. No unexpected features were observed.

6.5 2D-HPLC-CE analysis of multicomponential mixture

The performance of the 2D HPLC-CE system for accomplishing a comprehensive analysis was tested, using the artificial mixture of organic acids and phenols and a commercial product, tannin. The artificial mixture was analyzed with ion chromatography and a CE coupled system, and tannic acid with the reversed-phase chromatography-CE.

6.5.1 Comprehensive HPLC-CE analysis of model mixture

The standard mixture of phenols and organic acids (12 analytes) was separated to single components during the 2D experiment by combining the anion-exchange column, IonPac AG9-SC, 40 ×4 mm (Dionex, Sunnyvale, CA, USA) and the CE capillary through a pneumatic interface. The advantage of using ion chromatography in the case of 2D separation is in the fact that aqueous solutions of inorganic salts, which are frequently used as CE buffers, can be employed as a chromatographic effluent. In the current experiment 20 mM sodiumtetraborate solution was used both as a HPLC effluent and a CE buffer.

Consequently, the problem of matching/mismatching of buffers during the introduction of the sample to the second dimension was avoided. The results of the analysis are presented in Figure 15.

The sample zone in the LC column was approximately 5 minutes long. At different time moments, for 32 times a part of this zone ("a cut") was introduced into the CE capillary and further separated. The first sub-sample from the HPLC effluent was taken 20 s (the dead time of the HPLC column) after the sample was introduced into the HPLC column. Thereafter, the time between injecting the sample into the HPLC column and taking a sub-sample from the HPLC effluent was prolonged in each cycle by a 5-second interval. Figure 15a shows three large peaks of analytes exiting the chromatographic column, these peaks include 12 single components, 11 of which can be separated electrophoretically (peaks 8 and 9 coincide in the pherogram; Figure 15b). Figure 15c shows that with the two-dimensional method all 12 components of the mixture were separated into single peaks.

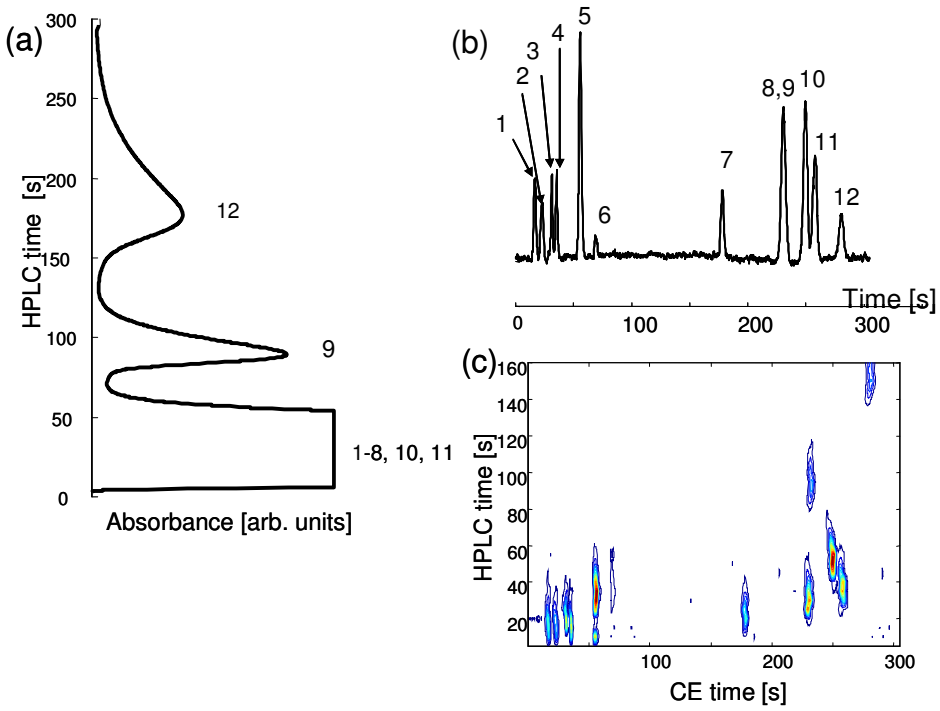


Figure 15. 2D HPLC-CE separation. Chromatographic effluent and CE buffer: 20 mM $Na_2B_4O_7*10H_2O$. (a), LC detector signal at 213 nm; (b), summed electropherogram, reconstructed from 2D data at 210 nm; (c), 2D chromatoferogram. Peaks: 1, 2,6-dimethylphenol; 2, 2,5-dimethylphenol; 3, 3-methylphenol; 4, phenol; 5, 5-methylresorcinol; 6, resorcinol; 7, hippuric acid; 8, 3-aminobenzoic acid; 9, 3-nitrobenzoic acid; 10, 2-aminobenzoic acid; 11, benzoic acid; 12, salicylic acid

6.5.2 Comprehensive HPLC-CE analysis of a commercial product

For the 2D separation of the heterogenic tannin sample, reversed-phase column Bio-Rad Hi-Pore RP-304 was used as the first dimension (Figure 16). 12.5 mM potassium dihydrogen phosphate in the $H_2O/CH_3CN/CH_3OH$ (77.5:20:2.5) mixture was used as a LC effluent, and 20 mM sodium tetraborate was used as a CE buffer. As a result of the use of different buffer systems, complicated system peaks appeared (Figure 16b). In the case of the sample introduction and separation in the 2nd dimension is strictly limited to a certain time frame, the summed electropherogram might be seriously influenced by those peaks. The performance of the 2D analysis in a stroboscopic regime, where the separation time in the CE is not limited, enables us to overcome the above described problem and achieve the separation of analytes without the interference of system peaks.

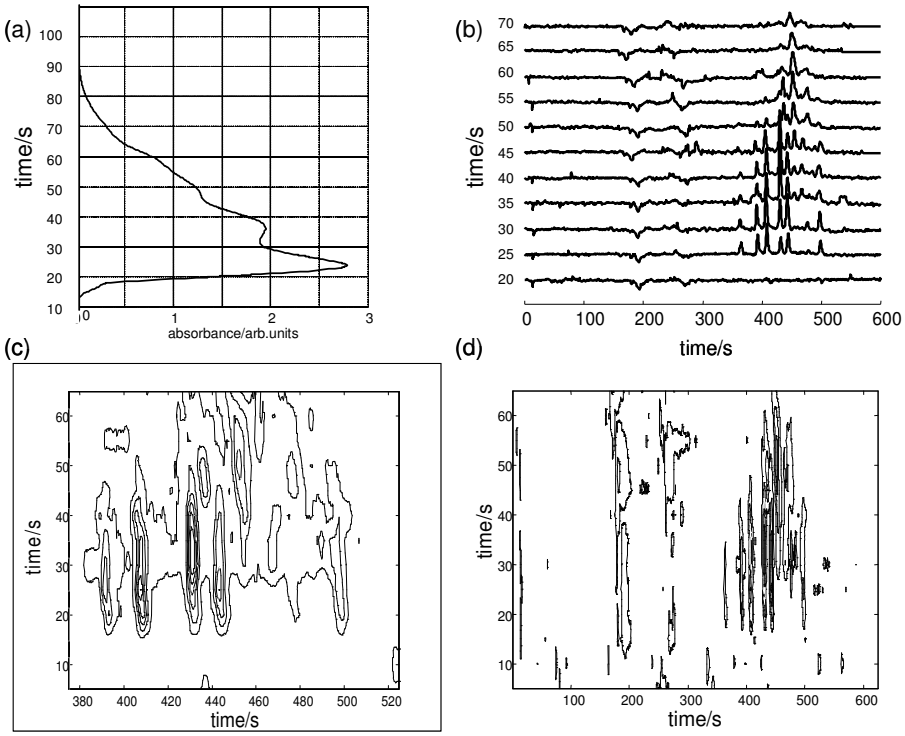


Figure 16. 2-D separation of tannic acid (0.5 mg/mL). (a) HPLC detector signal; (b) electropherograms recorded at particular time moments; (c) part of the contour plot ; (d) contour plot

The peak of tannin in the first-dimension was rather narrow (Figure 16a), thus only 11 sub-samples were introduced into the CE column. In each cycle, the interval of taking sub-samples from the HPLC effluent was prolonged by 5 s. The CE separation took about 10 minutes. The recorded electropherograms are

presented in Figure 16b. Figure 16d shows a contour plot of two-dimensional separation of tannin, part of the contour plot is zoomed in Figure 16c.

