THESIS ON NATURAL AND EXACT SCIENCES B88

Determination of Phenolic Compounds and their Antioxidative Capability in Plant Extracts

KATI HELMJA

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TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Chemistry

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Supervisors: Professor Mihkel Kaljurand, Department of Chemistry, Faculty of Science, Tallinn University of Technology, Estonia

Dr. Merike Vaher, Department of Chemistry, Faculty of Science, Tallinn University of Technology, Estonia

Opponents: Professor Ursel Soomets, Department of Biochemistry, Medical Faculty, University of Tartu, Estonia

Adjunct professor Matti Elomaa, University of Helsinki, Finland

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Declaration:

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

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by Roman numerals throughout the text:

I K. Helmja, M. Vaher, T. Püssa, K. Kamsol, A. Orav, M. Kaljurand. Bioactive components of the hop strobilus: comparison of different extraction methods by capillary electrophoresis and chromatographic methods. *J. Chromatogr. A* 2007, 1155, 222-229.

II K. Helmja, M. Vaher, J. Gorbatšova, M. Kaljurand. Characterization of bioactive compounds contained in vegetables of the Solanaceae family by capillary electrophoresis. *Proc. Estonian Acad. Sci. Chem*. 2007, 4, 172-186.

III K. Helmja, M. Vaher, T. Püssa, P. Raudsepp, M. Kaljurand. Evaluation of antioxidative capability of tomato (*Solanum lycopersicum*) skin constituents by capillary electrophoresis and high-performance liquid chromatography. *Electrophoresis* 2008, 19, 3980-3988.

IV K. Helmja, M. Borissova, T. Knjazeva, M. Jaanus, U. Muinasmaa, M. Kaljurand, M. Vaher. Fraction collection in capillary electrophoresis for various stand-alone mass spectrometers. *J. Chromatogr. A* 2009, 1216, 3666-3673.

V K. Helmja, M. Vaher, T. Püssa, M. Kaljurand. Analysis of the stable free radical scavenging capability of artificial polyphenol mixtures and plant extracts by capillary electrophoresis and liquid chromatography-diode array detection-tandem mass spectrometry. *J. Chromatogr. A* 2009, 1216, 2417-2423.

THE AUTHOR´S CONTRIBUTION TO THE PUBLICATIONS

The contribution by the author to the publications referred to above consisted in the following:

I The author was responsible for sample preparations and performed all the by capillary electrophoretic (CE) analyses. She interpreted the results obtained and prepared the manuscript.

II The author performed all the sample preparations and the analyses by CE. She interpreted the obtained results and wrote the manuscript.

III The author performed all the sample preparations and the analyses by CE. She interpreted the obtained results and wrote the manuscript.

IV The author was responsible for the sample preparation and performed the fractions collection by CE. She participated in data interpreting and collecting of the results. She participated in preparation of the manuscript.

V The author performed sample preparation and all the analyses by CE. She participated in data interpreting and discussions of the results. She had the major role in writing the manuscript.

ABBREVIATIONS

ABTS	2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
APCI	atmospheric-pressure chemical ionisation
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
cIEF	capillary isoelectric focusing
cITP	capillary isotachophoresis
CZE	capillary zone electrophoresis
DAD	diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	half maximal effective concentration
EOF	electroosmotic flow
ESI	electrospray ionisation
FC	Folin-Ciocalteau method
FRAP	ferric ion reducing antioxidant power
GA	gallic acid
GC	gas chromatography
HAT	hydrogen atom transfer
HO•	hydroxyl radical
HPLC	high performance liquid chromatography
ICP	inductively coupled plasma mass spectrometry
LC	liquid chromatography
LED	light emitting diode
LOD	limit of detection
MALDI	matrix-assisted laser desorption/ionisation
MEEKC	microemulsion electrokinetic chromatography
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
NACE	non-aqueous capillary electrophoresis
NMR	nuclear magnetic resonants
NO•	nitric oxide
O•	superoxide anion radical
O_2	oxygen
$ONOO^-$	peroxynitrite
ORAC	oxygen radical absorbance capacity
Q-TOF	quadrupole time of flight
RNS	reactive nitrogen species
ROO•	peroxyl radical
ROS	reactive oxygen species
rp	reversed phase
RSD	relative standard deviation

SDS	sodium dodecyl sulfate
TEAC	Trolox equivalent antioxidant capacity
TLC	thin-layer chromatography
TRAP	total radical trapping antioxidant parameter
μ	experimental mobility

INTRODUCTION

Nowadays, phenolic compounds, such as phenolic acids, stilbenes, lignins, coumarins and flavonoids, have aroused interest due to their remarkable spectrum of biochemical and pharmacological properties. The compounds are present in various vegetables, fruits and many food supplements. As of today, there have been isolated and identified a multitude of natural polyphenols in a wide variety of forms. Particular attention has been devoted to the most numerous groups of phenolic compounds, such as flavonoids and phenolic acids, which have also brought into focus in the current study.

Due to their crucial ability to act as antioxidants, polyphenols have become an important object of medical research. Polyphenols are secondary metabolites, which are synthesized by plants and share a common structural feature – they all have an aromatic ring bearing at least one hydroxyl group. Also, naturally occurring antioxidants are more favoured than their synthetic analogs because of the low toxicological safety of the latter. The ability of polyphenols to act as antioxidants is attributed to the following properties of the compounds: 1) ability to donate hydrogen; 2) ability to chelate metal ions; 3) ability to inhibit selectively enzymes responsible for the catalysis of various oxidation processes. The effectiveness of polyphenols in all the three processes is strongly dependent on their structure. Due to this, polyphenols are able to prevent the damages caused by the excessive production of the reactive oxygen species, ROS, or the reactive nitrogen species, RNS, which leads to the impairment of cell structures, including proteins, carbohydrates, lipids and nucleic acids. Moreover, the excessive production of ROS or RNS is considered to have an influence on several pathologies, including cardiovascular diseases, cancer, inflammatory disorders, neurological degeneration (Parkinson's and Alzheimer's diseases), and premature aging. Hence, plant extracts have aroused interest as a potential source of medicines. Since a high number of phenolic compounds found in crude plant extract are still unknown, thus, the latter are attractive objects for pharmacy. Moreover, medicines of plant origin may be used as supplements to synthetic pharmaceuticals as well.