Conclusions

- The above described applications show that complicated mixtures, which are not separable only by HPLC or CE, can be successfully analyzed with the proposed HPLC-CE instrument.
- The current interface enables us to perform electrokinetic or hydrodynamic injection of sub-sample from the HPLC system into the CE capillary without the use of flow splitters.
- Unlike previously described LC-CE systems, in the case of the proposed interface, analysis in the first dimension (HPLC part) is performed rather quickly, in the second dimension (in CE part), however, it is more important to have a good separation.
- Stroboscopic sampling, which was employed in the proposed HPLC-CE device, enables us to scan the whole chromatogram and was proved to be well suited for the constructed HPLC-CE instrument. Moreover, the interface described here is not specific for stroboscopic sampling, thus also other interfaces, e.g., flow-gating, could be used for this purpose.
- The stroboscopic sampling is performed automatically (under computer control) without intervention or supervision of an operator. This sampling mode sets no limitations on the analysis time in the second dimension, which helps to avoid the interfering influence of system peaks.
- Drawbacks of the described LC-CE device are long analysis time and unsuitability for applications where only minute amounts of a sample are available.

7 CAPILLARY ELECTROPHORESIS AND SPECTROPHOTOMETRY, TOOLS FOR DETERMINATION OF BIOACTIVE PHENOLS AND THEIR ANTIOXIDANT ACTIVITY

In this study classical spectrophotometric methods as well as capillary electrophoresis were applied to characterize antioxidant activities of commonly consumed Northern European berries and their phenolic profiles.

7.1 Sample preparation

All berry samples were either purchased from a local grocery store or collected ripe near Tallinn, Estonia at the end of August 2004. The berries were stored in a freezer (−18°C) until analysis. The species studied included bilberry (*V. myrtillus*), cowberry (*V. vitis-idaea*), cranberry (*V. oxycoccus*), strawberry (*F. ananassa*), black currant (*R. nigrum*), and red currant (*R. rubrum*).

Two different methods were tested for the extraction of berries. The methods used were the microwave-assisted extraction (MAE) and ultrasonic extraction. For the analysis, a weighed portion (12.5 g) of frozen berries was squeezed and extracted applying microwave power 180 W for 3 min with 25 mL of a C_2H_5OH/H_2O (70:30) mixture. For each berry, the final amount of extract obtained was 37.5 mL. After extraction, all berry samples were filtered and kept at +4°C until analyzed.

The ultrasonic extraction was performed with mixtures of CH_3OH/H_2O (50:50), CH_3OH/H_2O (70:30), and C_2H_5OH/H_2O (70:30). To all extraction mixtures, 1% HCl was added. For the analysis, a weighed portion (50 g) of frozen berries was squeezed and extracted in an ultrasonic bath at room temperature for 8 min, with 100 mL of extraction mixture. The extracts were kept for 16 h at +4°C, and then filtered and stored at +4°C until analyzed. To the extracts prepared for the CE measurements, 20 mg of L-ascorbic acid was added to the extraction mixture to reduce the degradation of polyphenols in the extracts over time. For each berry species, the final amount of the extract obtained was 150 mL.

The yields of flavonoids and phenolic acids by the ultrasonic extraction were higher than by MAE (results not shown). The yields of flavonoids and phenolic acids increased with the increasing amount of the methanol in the solvent. The highest yield of polyphenols was achieved by the ultrasonic extraction with 70% methanol, which was chosen for further study. The extracts obtained were used for the TPH and the AC analysis without further concentration. To study whether the first extraction of flavonoids and polyphenolic acids was exhaustive, the berry samples were extracted for the second time in the same conditions. The concentrations of phenolics determined in both of the extracts were summed, and the amount of each phenolic in the first extraction mixture was compared to the total. This procedure was performed in triplicate for each berry species. The recoveries varied from 60% for quercetin to about 100% for *trans*-resveratrol, *p*-coumaric acid, and ferulic acid (Table 2).

Table 2. Recoveries of quantitatively determined polyphenols after the solvent extraction

analyte	recovery (%)[a]	R.S.D. (%)
trans-Resveratrol	97.8	5.57
cinnamic acid	75.5	6.07
ferulic acid	96.8	4.11
p-Coumaric acid	98.5	6.63
quercetin	59.9	6.22
morin	90.9	3.58

[a] To determine the recoveries of individual polyphenols, the berry samples were extracted twice in an ultrasonic bath, and their content in both extracts was determined by CE. Finally, the amount (%) of individual phenolics found in the first extract in relation to their amount in a combined extract was determined. *n=3*

7.2 Determination of antioxidant potential and total phenolic content by spectrophotometric methods

The concentration of total phenolics in the extracts was measured according to the Price and Butler method, which is based on the colorization reaction [liv], using tannic acid as a standard.

In short, 250 μL of the berry extract standard was added to 25 mL of the deionized water and mixed. Then, 3 mL of the ferric chloride ($FeCl_3$) reagent was added, and the mixture was mixed again. After 3 min, 3 mL of potassium ferricyanide ($K_3Fe(CN)_6$) reagent was added with mixing. The samples were incubated at room temperature for a total of 18 min. The absorbance was read at 720 nm using a Jasco V-530 UV-vis spectrophotometer. The total phenolic content of the berries studied is expressed in milligrams of tannic acid equiv per 100 g of frozen berries (mg of TAE/100 g of FW), shown in Table 3.

To determine the antioxidant activity of berry extracts, an ABTS radical cation decolorization assay was used. The assay was carried out using a Jasco V-530 UV-vis spectrophotometer. The radical was generated through the reaction between ABTS and potassium persulfate [lv]. A stock solution of 7 mM ABTS was prepared in water. To this solution, potassium persulfate (the final concentration 2.45 mM) was added, and the solutions were allowed to react at room temperature in the dark for 12 h. The ($ABTS^{\bullet+}$) solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. The L-ascorbic acid solution and berry extracts were separately mixed with the ($ABTS^{\bullet+}$) solution and allowed to react until the absorbance reached a plateau (15 min). The antioxidant activities were determined by comparing the change in absorbance at 734 nm in a reaction mixture containing the berry extract with that containing L-ascorbic acid (AA). The results were expressed as μM AA equiv per 100 g of frozen berries (μM AEAC/100 g of FW). The relative antioxidant activities of berries are presented in Table 3.

Table 3. Total phenolic (TPH) content and antioxidant activity (AC) of various berries[a]

berries	TPH[b] (mg of TAE/100 g)	AC[c] (uM of AEAC/100g)
red currant	10.33±1.63	0.57±0.06
cranberry	18.08±1.13	0.84±0.03
strawberry	15.74±0.90	0.86±0.10
black currant	25.80±3.59	1.27±0.03
cowberry	35.95±2.02	1.76±0.05
bilberry	43.43±583	1.89±0.04

[a] The results are presented as mean ± standard deviation (SD) for triplicate analysis. [b] TPH results are expressed in tannic acid equivalents (TAE). [c] AC results are expressed in L-ascorbic acid equivalents (AEAC).

Correlation of TPH and AC

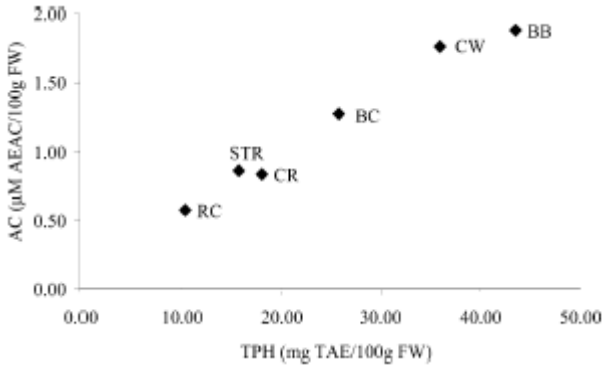


Figure 17. Linear correlation between total phenolic content and antioxidant activity of berries. AC (antioxidant activity) and TPH (total phenolic content) are expressed in tannic acid equivalents (TAE) and L-ascorbic acid equivalents (AEAC), respectively. RC, red currant; STR, strawberry; CR, cranberry; BC, black currant; CW, cowberry; and BB, bilberry

As seen in Figure 17, the total phenolic content is well correlated to the antiradical measures (TPH vs AC, R^2=0.981), which is in accordance with the previously reported results [lvi]. Therefore, it is according to expectations that bilberry and cowberry, which were found to contain the highest TPH levels, would also exhibit the greatest antiradical behaviour. Conversely, red currant exhibited the lowest levels of TPH and antiradical activity. The high correlation between TPHs and antioxidant activities suggests that the antioxidant activity of berries is derived mainly from the content of phenolic compounds in fruits.

7.3 Monitoring the degradation of various antioxidants in the presence of H_2O_2 by capillary electrophoresis

7.3.1 Degradation of L-ascorbic acid by H_2O_2

To monitor the degradation of L-ascorbic acid by H_2O_2, two slightly different systems were applied in this study. In one system, the reaction was performed in a special reactor and in the other it was performed inside the CE capillary. The pros and cons of both systems will be described below.

7.3.1.1 Reaction in separate reactor

The main component of the system, a pneumatic sampler, was the same as the one used in the HPLC-CE set-up already described in section 6.1 with an exception that the HPLC effluent vessel (waste) was replaced by a reagent vessel (Figure 18). In the reagent bottle, the vessels of ascorbic acid and H_2O_2 were placed so that equal amounts of both solutions could be injected into the reactor at the same time. The small reactor consisted of a Teflon tubing (1 m × 0.7 mm), where the H_2O_2 and L-ascorbic acid solutions were mixed and the

reaction was performed. It was set to a "serpentine" form, which has 48 turns, to achieve good mixing of the reactants. After selected time intervals, the sample from the reactor was injected hydrodynamically into the CE capillary.

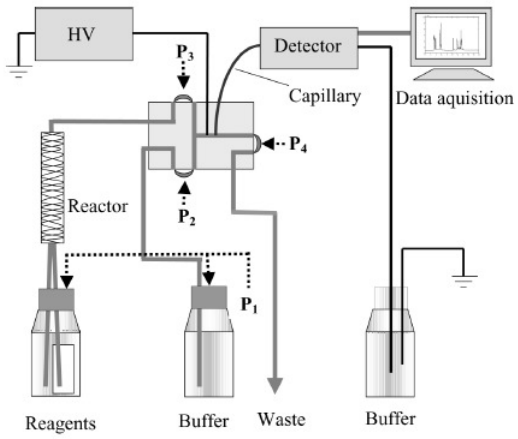


Figure 18. The schematic of the set-up

Separation was performed in an uncoated capillary (Polymicro Technologies, Phoenix, AZ, USA) with dimensions of 60 cm × 50 μm (41 cm to detector). 20 mM sodium tetraborate buffer (pH 9.3) was used to separate the analytes. All the experiments were conducted with an applied voltage of 20 kV. The separation was performed at 265 nm. The capillary was conditioned prior to use with 1 M NaOH for 10 min and with H_2O and separation medium for 5 min, between the analyses, the capillary was washed with background electrolyte for 3 min.

The measurement was started with releasing pressure P_3 in the sampler for 1-2 s. This yielded equal amounts of 7.5% (2.45 M) H_2O_2 in 15 mM disodium hydrogen phosphate (pH 7.4) and 5 mM L-ascorbic acid or selected polyphenol (1 mM) solutions to be mixed in the reactor. The reaction mixture was let to incubate at room temperature for different periods of time. Then pressure P_3 was released again for 1-2 s and the mixture of reactants and products was transported before the inlet end of the capillary for sampling. During the introduction of the sample, pressure P_4 was applied. The separation of the analytes was started when the inlet channel was filled with the buffer solution (by releasing pressure P_2). When the separation was completed, a new reaction and analysis cycle was started but with a different incubation time. By increasing the time of incubation from experiment to experiment, the decrease of L-ascorbic acid concentration was recorded.

7.3.1.2 Reaction in CE capillary

Besides performing a reaction in a separate reactor, an on-column assay for the monitoring of oxidation kinetics of polyphenols by H_2O_2 using CE was proposed in this work. The same reaction was initiated many times with an increasing incubation time, to monitor the reaction kinetics. Unlike the EMMA method, which was described in section 3.1.1, here the reactants were rapidly mixed (within a fraction of a second) before the capillary and introduced into the capillary as a short sample plug of the reacting mixture. After the predetermined contact time, high voltage was applied and common

electrphoretic separation in the capillary took place. When the separation of the reactants / products was completed, the reaction was initiated again.

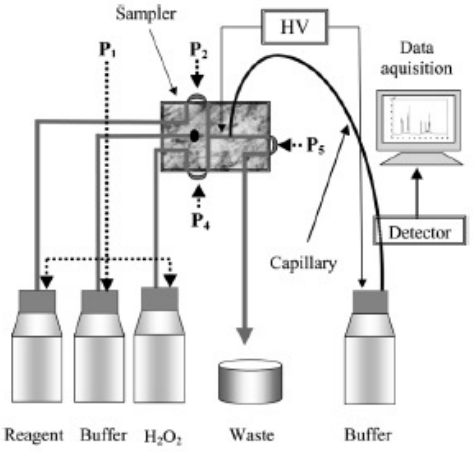


Figure 19. Equipment for performing reactions in capillary

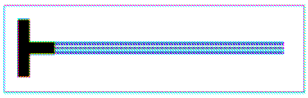
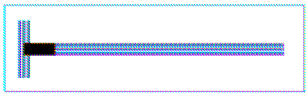
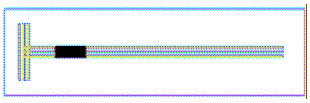
Once again, the above described pneumatic sampler (section 6.1) with slight modifications was used. In this set-up, another L-shaped channel was employed. Thus, the system consisted of three pressurized vessels, which contained a reactant, H_2O_2, and separation buffer (or sodium hydroxide for capillary washing), respectively. Buffer, reagent and H_2O_2 reservoirs were connected to L-shaped channels via a Teflon tubing (20 cm × 0.7 mm). The buffer vessel connection and the corresponding pressure P_3 is not shown in Figure 19 to avoid crowding it with details. The pressure applied to the membranes that closed the channels (P_2, P_3, P_4, P_5) was 1.2 atm, while that applied to the reagent, H_2O_2 and buffer vessels (P_1), was somewhat lower, being about 0.5 atm. This pressure generated liquid flow through the inlet channel equal to 0.45 mL/s. Taking into account the inlet channel volume (40 μL), it means that the inlet channel contents were replaced within 0.1 s.

The capillary dimensions, separation conditions, and capillary rinsing procedures were the same as in the system with a separate reactor. Only here the capillary was thermostated by ambient forced air flow at 25 ± 0.5 °C and detection was performed at 210 and 265 nm.