The determination of phenolic compounds in natural products is a highly challenging task. For this, contemporary analytical methods should be applied. The procedure encompasses a number of aspects, for instance, the nature of a sample and an analyte and also the aim to be achieved. Since phenolic compounds are synthesized in the plants in response to stress factors, such as unfavourable environmental conditions (infection, UV radiation), the qualitative and quantitative variations in their concentration in the plant are strongly influenced by environmental, as well as physiological and genetic factors. Polyphenols are distributed in natural products unevenly. Principally phenolics have been accumulated in the outer layers of the tissue of plants[1,2], while insoluble phenolics are mostly found in the cell walls and soluble phenolics in cell vacuoles[1]. Despite the fact, that due to the existence of their glycosidic and/or other conjugated forms, the analysis of phenolic compounds is

complex, the methods to be employed for their analysis must be comprehensive, rapid and provide maximum information about an analyte under study[3].

Several analytical methods have been applied to the analysis of phenolic compounds. Chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), have occupied a leading position in the analysis of natural phenolics. HPLC coupled with different types of detectors, including a UV one and especially, a diode-array detector (DAD), fluorescence and electrochemical detectors have found wide applications for this purpose. However, currently, the most powerful technique is definitely mass spectrometry (MS), which enables the identification of minor and unknown compounds by structural moieties of an analyte. Also, hyphenated techniques, like LC coupling to MS have gained remarkable application in the analysis of polyphenols. Thus, MS was also an indispensable tool throughout the current study, allowing the analysis of phenolic compounds in various plant extracts. Besides chromatographic techniques, also electrophoretic methods, such as capillary electrophoresis (CE), have gained in popularity in the analysis of polyphenols. However, the opportunities of CE in this field have not fully explored yet. The main advantages of CE over the other separation techniques are, for instance, high efficiency, reduced sample and solvent requirements, ease of operation and simple instrumentation. Likewise, CE coupled to MS has been demonstrated to be a sensitive and powerful method but, still, system's drawbacks are the interface between CE and MS and the choice of an appropriate buffer. To overcome these problems, in the current study it was proposed employing an off-line CE-MS for the fraction collection system, which was to be used in the analysis of small molecules like polyphenols.

In general, a parameter to evaluate potent antioxidants from the point of view of their scavenging effect against free radicals is antioxidative capability, which is defined as a sum of all antioxidant activities in a mixture containing antioxidants. The antioxidative capability of polyphenols is strongly dependent on their structure. Among plant phenolics, especially flavonoids are considered to have the highest antioxidative capability. Traditionally, in order to determine total phenolic content and estimate relative antioxidative capability, spectrophotometric assays which are based on the oxidizing and reducing properties of polyphenolics have been performed[4,5]. As a result, a degree of total antioxidative capability is given. In spite of the simplicity for the determination of antioxidative capability, it takes into account of all the antioxidants found in a sample. Also, the dependence of the antioxidative capability of individual phenolic compounds on their structure has been neglected. Therefore, methods enabling monitoring and evaluating the relative antioxidative capability of a single component separately in a mixture are highly necessary. Highly efficient separation methods, such as HPLC or CE, can provide a solution, but their possibilities have not fully been explored yet and no comparative studies of both the methods have been performed either. Hence, this fact has set goals of the present work.

AIMS OF THE STUDY

The main goals of the present thesis were to profile phenolic compounds found in various crude plant extracts using a CE method and to compare the CE results obtained by a conventional HPLC. Special emphasis was placed on developing a simple method for the assessment of the antioxidative capability of the individual phenolic compounds present in plant extracts, as well as on elucidating the factors influencing this capability.
Individual studies aimed at the following:

● analysing the phenolic compounds found in crude plant extracts

– developing a suitable separation protocol for profiling qualitatively the constituents of the phenolic compounds found in crude plant extracts, including sample preparation methods for CE and a comparative study by HPLC.

● employing a capillary electrophoretic method for fraction collection in CE as an accessible alternative identification tool to an on-line CE-MS

- developing a fraction collection system for an off-line CE-MS;
- applying CE in conjunction with LC-MS as a second dimension.

● evaluating the relative antioxidative capability of phenolic compounds

- developing a simple and efficient assay for the assessment of the relative antioxidative capability of phenolic compounds by both CE and LC;
- monitoring the free radical scavenging capability of phenolic compounds by the oxidation reaction conducted by both a stable free DPPH and hydroxyl radicals. The latter is considered as the most aggressive one among reactive oxygen species.

● proposing a new quantitative approach for a “half maximal effective concentration” - EC_{50} to estimate antioxidative capability of individual compounds in extracts based on radical scavenging capability.

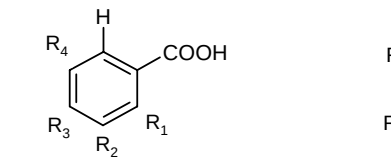
● elucidating the relationship between the structure and antioxidative capability of phenolic compounds.