To initiate the reaction, pressures P_2, P_4 and P_5 (closes the input channel) were released simultaneously, and the input channel was filled with a mixture of the reactant and H_2O_2. To rinse the input channel, the reaction components were allowed to flow for 3 s. Then pressure P_5 was applied for 0.8 s to inject the reaction mixture into the capillary (pressures P_2 and P_4 remained released). Thereafter pressures P_2 and P_4 were applied and the input channel was filled with the separation buffer by releasing pressures P_3 and P_5 for 3 s. The buffer zone was introduced into the capillary by applying pressure P_5 for 0.8 s (pressure P_3 remained released). Now the reaction zone was located between two buffer zones near the capillary inlet. Thereupon pressure P_3 was applied and P_5 was released simultaneously (other pressures remained in "on" position). The pressures remained unchanged during the CE analysis. The above described sampling procedure is thoroughly explained in Table 4. After a pre-determined incubation period, high voltage was applied. This action caused the termination of the reaction as the reactants and products were separated due to their

different electrophoretic mobilities. To study the kinetics, the reaction was stopped at different time moments before the CE analysis.

Table 4. Sampling procedure

time (s)	duration (s)	pressures [a]				action	schematics of the situation in input channel and capillary inlet [b]
		P_2	P_3	P_4	P_5		
0.00	3.0	0	1	0	0	filling of the input channel with reaction mixture	
3.00	0.8	0	1	0	1	introduction of reaction mixture into capillary	
3.80	3.0	1	0	1	0	filling of the input channel with buffer	
6.80	0.8	1	0	1	1	introduction of the buffer into capillary inlet	
variable	variable	1	1	1	0	incubation	
variable	1200	1	1	1	0	application of high voltage and separation of analytes	

[a] 1 denotes pressure "on" and 0, denotes pressure "off";
[b] Vertical bar denotes sampler input channel, horizontal bar means capillary, triple line bar denotes buffer, black and grey bars mean H_2O_2 and reactant mixture.

The same principles were applied when the degradation of other antoxidants, polyphenols in the presence of H_2O_2, either individually or in mixtures, was monitored. The reaction mixture contained equal amounts of H_2O_2 (1.2 M in 15 mM borate buffer) and L-ascorbic acid or polyphenolic solution (0.5 mM in 1.25 mM borate buffer).

Besides different standard mixtures of phenolics, degradation of L-ascorbic acid by the reaction with H_2O_2 was monitored in the extract of sea buckthorn berry (*Hippophae rhamnoides* L.). Sea buckthorn berries were collected near Tallinn, in August 2004. The berries where stored in a freezer (-18 °C).

Extraction was performed as follows. The weighed portion (50 g) of raw material was squeezed and extracted for 1 h with CH_3OH/H_2O (50:50). Then the extract was filtered and stored at +4°C until analyzed.

7.3.2 Degradation kinetics of various antioxidants

The reactions were performed in a way that one reactant, H_2O_2 was in large excess compared to the antioxidant (L-ascorbic acid or polyphenols). Thus, the reactions were pseudo first-order with respect to the antioxidant (AH). The order of the reaction can be given as an overall order or with respect to a specific reactant (a partial order). In this study, the reaction rate was given with respect to AH. The integration of the rate equation for a reaction that is the first order with respect to AH

$$-\frac{d[AH]}{dt} = k_A[AH] \tag{5}$$

yields

$$\ln[AH]_t = \ln[AH]_0 - k_A t, \tag{6}$$

where $[AH]_0$ and $[AH]_t$ are the initial concentration of species AH and that at reaction time t, respectively, and k_A is its rate constant. Equation (6) is called the "integrated rate equation" and allows one to determine the order with respect to AH. According to this equation, a plot of $\ln[AH]_t$ against time should be linear if the reaction is the first order in AH. Thus, the rate of the reaction is only dependent on the concentration of AH.

The degradation of L-ascorbic acid and polyphenols by H_2O_2 was fitted to the first-order reaction kinetics during a period of time where a function $\ln[AH]=f(t)$ remained linear (R^2 was higher than 0.9). The rate constant of the reaction and its standard deviation were calculated by the least-squares method. $\ln[AH]$ was plotted as a function of time and the rate constant (k) at a given H_2O_2 concentration and temperature with respect to AH was determined from the slope of the obtained straight line. As the reactions in a separate reactor and the CE capillary were performed in different conditions, also, the obtained constants differed, being 73×10^{-4} s^{-1} and 13.2×10^{-4} s^{-1}, respectively. The higher constant was obtained when performing the reaction in a serpentine-shaped reactor compared to when it was performed inside the CE capillary. The reason for that might be a better mixing obtained in the reactor.

In the CE capillary reactions with other antioxidants besides L-ascorbic acid were also performed. The first-order rate constants for L-ascorbic acid and polyphenols degradation separately and in mixtures, in the presence of H_2O_2, are presented in Table 5 and Figure 20. In this figure, the oxidation rates of various individual antioxidants (x-axis) are represented according to which mixture they belong to (y-axis). Not all of the possible combinations were

covered, as the purpose of the work was set to be analytical (*i.e.* proof of the concept). It is evident from Table 5 and Figure 20 that from the studied antioxidants, quercetin oxidizes the fastest, followed by chlorogenic and L-ascorbic acids.

Table 5. Reaction rate constants (k) for antioxidant degradation in the presence of hydrogen peroxide

mixture	component	$k \times 10^{-4}$ [a] (s^{-1})	$dk \times 10^{-4}$ [b] (s^{-1})	RSD [c]	R^2 [d]
mixture with	rutin	3.7	0.27	7.28	0.987
p-coumaric	chlorogenic acid	2.6	0.27	10.39	0.968
acid	gallic acid	6.4	0.74	11.53	0.917
	caffeic acid	3.4	0.44	13.00	0.969
	quercetin	51.8	3.02	5.79	0.996
mixture	caffeic acid	2.6	2.77	10.48	0.929
	quercetin	15.4	2.30	14.97	0.922
	ascorbic acid	37.4	2.44	6.52	0.991
mixture	chlorogenic acid	6.2	0.56	9.10	0.967
	caffeic acid	8.4	1.30	15.45	0.918
	quercetin	50.2	6.93	13.82	0.978
mixture	gallic acid	9.1	1.08	11.92	0.930
	quercetin	28.8	2.76	9.59	0.949
	ascorbic acid	85.0	2.84	3.34	0.986
mixture	gallic acid	3.1	0.24	7.89	0.958
	caffeic acid	3.4	0.22	6.45	0.984
	ascorbic acid	36.2	2.01	5.55	0.997
mixture	gallic acid	12.1	1.06	8.75	0.956
	caffiec acid	3.3	0.31	9.40	0.945
	quercetin	50.7	2.1	4.14	0.978
mixture	chlorogenic acid	6.3	0.94	14.91	0.910
	quercetin	55.7	1.63	2.93	0.998
mixture	gallic acid	9.5	0.71	7.49	0.982
	quercetin	50.6	6.55	12.95	0.958
mixture	gallic acid	14.2	2.14	15.05	0.905
	ascorbic acid	11.6	0.98	8.42	0.972
mixture	quercetin	24.9	2.02	8.11	0.980
	ascorbic acid	66.2	3.49	5.27	0.996
methanol extract of sea buckthorn	ascorbic acid	7.26	0.77	10.61	0.945
individual	ascorbic acid	13.2	1.00	7.57	0.979
individual	quercetin	61.5	5.57	9.04	0.991
individual	caffeic acid	9.5	0.29	3.04	0.998
individual	gallic acid	6.7	0.76	11.44	0.929
individual	chlorogenic acid	18.8	2.30	12.25	0.958

[a] Rate constant; [b] standard deviation of rate constant; [c] RSD = 100 × (dk/k); [d] squared correlation coefficient of dependence, ln(peak area) *versus* incubation time.

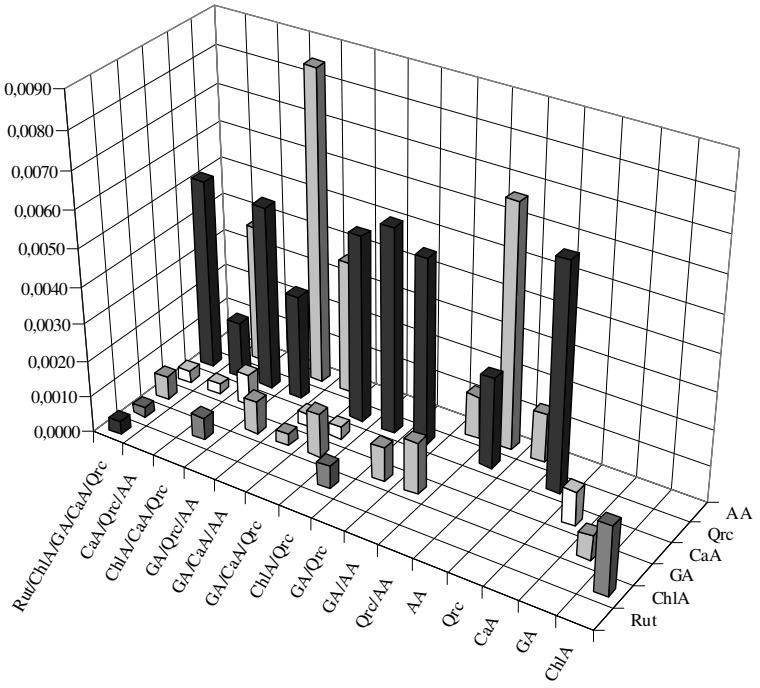


Figure 20. Oxidation rate constants of various plant polyphenols and L-ascorbic acid combinations in the presence of H_2O_2. x-axis, individual compounds; y-axis, various mixtures; z-axis, rate constant value (s^{-1}). AA, L-ascorbic acid; Qrc, quercetin; CaA, caffeic acid; GA, gallic acid; ChlA, chlorogenic acid; Rut, rutin

After a longer incubation period, the degradation of antioxidants in different mixtures did not fit anymore to a first-order reaction kinetic. Over time, even an increase in the concentration of some phenolics was observed (not shown here). In this case, the reaction products were probably not separated from the reactants. The behaviour of antioxidants in the AA/Qrc/GA mixture, where the degradation of AA followed first-order kinetics, was particularly interesting. The oxidation of Qrc was strongly inhibited until AA had been completely reacted, after which the rapid oxidation of Qrc started. GA started to degrade when both AA and Qrc had been completely oxidized.

During the oxidative degradation of L-ascorbic acid and quercetin, a synergism between them has been observed also by others. Takahama *et al.* and Makris *et al.* stated that L-ascorbic acid inhibited the oxidation of quercetin [lvii,lviii]. Quercetin, in turn, enhanced the oxidation of L-ascorbic acid, as the oxidation intermediate of quercetin, which may be quercetin radical, is reduced by L-ascorbic acid [lvii]. Our findings strongly support these observations: quercetin oxidation remains slow in the L-ascorbic acid/quercetin mixture until L-ascorbic acid has completely reacted. On the other hand, in binary H_2O_2/antioxidant mixtures, the oxidation rate of quercetin is 4-5 times higher

than that of L-ascorbic acid and more than for other plant polyphenols studied by us.

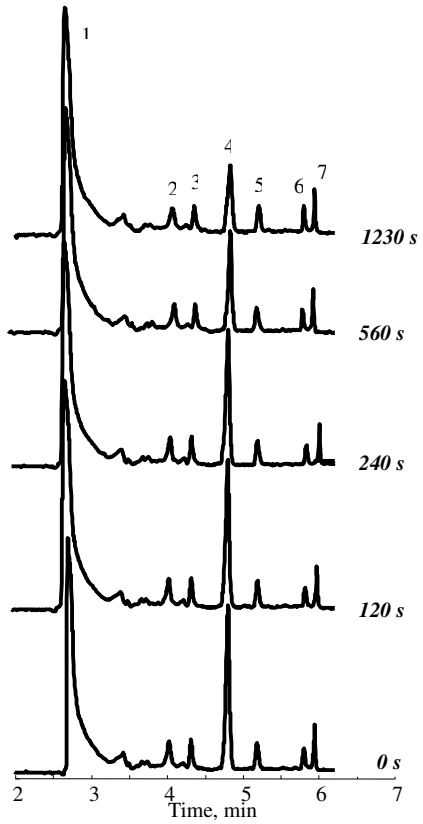


Figure 21. Monitoring of oxidation kinetics of sea buckthorn berry extract. Equal amounts of H_2O_2 (1.2 M in 15 mM borate buffer) and of sea buckthorn berry methanolic extract in 1.25 mM borate buffer. Peaks: 1, H_2O_2; 2, unknown; 3, (+)-catechin/rutin; 4, L-ascorbic acid; 5, myricetin/quercetin; 6, unknown; 7, caffeic acid. Experimental conditions: 20 mM sodium tetraborate, pH 9.3, voltage 20 kV, detection wavelength 265 nm. Numbers by electropherograms indicate the incubation time (filling/mixing time is neglected)

The identification of the target analytes in the sea buckthorn berry extract was done by a standard addition method. The largest peak was identified as L-ascorbic acid. It can be seen in Figure 21 that AA degrades the fastest in the berry extract and that the concentration of other components remains approximately the same during the monitored time. This indicates again that L-ascorbic acid inhibits the oxidation of polyphenols. The oxidation rate constants of L-ascorbic acid in the sea buckthorn berry extract and in standard mixtures of polyphenols differ remarkably. This has also been noticed by other researchers, for example, Özkan *et al.* [lix] have reported that in various fruit juices, the rate constant of L-ascorbic acid degradation varies within a large range, depending on the content of flavonols and anthocyanins in a particular juice.

7.4 Solid-phase extraction coupled to capillary electrophoresis

The determination of polyphenolic compounds in berry samples requires preconcentration procedures prior to the CE analysis. This is because berry matrices are very complex and many phenolic compounds are present in them at very low concentrations. Besides, berries contain high amounts of sugar, organic acids, and pectin. The solid-phase extraction (SPE) has been rather widely used to separate these compounds and to obtain the desired extract

[lvi,lx]. Also, in this work the SPE treatment was used before the CE analysis, to decrease the matrix effect and simplify the electropherograms.

The off-line concentration process before the CE analysis was carried out using 2 g of C-18 cartridges (Mega Bond Elut, Varian). The C-18 cartridge was conditioned with 15 mL of CH_3OH and washed with H_2O. Then, 5 mL of the sample solution, obtained after ultrasonic extraction of berries, was loaded into the cartridge and, using a syringe attached to the outlet, pulled with a low flow rate (3-5min). After washing the cartridge with H_2O, the phenolic compounds retained were eluted with methanol. The first fraction with a volume of 0.5 mL was introduced for analysis to CE. The effect of the SPE treatment was first tested on a standard mixture of phenolics. It was found that after the SPE treatment, the content of flavanol (±catechin), some of the hydroxy-cinnamic acids (caffeic and chlorogenic acid) and hydroxybenzoic acid (gallic acid) in the sample was either remarkably reduced or the compounds were completely lost. However, cinnamic acid, quercetin, and morin were concentrated during the SPE process (Table 6). SPE proved to be well-suited also for the determination of *trans*-resveratrol, ferulic acid, and *p*-coumaric acid. Recoveries of these compounds varied from 88 to 96% (Table 6). Therefore, only these compounds were determined quantitatively by the CE. All the results were corrected for recoveries.