1. LITERATURE OVERVIEW

Polyphenolic compounds embrace a wide range of secondary metabolites synthesized from carbohydrates via a specific biosynthetic route restricted to plants[6-8]. Polyphenols are associated with a beneficial effect on humans in terms of their potential to scavenge ROS/RNS, such as a superoxide anion radical (O•), hydroxyl radical (HO•), peroxyl radical (ROO•), peroxynitrite ($ONOO^-$), nitric oxide (NO•), and also scavengers of non-radical like singlet oxygen (O_2)[9-13]. Defence mechanisms against the effects of excessive oxidation are provided by an efficacious action of antioxidants in retarding the oxidation[7,12,13]. The capability to donate an electron to free radicals is primary assigned to the structure of polyphenols, which differs in the degree of hydroxylation, substitutions, conjugations and polymerization[14-17]. A correlation has been established between the structure of polyphenols and their antioxidant capability.
The diversity of phenolic compounds complicates their identification in plant matrices. In general, assays used for the analysis of polyphenols can be classified into batch-type techniques, by which total phenolic content is determined, and those, qualifying/quantifying a specific group or class of phenolic compounds. In order to elucidate the most potent antioxidants, a wide array of the respective assays is available.

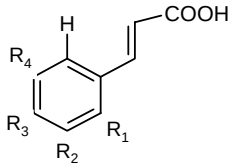
1.1. The structure of polyphenols

According to the structure of phenolic compounds are divided into several groups, such as simple phenols, hydroxybenzoic and hydroxycinnamic acids, coumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids and lignins[6,7,11,16-18]. The chemical structures of the main group of phenolic compounds, phenolic acids, stilbenes and flavonoids, including their subclasses, which were the objects of the present study, are shown in Figure 1.



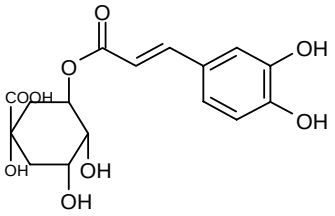
Hydroxybenzoic acid

Gallic acid R_2=R_3=R_4=OH



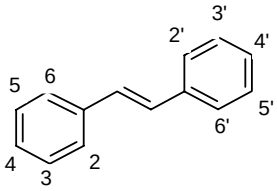
Hydroxycinnamic acid

Cinnamic acid R_1=R_2=R_3=R_4=H
p-Coumaric acid R_2=R_3=R_4=H; R_5=OH
Caffeic acid R_1=R_4=H; R_2=R_3=OH



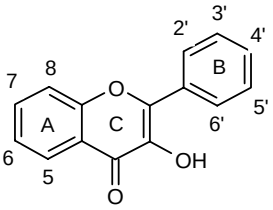
Cinnamic acid esters

Chlorogenic acid (5-caffeoylquinic acid)



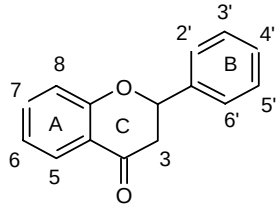
E-Stilbenes

trans-Resveratrol 3=5=4'=OH
Astringin 3=OGlu; 5=3'=4'=OH



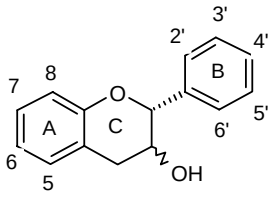
Flavonols

Kaempferol 5=7=4'=OH
Quercetin 5=7=3'=4'=OH
Myricetin 5=7=3'=4'=5'=OH



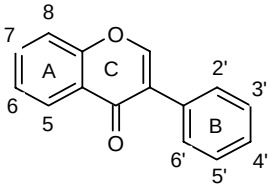
Flavanones

Naringenin 5=7=4'=OH
Eriodictyol 5=7=3'=4'=OH



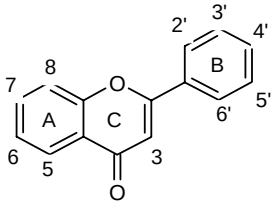
Flavanols

Catechin (2R, 3S) 5=7=3'=4'=OH



Isoflavones

Genistein 5=7=4'=OH



Flavones

Apigenin 5=7=4'=OH
Luteolin 5=7=3'=4'=OH

Figure 1. The structure of phenolic acids, stilbenes and flavonoids

Flavonoids are derivatives of benzopyrone parent compounds composed of two aromatic rings labelled as an A - and B - ring linked by a heterocycle referred as a C-ring (Figure 1)[6,11,15-20]. Flavonoids belonging to various classes differ in the presence

or absence of an unsaturated C_2-C_3 bond and the 4-oxo group in conjugation with 3-OH, while individual compounds within classes vary in the pattern of substitution in the A- and the B-ring.
The structural differences determine the effectiveness of a phenolic compound as an antioxidant. The structural element, which is responsible for the antioxidant activity, is the difference in the spatial arrangement and the number of hydroxyl, methoxy, and glycosidic groups[7,14,16-19]. Flavonoids lacking even one of these features are less potent antioxidants than those with all crucial structural patterns[17].
Hydroxyl substituents are the most significant determinant to stabilise a radical species. They undergo the following reaction[11,17]:

$$Ph - OH + R\bullet \rightarrow Ph - O\bullet + RH$$

Especially the *ortho*-dihydroxy groups (catechol structure) in the A- and the B-rings and as well as the 3-OH in the C-ring are considered to be the most essential for effective radical scavenging[9,11,14,16,17,19,21]. However, the presence of only one hydroxyl group in the B-ring may diminish the activity[18,21]. The arrangement of hydroxyl groups, especially at the positions of C_5 and C_7 in the A-ring, has been considered to have a low impact[17,18] on antioxidant capability, but, yet, increase in the number of hydroxyl groups, the A-ring substitution has been thought to increase in antioxidant capability[17]. The substitution of methoxy groups has been found to decrease antioxidant capability[17]. Quercetin (Figure 1) as the most abundant dietary flavonol is expected to be the most potent antioxidant because it owns all structural patterns for an efficient free radical scavenging activity. But, for instance, the radical scavenging ability of luteolin significantly exceeds that of kaempferol; the hydroxyl groups of the compounds have an identical configuration, but kaempferol lacks of *ortho*-dihydroxy groups in the B-ring. Flavanones, such as naringenin, which have a single bond at C_2-C_3 and only one hydroxyl group in the B-ring at $C_{4'}$ have a low antiradical activity[19]. Flavonols and flavones containing a catechol group in the B-ring are highly active, but the former are more potent antioxidants because of the presence of the 3-OH in the C-ring. But flavanols and flavanones, due to the lack of conjugation provided by the C_2-C_3 double bonds with the 4-oxo group, are considered weak antioxidants. In the case of isoflavones, in which the phenyl ring (the B-ring) is attached to the C_3 position in the heterocycle C-ring, is known to affect radical scavenging capability[11,22]. So, genistein is potentially a more efficient antioxidant than, for example, naringenin.
Flavonoids are present in plants mainly in the glycosylated form. One or more sugar moieties are linked through either an OH group (*O*-glycosides) or C-C bonds (*C*-glycosides)[17,21]. The most common glycoside is glucose, but other examples include rhamnose, glucose, galactose and arabinose[7,21]. The sugar substituent is usually bound to the hydroxyl group (*O*-glycosides) located at the position C_3 or C_7, whereas in the case of the *C*-glycosides, the sugar group is bound to carbon at the position of

C_6 or C_8 in the respective aglycone nucleus[14,17]. It has been noticed that glycosylation diminishes antioxidative capability compared to the corresponding aglycone of the phenolic compound[16], but the sugar in the A-ring leads to more negative influence on antioxidativity than 3-glycosylation in the heterocycle C-ring.
Flavonoids also possess the ability to efficiently chelate trace elements, for instance, Fe^{2+}, Cu^{2+}, Zn^{2+}, which potentially participate in the formation of the reactive oxygen species[9,11,17,23,24].
Naturally occurring phenolic acids (Figure 1) contain two distinctive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures[6,25] albeit the content of the latter in plants is less common than that of hydroxycinnamic acids. Again, the number and position of hydroxyl and methoxy substituents in the structure influence antioxidative capability[15,17,18]. A *para* hydroxyl group enhances the antioxidative capability, while in *meta* or *ortho* positions it has little or no effect[18]. The majority of phenolic acids occur in various conjugated forms, usually linked through ester, ether, or acetal bonds to cellulose, proteins, lignin, flavonoids, glucose, terpenes, etc [3,6,7] and only a minor phenolic acid exists in a free acid form.
Another important group of polyphenols is stilbenes, although in plants these compounds are less represented[26]. The major compound among stilbenes is resveratrol (3, 5, 4´- trihydroxy-*trans*-stilbene), which is one of the most extensively investigated phenolic compound at all. Although stilbenes are usually characterized by the presence of the 1, 2-diphenylethylene nucleus substituted with the hydroxyl or methoxy group(s), yet those found in most plants are derivatives of the *trans*-resveratrol (Figure 1)[27]. The potential sites for hydroxyl groups are at the positions $C_{3'}$, $C_{4'}$, C_3 and C_5. It is considered that the OH group at the $C_{4'}$ position undergoes oxidation more easily than the other OH groups[28]. Like the other polyphenols, stilbenes exist in plants mainly in the glycosylated form, which alters their bioactivity. A high number of stilbenes are present as oligomers[27]. Stilbenes are found in two geometrical isomers *cis*-(Z) and *trans*-(E) form. The latter is thought to be responsible for bioactivity[20]. However, the *trans* form of stilbene easily undergoes isomerisation to the *cis* configuration by the irritation with UV radiation[20].

1.2. Methods for the determination of bioactive phenolic compounds in plant extracts and antioxidative capability

Due to the immense variety of polyphenols, there is no universal approach for the determination of their content in plants, as well as their antioxidative capability. The quantification of phenolic compounds in plant extracts is largely influenced by their chemical nature, the extraction method employed, interfering substances, as well as analytical method used. Although both the qualitative and quantitative determination of individual flavonoid glycosides in food would be desirable, most reference compounds are not commercially available. Moreover, plants differ in flavonoid glycosides composition, although the compounds have been derived only from a few aglycones only. Hence, the analysis of flavonoids should involve the determination of

either target analytes in their various conjugated forms, or aglycones. In order to facilitate the analyses, the conversion of glycosides into aglycones by alkaline, acid or enzymatic hydrolysis[6,7,29-31] has been described. So, there is a need to exploit selective and sensitive analytical methods for the analysis of phenolic compounds of plant origin.

1.2.1. Spectrophotometric assays for total phenolic content

Traditionally spectrophotometry has been applied to determining the total amount of phenolic compounds in a sample by using the Folin-Ciocalteau[6,32-37] method (FC), which is straightforward and highly reproducible. The method is based on the reaction of phenolic compounds with a colorimetric reagent, specifically, on the reduction (electron-donating) of the phosphotungsten–phosphomolybdates complex by reductants (antioxidants) to a blue chromogen[32-35]. The FC method described in[38] is based on the multi-syringe flow injection. Strict control of reaction conditions is necessary in case of this method. Little information is available about another, the Price and Butler method (PB)[36], used for the analysis of total phenolics. FC has been proven to be more convenient and reproducible because of the stability of the formation of the complex between the phenolic compound and the reagent. Little data are available on the determination of total phenolics by the spectrophotometric-enzymatic method[39,40]. There have also been developed protocols for total flavonoid and anthocyanin content[7,8,35,41,42].