Table 6. Recoveries of quantitatively determined polyphenols after the SPE procedure [a]

analyte	recovery (%)	R.S.D. (%)
trans-resveratrol	95.6	3.50
cinnamic acid	221.9	4.94
ferulic acid	92.4	3.80
p-coumaric acid	88.3	3.33
quercetin	236.6	4.95
morin	151.2	3.41

[a] To determine the influence of SPE treatment, the standard mixture of polyphenols was analysed by CE before and after passing it through a SPE cartridge. Then, the recoveries of individual phenolics were calculated using peak areas. The concentration of each polyphenol in the standard solution was 5 μg mL^{-1}. *n=3*.

7.5 Determination of polyphenolic profiles by capillary zone electrophoresis

Several research groups have successfully used borate buffer for the separation of phenolic compounds by capillary electrophoresis [lxi,lxii]. The separation of polyphenols in CE is based on differences in the mass-to-charge ratios of these compounds and on their complex formation with tetraborate molecules when the phenolic group has *o*-hydroxy groups [xxxiii]. It is also known that differences in the conductivities between the sample and the separation buffer may cause the widening of the sample zone. Therefore, to avoid the latter and to improve the resolution of the analytes, we added 5% of methanol to the separation buffer as the berry extracts also contained methanol. Thus, the CZE

separation was performed with 35 mM sodium tetraborate (pH 9.3), containing 5% v/v methanol at an applied voltage of 20 kV at 25 °C. The detection was performed at 210 nm. The standards and samples were injected into the capillary gravitationally at a fixed time of 20 s from 15 cm. The capillary was conditioned prior to use with 1 M 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 M NaOH, H_2O, and the separation buffer for 5 min each.

To evaluate the precision of the method, the reproducibility of the migration time and peak area were determined for each compound for run-to-run and day-to-day by multiple injections of a single solution of all phenolic compounds (5.0 μg mL^{-1} each analyte). The relative standard deviations (R.S.D.) of the migration times and peak areas obtained are presented in Table 7.

Table 7. Reproducibility of the CZE method for the determination of polyphenols[a]

analyte	R.S.D.[b] (%)			
	peak area		migration time	
	run-to-run	day-to-day	run-to-run	day-to-day
trans-resveratrol	1.96	6.34	2.02	2.32
cinnamic acid	1.67	5.74	1.74	2.60
ferulic acid	1.48	4.95	2.06	2.10
p-coumaric acid	3.19	3.90	2.77	1.89
quercetin	3.68	5.74	2.43	1.85
morin	1.23	6.72	2.02	1.71

[a] The separation was performed with 35 mM borate buffer, pH 9.3 in 55 cm (effective length 39 cm) × 50 μm capillary. [b] *n=5*.

Table 8. Quantitative results of the polyphenols studied by CE

analyte	linear range	$y=b_0+b_1c$	R^2	LOD	LOQ
trans-resveratrol	0.60-90.0	b_0=–0.0038±1.61*10^{-3} b_1=3.4750±4.03*10^{-2}	0.9989	0.40	1.32
cinnamic acid	0.40-0.70	b_0=–0.0032±4.17*10^{-3} b_1=8.6019±0.14	0.9981	0.12	0.42
ferulic acid	0.50-50.0	b_0=–0.0066±8.49*10^{-3} b_1=4.0330±0.37	0.9982	0.15	0.50
p-coumaric acid	0.60-70.0	b_0=–0.0041±4.62*10^{-3} b_1=7.9850±0.16	0.9973	0.25	0.85
quercetin	0.60-70.0	b_0=–0.0034±5.54*10^{-3} b_1=8.5288±0.18	0.9966	0.27	0.90
morin	0.60-90.0	b_0=–0.0012+6.05*10^{-3} b_1=8.4565±0.15	0.9974	0.30	0.99

[a] b_0, the intercept; b_1, the slope; LOD, the limit of detection; and LOQ, the limit of quantification. LOD and LOQ are in μg mL^{-1}. Experimental conditions as in Table 7.

For a quantitative determination, the calibration graphs were produced from the results obtained by injecting standard solutions in the range of 0.10-90 μg mL^{-1}. Each point of the calibration graph corresponded to the mean value obtained from three independent area measurements. The limits of detection (LOD) and quantification (LOQ) were obtained by multiplying the standard deviation of five measurements of the standard solution (10.0 μg mL^{-1}) by three and ten, respectively. The results of the detection limit and other characteristic parameters for the determination of phenolic compounds are shown in Table 8.

The representative electropherograms of red currant, strawberry, black currant, cranberry, bilberry, and cowberry are presented in Figure 22. Individual flavonoids, phenolic acids, and stilbene in the electropherograms obtained were identified by spiking experiments, in which the addition of standards to the sample solution resulted in an increase of the analyte peak without the appearance of shoulders or split peaks. The compounds of interest were quantified using calibration standards, and the concentrations were verified by the standard addition method. The analytical results for the presence and the quantity of the phenolics studied by CE are reported in Table 9 and are expressed as μg per g of frozen berry extract.

In general, our qualitative and quantitative data for the phenolic content in berries are in accordance with earlier reports [lxiii,lxiv,lxv]. According to Häkkinen *et al.* [lxvi], *p*-coumaric acid, ferulic acid, and quercetin were the most abundant phenolics in bilberries. The same compounds were found in our studies (6.00, 23.01 and 12.75 μg/g of FW, respectively). The main phenolic compounds identified in cranberry were *p*-coumaric acid (20.28 μg/g of FW), *trans*-resveratrol (19.29 μg/g of FW), and quercetin (5.15 μg/g of FW). Taruscio *et al.* [lxv] have also reported high levels of quercetin and *p*-coumaric acid in cranberries. However, the contents of these compounds were reported to be higher as compared to the levels given in this work. These diversities in quercetin content may be due to differences in varieties or cultivars. According to Bilyk [lxiii], the variation in flavonol content between six cranberry varieties was large (50-70 %). The level of *p*-coumaric acid varies, according to different studies, between 0 and 100 μg/g of fresh weight [lvi,lxv]. According to Häkkinen *et al.*, the main phenolic compound in strawberry is ellagic acid, followed by *p*-coumaric acid [lxvi]. In this study, the concentration of ellagic acid was not determined. The concentration of *p*-coumaric acid (12.48 μg/g of FW) was found to be similar to that reported earlier [lxvii,lxviii]. Strawberry was the only berry species among those studied in which the flavonol morin (0.6 μg/g of FW) was detected. In red currant, an abundant phenolic was *trans*-resveratrol (15.72 μg/g of FW), followed by *p*-coumaric (1.89 μg/g of FW) and cinnamic acid (1.03 μg/g of FW), but this is not in agreement with the study of Häkkinen *et al.* who found the content of quercetin to be the highest [lxvi]. However, the *p*-coumaric acid content determined in this study was similar to that reported by Schuster [lxviii]. In the study of Justensen [lxix], similar to cranberry, high levels of quercetin were detected in cowberry. This is not in agreement with our results. In our study, cinnamic acid (41.2 μg/g of FW),

trans-resveratrol (30.00 µg/g of FW), and *p*-coumaric acid (17.1 µg/g of FW) were the main phenolics