1.2.2. Separation methods

Since all the above methods for the assessment of total phenolics or flavonoids preclude evaluating specific phenolic profiles of a sample and the structure-activity relationship, there is a demand for more selective and sensitive methods to identify individual phenolic compound in a sample. Among separation methods, classical chromatographic techniques, such as thin-layer chromatography (TLC), GC and especially HPLC with various detection modes (for example, amperometry, photodiode array, fluorimetry) [6,7,25,29,31,43-45] are very powerful tools for the analysis of phenolic compounds in plant extracts. However, most chromatographic methods for a simultaneous determination of different classes of flavonoids as aglycones are time-consuming and require complex instrumentation. Hence, the electromigration technique like CE with its various modes has gained in popularity as an alternative and complementary tool for the analysis of phenolic compounds[46-56].

1.2.2.1. Liquid chromatography

In general, a liquid chromatography (LC) with its several modifications has proven to be highly effective analytical tool in the research of phenolic compounds. All phenolic compounds absorb in the UV or UV/Vis region due to the presence of at

least one aromatic ring and the double-bound conjugation in the molecule. Therefore in LC, a UV/Vis detector is used most widely. Phenolic compounds may have more than one absorbance maximum in the UV region; thus a variable-wavelength detector, such as diode array detector (DAD) is more preferable. Moreover, DAD has the ability to register multiple spectras, which is highly advantageous in the identification of compounds and determination of their purity. For example, among phenolic acids, most derivatives of benzoic acids usually have their absorbance maximum at 246–262 nm, but hydroxycinnamic acids possess two characteristic absorbance maxima, one maximum is in the range of 200–235 nm and the other in the range of 290–330 nm. In the case of flavonoids, again, two specific absorbance maxima may be observed. The first maximum in the range of 210-285 nm is due to the A-ring and the second between 300-385 nm corresponds to the B-ring. The shift in absorbance is attributed to the substitution pattern and the conjugation system in the molecule[15].

The main limitations in the UV detection are low sensitivity and selectivity, therefore, in some applications fluorescence or electrochemical detections are used. Albeit, MS as a detector has proven to be an excellent tool for the determination of the structure of polyphenols.

Typically LC consists of a normal-phase system, in which less polar analyte eluate first. As in the normal-phase LC the column is polar, highly polar compounds may be retained in it due to the interactions between polar molecules. Since phenolic compounds are weak acids and can be separated as relatively hydrophobic compounds, reverse-phase (rp) columns comprising a non-polar C_{18} stationary phase with an internal diameter of from 2 to 5 mm and a particle size of from 3 to 10 μm are applied to their analysis. A suitable polar solvent system consists of the aqueous and organic phases (methanol or acetonitrile). Typically an acid, for example, acetic or formic acid is added to the solvents. Preferably the gradient elution is applied. It is typical of rp-LC that polar compounds eluate first, followed by those of decreasing polarity. Hence, phenolic acids eluate first and then followed by flavonoid as aglycones. Phenolic acids are eluated from the column by decreasing polarities. The increase in retention time is caused by the loss of the hydroxyl group or the presence of methoxy groups or the ethylene side chain[7]. In case of flavonoids containing equivalent substitution patterns flavonone glycoside eluates first, followed by flavonol and flavone glycosides and then aglycones in the same order, although the overlapping of individual members of different classes is inevitable because of the diversity of compounds.

1.2.2.2. Capillary electrophoresis

Such factors as fast and efficient separation, minimum sample and reagent requirement, ease of operation, non-use or use of very small quantities of organic solvents and simple instrumentation have increased the use of CE for the analysis of pharmaceutically and biomedically important compounds in natural products[46-48,53-

[56]. However, the drawbacks of CE are lower sensitivity and reproducibility compared to HPLC.
Different modes of CE, for example, capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary isoelectric focusing (cIEF), capillary isotachophoresis (cITP), capillary electrochromatography (CEC), microemulsion electrokinetic chromatography (MEEKC) and a novel technique, non-aqueous capillary electrophoresis (NACE)[56,57], possesses their own principle of separation. However, in all the above CE modes separation is based on differences in electromigration between the analytes in a given electric field.
The simplest and typical mode of CE, CZE, is applied to the separation of both anions and cations, including small ions and particles. The sample is introduced at the anodic end of the capillary and separation is governed by the difference in the charge-to-size ratio between analytes in a conductive liquid in the capillary under the influence of applied high-voltage. The movement of solutes in the silica capillary is affected by the electrophoretic migration and electroosmotic flow (EOF). The EOF is the bulk flow of the liquid inside the capillary that originates from the negatively charged silanol groups ($Si\text{-}O^-$) of the inner wall of the capillary during the application of an electric field. The silanol groups are ionized above pH 3, which results in the negatively charged inner wall. Cations in the solution are attracted by the negatively charged inner wall to form a fixed layer; upon applying voltage the cations drag the solution, even the anions, toward the cathode, which increases the EOF. Since the rate of the EOF is higher than the electrophoretic mobility of negatively charged solutes, which originally tend to migrate toward the anode, both the negatively and positively charged solutes can be analyzed within one run. All neutral analytes migrate with the rate of the EOF and remain inseparable.
In the optimization of the CZE separation, the pH of the background electrolyte is the most important factor and the use of additives interacting with analytes may improve selectivity.