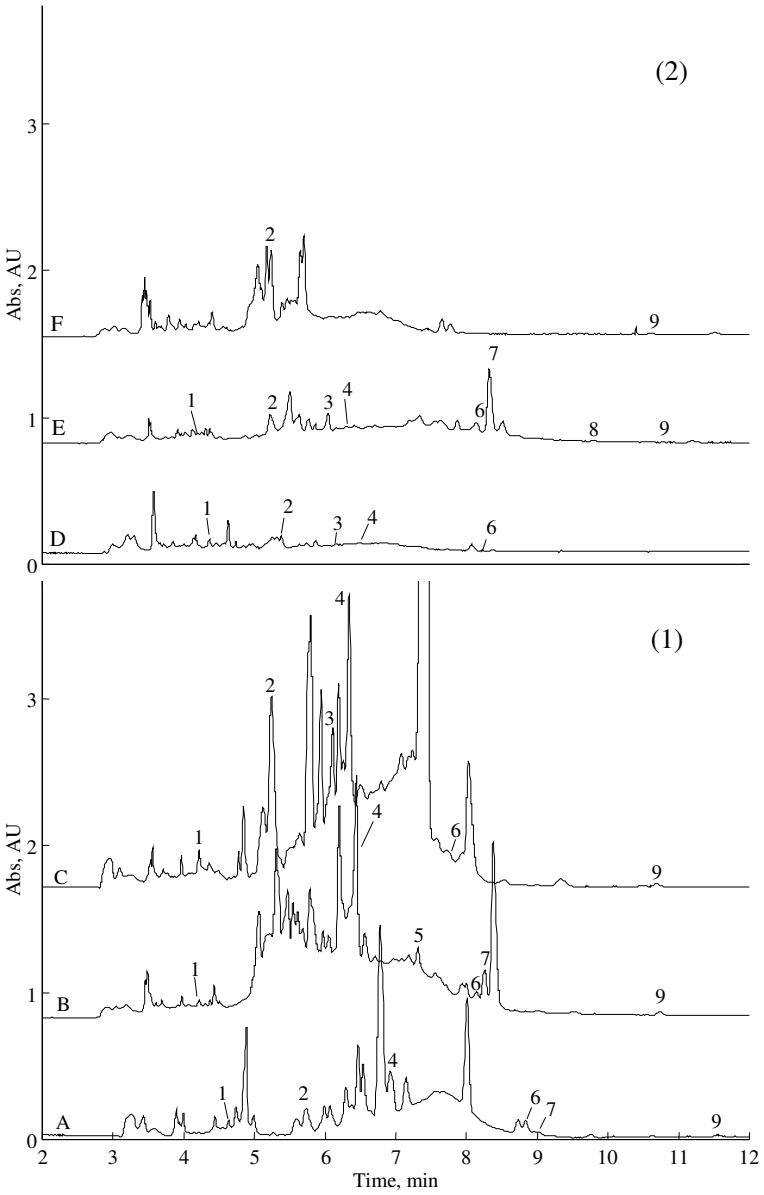


Figure 22. Electropherograms of wild (1): A, cranberry; B, bilberry; and C, cowberry and cultivated berries. (2): D, red currant; E, strawberry; and F, black currant. The analytes are numbered as in Table 9

quantified in cowberry. In black currant, none of the phenolics determined quantitatively in other berries were found. However, in black currant, catechin and caffeic acid, which have also been reported by others, were detected

[lxvi,lxviii]. *trans*-Resveratrol, which was found in high levels in cowberry, cranberry, and red currant, and cinnamic acid whose content was high in cowberry and strawberry, have not been reported in earlier studies.

Table 9. Stilbene, flavonoid, and phenolic acid content of berries [a]

analyte, (μg/g) of FW	berries					
	bilberry	cowberry	black currant	cranberry	strawberry	red currant
trans-resveratrol	6.78±0.18	30.00±2.8	n.d.	19.29±1.53	3.57±1.05	15.72±2.05
catechin	n.d.	n.q.	n.q.	n.q.	n.q.	n.q.
cinnamic acid	n.d.	41.2±2.36	n.d.	n.d.	10.81±1.36	1.03±0.32
chlorogenic acid	n.q.	n.q.	n.d.	n.q.	n.q.	n.q.
ferulic acid	23.01±1.69	n.d.	n.d.	n.d.	n.d.	n.d.
p-coumaric acid	6.00±0.18	17.1±1.68	n.d.	20.28±1.35	12.48±0.76	1.89±0.51
quercetin	12.75±0.70	n.d.	n.d.	5.15±0.40	n.d.	n.d.
morin	n.d.	n.d.	n.d.	n.d.	0.60±0.07	n.d.
caffeic acid	n.q.	n.q.	n.q.	n.q.	n.q.	n.d.

[a] The results are presented as mean ± SD for triplicate analysis. n.d., not detected and n.q., not quantified. Experimental conditions as in Table 7.

As seen from the previous discussion, the contents of flavonoids and phenolic acids in berries vary considerably. The variation in the content of phenolic compounds within one species may be explained by the occurrence of different berry varieties in different countries [lxiii,lxvii,] or by different growing conditions (soil nutrients, temperature, light) [lxx]. Also, differences in berry ripeness may contribute to the variability of the reported flavonoid and phenolic acid concentrations [lxvii].

Conclusions

- Besides spectrophotometry, other methods, like CE enable us to study the antioxidative properties of certain compounds, either in standard mixtures or in plant extracts.
- An important advantage of CE over spectrophotometric methods, where the total antioxidative potential of specific mixture or plant extract can be evaluated, is the possibility to determine the antioxidant activities of single antioxidants simultaneously. In this way, CE offers an alternative to HPLC.
- The above described CE system enables us to control the reaction start- and endpoint better than the EMMA methods, however, the set-up of the system is more complicated than with EMMA
- Bilberries, cowberries, black currants, cranberries, strawberries, and red currants contain large amounts of phenolics and have also high antioxidant activity. The wild berries have significantly higher antioxidant activities than domestic berries and also higher content of phenolics (except for cranberry).

- The polyphenolic profiles of berries obtained are the result of analysis of the antiradical components contained in berries and thus may help to provide a better understanding of the health benefits of different berries.

8 CONCLUSIONS

The aim of the present work was to develop multidimensional methods for the analysis of bioactive compounds in natural mixtures. Special focus was put on the analysis of biophenols in plant extracts.

The principal findings of the work can be summarized as follows:

1) Three different two-dimensional methods were developed for the analysis of bioactive compounds. These methods involve the known separation methods, such as HPLC and CE, and a very powerful detection method – MALDI mass spectrometry.
2) A method where a liquid flow system was coupled with AP MALDI MS was developed. We presume that the same set-up could also be used in combination with HPLC.
3) Several known solid MALDI matrices in different solvents were studied to evaluate their suitability for a liquid flow-AP-MALDI MS system.
4) The application of stroboscopic sampling for the HPLC-CE analysis enabled us to solve the known problem with two-dimensional separation methods, which is the necessity of fast separation in the second dimension. This approach enables us to take samples from the HPLC column after every few seconds, which would be especially useful when mixtures of hundreds of compounds are being analyzed simultaneously.
5) Special emphasis has been laid upon the study of polyphenols and their antioxidant activity in commonly consumed berries in Northern Europe. The SPE-CE method has been applied to determine the phenolic profiles of the studied berry extracts. The antioxidant activity of these compounds was evaluated by studying kinetics of their oxidation by H_2O_2.
6) In addition, spectrophotometry was employed to study the antioxidant activity of the same berries.

ABSTRACT

Multidimensional methods are most frequently applied for the analysis of peptides and proteins in biological samples. This study suggests that they could successfully be employed also for the analysis of plant extracts that often contain hundreds of compounds belonging to different classes of substances.

The main goal of this study was to develop new and improve existing methods for the analysis of complicated biological samples, like peptide hydrolysates and plant extracts. To evaluate the suitability of the proposed methods for such applications, standard mixtures of peptides and polyphenols as well as content of latter compounds in real samples were studied. From separation methods CE and HPLC were applied in this work. Coupling of the latter method with MALDI MS was one of the purposes of the current work. To perform this coupling a method is proposed, where one link is a liquid flow system, which could potentially be a liquid chromatograph and the other link is AP MALDI MS. To couple these methods, a special interface is proposed and further improved. Moreover, substances that are usually used as solid matrices in different solvents were investigated to find their suitability for the developed liquid flow-AP MALDI system. Besides, it was found that the proposed system is applicable for quantitative analysis. In addition to MALDI MS, HPLC coupled to CE was used in this work. The new development of the HPLC-CE method was the application of stroboscopic sampling. Unlike the previously proposed systems, this sampling mode does not set any limit for the analysis time in the second dimension. Besides, it enables us to take samples from the HPLC column every few seconds, thus, making it possible to scan the whole chromatogram.

If only a specific group of compounds in plant extract is analyzed, then application of SPE and choosing of suitable extraction conditions often helps to remove most of the interfering compounds. Depending on the solvents used, SPE also enables a remarkable concentration of some of the compounds, which was also shown in this work. Unlike the above described two-dimensional methods, the SPE-CE method was performed off-line. First the SPE fractions were collected and then analyzed by CE. In addition to standard mixtures, real samples, extracts of most commonly consumed berries in Northern Europe were studied. The SPE-CE method was used to determine biophenols in these berries. The antioxidant activity of the polyphenols identified in berries was studied with capillary electrophoresis. During these experiments, the oxidation of polyphenols by H_2O_2 was monitored and the corresponding rate constants were calculated. Different oxidation rates were observed for the same compound depending on whether it was oxidized separately or in a mixture with other phenolics or L-ascorbic acid. The latter circumstance is in accordance with the data presented in literature about the synergistic effects of polyphenols. Besides the SPE-CE and CE methods, spectrophotometry was used to study the berry extracts, to determine the total phenolic content and antioxidant activity.

KOKKUVÕTE

Antud töös leiti, et lisaks peptiidide ja valkude määramisele bioloogilistes proovides, mis on kõige tavapärasem multidimensionaalsete meetodite kasutusvaldkond, võiks neid meetodeid edukalt rakendada ka taimeekstraktide analüüsil, kuna viimased sisaldavad sageli sadu erinevatesse aineklassidesse kuuluvad komponente.

Antud töö peamiseks eesmärgiks oli välja töötada uusi ning täiustada olemasolevaid meetodeid keeruliste bioloogiliste proovide nagu peptiidide hüdrolüsaatide ja taimeekstraktide analüüsi jaoks. Väljapakutud meetodite sobivuse hindamisel analüüsiti nii peptiidide ja biofenoolide standardsegusid, kui ka uuriti viimaste sisaldust reaalsetes proovides. Lahutusmeetoditest kasutati antud töös kapillaarelektroforeesi (CE) ja vedelikkromatograafiat (HPLC). Viimatinimetatud meetodi ühendamine MALDI mass spektromeetriaga oli antud töö üks eesmärkidest. Selle teostamiseks pakutakse antud töös välja meetod, mille üheks lüliks on vedelikku transportiv süsteem, milleks võiks olla ka vedelikkromatograaf ja teiseks lüliks atmosfäärirõhu MALDI mass spektromeetria (AP–MALDI). Nende meetodite ühendamiseks on töös esitatud spetsiaalne liides, mida on töö käigus täiustatud. Töös on uuritud ka tavaliselt tahkete maatriksitena kasutatavaid aineid erinevates solventides, et teha kindlaks nende sobivust väljapakutud vedelikku transportiva süsteemi–atmosfääri rõhul toimuva ionisatsiooniga MALDI mass spektromeetria jaoks. Leiti, et töös esitatud meetod sobib ka kvantitatiivseks analüüsiks. Lisaks MALDI-le kasutati antud töös vedelikkromatograafiat ühendatuna kapillaarelektroforeesiga. HPLC-CE meetodi puhul oli uudseks momendiks stroboskoopilise sisestuse kasutamine. Erinevalt teiste teadusgruppide poolt varem välja töötatud süsteemidest ei sea selline proovi sisestamine piiranguid analüüsi kestvusele teises dimensioonis. Lisaks võimaldab ta võtta HPLC kolonnist proove vaid mõnesekundiliste intervallide tagant, mis omakorda annab võimaluse uurida põhjalikult kogu kromatogrammi.

Kui taimeekstraktides soovitakse analüüsida ainult spetsiifilisse gruppi kuuluvaid aineid, siis sageli piisab segavate komponentide eemaldamiseks proovist ka tahke faasi ekstraktsiooni kasutamisest enne vedelikkromatograafilist või kapillaarelektroforeetilist analüüsi. Sõltuvalt lahusti valikust võimaldab SPE teatud ainete kontsentreerimist, mida on näha ka antud töö tulemustest. Kui eelpoolkirjeldatud kahedimensionaalsete meetodite puhul toimus proovivõtmine esimesest dimensioonist reaalajas, siis SPE-CE meetodi puhul koguti esmalt SPE fraktsioonid ja alles seejärel analüüsiti neid CE-ga. Nimetatud meetodiga uuriti lisaks standardsegudele ka reaalseid proove, milleks olid Põhja-Euroopas enam levinud marjade ekstraktid. SPE-CE meetodit kasutati biofenoolide määramisel neis marjades. Marjades identifitseeritud polüfenoolide antioksüdatiivset aktiivsust uuriti kapillaarelektroforeesi abil. Nende eksperimentide käigus jälgiti polüfenoolide oksüdatsiooni vesinikperoksiidiga ning arvutati vastavate

oksüdatsioonireaktsioonide kiiruskonstandid. Sõltuvalt sellest kas kiiruskonstant oli arvutatud eraldi või segus teiste polüfenoolide või C-vitamiiniga, olid saadud konstandid ühe ja sama aine jaoks mõnevõrra erinevad. Viimane asjaolu on kooskõlas kirjanduses toodud andmetega polüfenoolide sünergistiliste efektide kohta. Lisaks SPE-CE ja CE meetodile kasutati nimetatud marjaekstraktide uurimiseks ka spektrofotomeetriat, mille abil määrati nii kogufenoolide sisaldus kui ka antioksüdatiivne aktiivsus.

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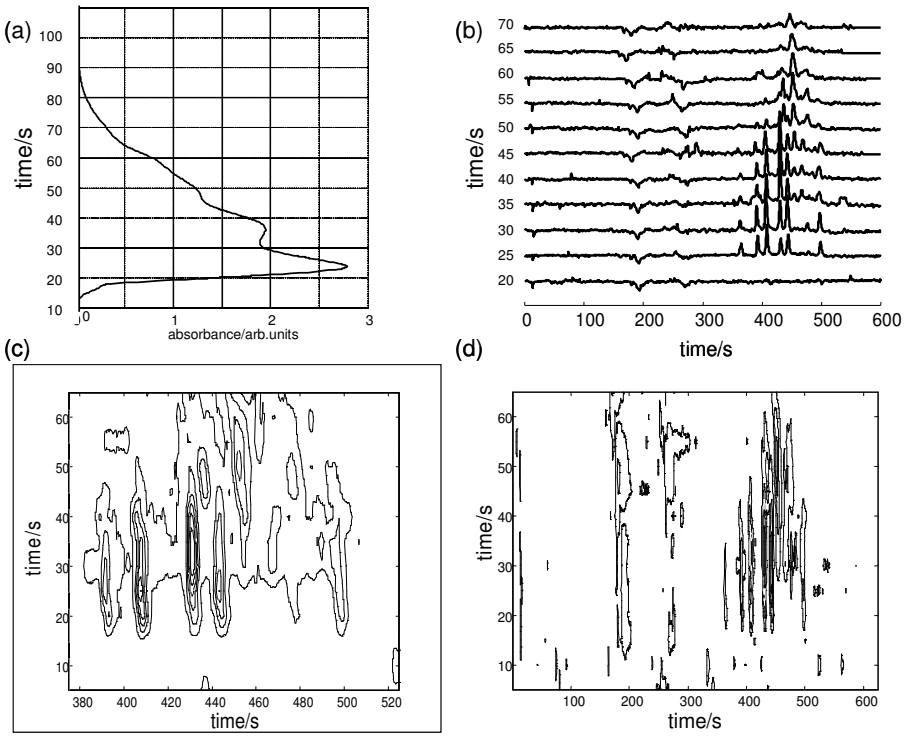
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