1.2.2.3. Hyphenated techniques

Considerable progress has been made in the analysis of polyphenols by the application of chromatographic or electromigration techniques coupled to MS[7,8,25,29-31,44,58] in the performance of the analysis of polyphenols. The method provides information about structural characteristics of an unknown component facilitating its identification in crude plant extracts.
The proper choice of an interface is highly important for the optimal performance of LC-MS. Electrospray (ESI) and atmospheric-pressure chemical ionization (APCI) interfaces have been used for polyphenols analysis because high sensitivity and low limits of detections (LOD in range of ng/mL). Commonly, polyphenols are analysed by LC-MS in a negative mode using an acid as a modifier to improve peak shape. As a mass analyzer ion trap MS to provide higher sensitivity has been proposed.

The coupling of LC to nuclear magnetic resonance (NMR) has attracted attention as well. The method enables, besides structural characteristics, also information about the differentiation of isomers and substitution patterns in the search of novel natural phenolic compounds to be achieved[7,59-61]. But, still, the main disadvantages of LC-NMR are expensive instrumentation and low sensitivity [59].
At present CE-MS has been successfully used in the analysis of a wide range of analytes (small molecules, metabolites, amino acids, etc.)[56,62-65]. However, particularly in the hyphenation of CE to MS the weakest points have been interfacing because of the necessity of the analytes to be ionized, relevant electrical contact for CE and small liquid flow introduced into the CE capillary[47,54,56,62-64]. Various interfaces have been proposed[62,63,66], but ESI with a sheath liquid flow configuration is the most commonly used[64,67]. Moreover, it is considered that the common background electrolytes required in CE, such as borate and phosphate, as well as additives, such as sodium dodecyl sulfate (SDS), are inappropriate if CE is coupled to MS in an on-line mode[64] because of non-volatility and the risk of MS source contamination causing the reduction in sensitivity. On the other hand, the buffers, such as acetic acid, ammonium carbonate and ammonium acetate, proven to be suitable for MS[63,64], may not always allow satisfactory separation by CE. Hence, attempts have been made to set up an off-line CE with fraction collection[68-70], which allows using, besides MS, the other types of ion sources, such as matrix assisted laser desorption/ionization (MALDI). However, the very low sample amount (nanoliter volumes) injected to CE may be the main disadvantage because it affects the concentration of analytes in the collected fractions for further analyses.

1.3. Assays for antioxidative capability

A numbers of studies on the determination of antioxidative capability of polyphenols have been reported[10,13]. Antioxidative capability is a measure of the amount of a given free radical scavenging by a sample. Based on the chemical reaction involved, there are two types of assays. In the reaction of the hydrogen atom transfer (HAT), the antioxidant and substrate, for instance a biomolecule, compete for the reactive species, thus inhibiting substrate oxidation[13,32,71-73]. Thereby the assay measures the ability of an antioxidant to quench free radicals described by oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP) [4,5,13,32,73]. The HAT assay is preferred in lipid oxidation and other biological systems.
The second approach is associated with electron transfer:

$$\text{Oxidant} + \bar{e} \text{ (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidized antioxidant}$$

In these assays the antioxidant interacts with the reactive species, causing a colour change of the reaction medium, which is used to measure the antioxidant's reducing capacity. The degree of colour change is considered to be proportional to the

antioxidant concentration[4,32,61]. The reaction end point is reached when the colour no longer changes. After the substrate is oxidized under standard conditions, either the rate or extent of oxidation is measured. This type of assays includes ferric ion reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazy (DPPH) colorimetric assays[5,13,34-37,71]. The above assays, especially ABTS and DPPH assays, have been widely used in the monitoring of antioxidative capability[4,5,34,36,42,74] because the handling of the above compounds is easier than that of the other reactive oxygen species. However, the overlapping of the compounds with the DPPH or ABTS spectrum is the main complication.

Traditionally, spectrophotometry has been applied to control the mixing of a stable radical with a sample. As a result, the potential of an individual compound in the overall antioxidative capability is neglected. To assess the role of a single compound in overall antioxidative capability, one possible alternative is to combine the separation of compounds by, for instance, LC, and the post-column detection of radical scavenging[75-80]. The method proposed is based on the on-line HPLC comprising the batch-type DPPH assay. However, this approach requires a special post-column reaction device, reaction medium control and fixed reaction time is highly needed. Besides the use of a stable free radical, like DPPH and ABTS, to evaluate antioxidative capability, assays on the determination of the scavenging activity of the HO• have been reported[81-88]. In these assays, the detection of HO• is indirect and the use of the trapping agents producing stable adducts is necessary. The oxidation of the substrate causes a decrease in absorbance, which is proportional to the antioxidative capability. In the case of spectro(fluoro/photo)metry, again, it measures overall antioxidative capability[82-84], although the method for CE[81,86,87] or LC-MS[85] enables to determine scavenging effect for every compound separately.

As follows from the above, mainly integral methods have been used to assess the antioxidative capability of extracts. Reports about the use of LC or CE are scarce and, moreover, it is difficult to decide whether LC or CE should be preferred. Such advantages of CE as minimum sample/solvent consumption, short analysis time, ease of performance, could be fully realized if there would exist a simple and cost-effective method for the identification of compounds in crude plant extracts. Besides, from the "green chemistry" point of view, due to its low eluent requirement, minimized waste generation, simple technology, which is applicable to a broad range of analytes in a continuous monitoring, CE fully qualifies as an environmentally friendly method of analysis. Moreover, the importance of CE arises from the shortage of a key chemical solvent, acetonitrile, particularly in LC. The goal of the present work was to investigate the possibilities of CE for opportunities for the assessment of the antioxidative capability of polyphenols and compare the technique with LC. Moreover, it was decided to develop an off-line MS technique based on fraction collection. Further, a method for the on-line and off-line monitoring of oxidation reactions by CE was developed and its use for the assessment of antioxidative capability was demonstrated.

2. EXPERIMENTAL
2.1. Instrumental

2.1.1. Capillary electrophoresis

CE analyses were performed using an Agilent CE System (Agilent Technologies, Waldbronn, Germany) equipped with DAD. A CE Chemstation (Agilent Technologies) for instrument control, data acquisition and data handling was used. The separation of polyphenols was performed in a fused silica capillary (Polymicro Technology, Phoenix, USA) with an effective length of 50 cm (a total length of 75 cm) and i.d. of 75 μm.
The injection of the sample was performed hydrodynamically over 10 or 15 s, respectively. Prior to all the experiments, the capillary was rinsed with a 0.1 M NaOH solution for 5 min and with the background electrolyte for 5 min. A positive electrical voltage of 15 up to 30 kV was applied to the inlet reservoir.

2.1.2. Fraction collection in capillary electrophoresis

The CE apparatus for fraction collection was constructed in-house equipped with a 1000R high-voltage power supply (Spellman High-Voltage Electronics, NY, USA) and UV detector (Prince Technologies, The Netherlands) at a wavelength of 210 nm. The signal was transferred to a personal computer via a self-made 16-bit analogue-to-digital-converter. The detector was placed at 13 cm from the cathode end of the capillary. A fused silica capillary (Agilent Technologies, USA) with an effective length of 57 cm (total length of 70 cm) and i.d. of 100 μm was used. Prior to use the capillary was rinsed with a 0.1 M NaOH solution for 3 min and with the background electrolyte, 25 mM sodium tetraborate (pH 9.3) for 3 min The injection of the sample was performed hydrodynamically (Δh = 20 cm), a positive electrical voltage of 15 kV was applied to the inlet reservoir. The data were handled by MATLAB Version 6.0 Release 12 (The MathWorks, USA).
In Figure 2 the schematic of the fraction collector is depicted.

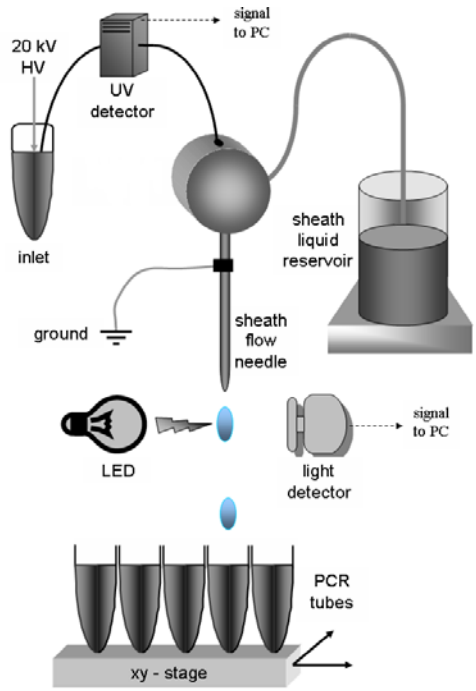


Figure 2. A schematic of the fraction collector

The cathode end of the capillary was inserted inside a stainless steel needle using a coaxial liquid-sheath flow configuration. The vessel of the sheath liquid was raised above the stainless steel needle endpoint by approximately 10 cm and as a sheath liquid the 5% (1 M) of acetic acid delivered by gravity at a flow rate of 2.5 µl/ s was used. The concentration of acetic acid was chosen to match the ionic strength of the background electrolyte in CE. The sheath liquid left the stainless steel needle as a sequence of 18.5 µl droplets. The resolved analytes in the CE capillary were sequentially fractionated into droplets, which were collected into individual 250 µl PCR tubes located in the wells on a moving *xy*-stage on a microtiter plate. The droplets were counted by a light detector with a built-in light emitting diode (LED) driver circuit (Optoswitch S4282-51, Hamamatsu). This only responds to the luminous flux of the connected LED, because the flux to the LED is modulated and the signal processor synchronizes the sensitivity of the light detector to the modulation. The unit can tolerate high background light levels. The output level is TTL/CMOS-compatible and readable using a parallel computer [IV].

2.1.3. Liquid chromatography-mass spectrometry

Chromatographic analyses were carried out using an LC-MS/MS (Agilent Technology, Palo Alto, USA) equipped with an Agilent 1100 Series UV-Vis DAD

and 1100 Series LC/MSD Trap-XCT with an ESI interface connected to an Agilent 1100 Series instrument. The DAD system was working at an interval of 200-600 nm. An rp-HPLC Zorbax 300SB-C18 column (2.1×150 mm; 3.5 µm; Agilent Technologies, Palo Alto, USA) was used in a stepwise mobile phase gradient of a 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min at 35° C. The elution was started with a linear gradient of B from 10 to 30% at 30 min, then to 90% at 40 min finishing isocratically with 90% of B for 10 min. The sample injection volume varied from 3 up to 10 µl. The HPLC 2D ChemStation Software with a ChemStation Spectral SW (Agilent Technology) module for process control was used. The conditions of the MS/MS detection were as follows: negative ionization in an m/z interval 50-1000, target mass 400, number of precursor ions 2, maximum accumulation time 100 ms, compound stability 100%, flow rate of the drying gas (N_2) 10 l/min, gas temperature 350° C, nebulizer pressure 30 psi, collision gas He pressure $6 \cdot 10^{-6}$ mbar.
To the analysis of the fractions collected by CE rp-micro-HPLC-MS a Q-Star Elite device (Applied Biosystems, Germany) equipped with an ESI and quadrupole time-of-flight (Q-TOF) was applied. An SB-C18 rp microcolumn (0.5 x 75 mm, 3.5 µm, Agilent Technology, USA) whose outlet was connected to an ESI fused-silica capillary (50 µm i.d. x 165 µm o.d.), was used. The samples (3 µl) were injected into the HPLC system without any pre-treatment. Mass spectra were obtained in a full scan mode (200–1000 amu). The instrument was operated in a positive ion mode under the following conditions: needle voltage, 4.5 kV; sheath gas (N_2), 80 psi; auxiliary gas (N_2), 29 psi. The collision energy for fragmentation was optimized by Q-Star Elite software depending on MS/MS ion intensity.

2.2. Chemicals and reagents

Phenolic compounds: All reagents were of analytical grade. *trans*-Resveratrol (3,4',5-trihydroxy-trans-stilbene), rutin (3,3',4',5,7-pentahydroxyflavone-3-rutinoside), 3-chlorogenic acid (3-*O*-caffeoylquinic acid), quercetin (3,3´,4´,5,7-tetrahydroxyflavonol), quercitrin hydrate (quercetin 3-rhamnopyranoside), naringenin (4´,5,7-trihydroxyflavanone), naringin (4,5,7-trihydroxyflavanone 7-rhamnoglucoside), kaempferol (3,4´,5,7-tetrahydroxyflavone), genistein (4',5,7-trihydroxyisoflavone), myricetin (3,3´,4´5,5´,7-hexahydroxyflavone), caffeic acid (3,4-dihydroxycinnamic acid), cinnamic acid ((E)-3-phenyl-2-propenoic acid), luteolin (3',4',5,7-tetrahydroxuflavone), *p*-coumaric acid (4-hydroxycinnamic acid), ferulic acid (3-methoxy-4-hydroxycinnamic acid), flavone, tannic acid, gallic acid (3,4,5-trihydroxybenzoic acid) and L-ascorbic acid were from Sigma-Aldrich (USA). Astringin (*trans*-piceatannol-3-glucoside) was purchased from Polyphenols Laboratories (Sandnes, Norway). Catechin (5,7,3´,4´-tetrahydroxyflavane) was from Fluka Chemie (Switzerland). Naringenin chalcone was isolated from the extract of *S. lycopersocum*.

The stock solutions of phenolic standards (5 mM) were prepared in methanol or the methanol/water (80:20, v/v) mixture. All the solutions were stored at +4˚ C in the dark.

Background electrolyte in CE: Sodium tetraborate was purchased from Sigma-Aldrich (USA). For the separation of analytes by CE, 25 mM of sodium tetraborate (pH 9.3) was prepared.

Reagents and solvents: Acetic acid, trifluoroacetic and formic acid, ferric chloride, potassium ferricyanide, aluminium chloride, sodium nitrite, DPPH, ferrous (II) sulphate, sodium hydroxide and hydrogen peroxide were purchased from Sigma-Aldrich (USA); methanol was obtained from Rathburn (Walkerburn, Scotland) and acetonitrile (ultra gradient) from Romil (Cambridge, UK).
For the preparation of all solutions and samples, the high purity deionized water (18.2 MΩ/cm) from the Milli-Q system (Millipore, Milford, USA) was used.

2.3. Sample preparation

2.3.1. Ultrasonic extraction

All the plants were air-dried and homogenized into powder. As an extraction solvent the methanol/water mixture (70:30 or 80:20, v/v) and methanol for the chromatographic analysis were used. Briefly, 5 ml of the extraction solvent was added to 0.5 g of the ground sample. After 60 min the samples were placed into an ultrasonic bath for 20 min at room temperature. The extraction procedure was performed in triplicate. The extracts were collected and concentrated to a 2 ml volume by a Laborata 4000/4001 vacuum rotator (Heidolph Instruments, Germany). All the extracts were filtered through a 0.45 μm filter and stored at +4 ˚C in the dark.

2.4. Determination of total phenolic and flavonoid contents

2.4.1. Total phenolic content

The determination of total phenolics by the Price-Butler[89] method was performed. In this approach, the sample (250 μl) was diluted with 25 ml of deionised water. Subsequently, 3 ml of $FeCl_3$ was added and after 3 min 3 ml of $K_3[Fe(CN)_6]_3$ was added. After the incubation for 18 min at room temperature, the absorbance was measured spectrophotometrically at 720 nm. Tannic acid was used as a standard (linear range of 0.01-2.5 mM). All the measurements were performed in triplicate.

2.4.2. Total flavonoid content

The determination of the total flavonoid content was based on the colorimetric assay[41]. The plant extract (50 µl) diluted with 0.5 ml of deionised water was mixed with 0.03 ml of $NaNO_2$ (5%). After 5 and 11 min, 0.06 ml of $AlCl_3$ (10%) and 0.2 ml of NaOH (1 M), respectively, were added. Finally, 0.21 ml of deionised water was added. The absorbance was recorded at 510 nm spectrophotometrically, using rutin as a standard. The measurements were performed in triplicate.

2.5. The radical scavenging of phenolic compounds

The antioxidative capability of phenolic compounds was assessed by the oxidation of an artificial mixture of phenolic compounds (*trans*-resveratrol, rutin, 3-chlorogenic acid, quercetin, gallic acid, caffeic acid and astringin) and crude plant extract by a DPPH radical or *via* the Fenton reaction. Additionally, comparative analyses of the standard mixture of compounds at different concentrations of quercetin (1 and 2 mM) by using the DPPH radical were performed.
The mixture of polyphenols (50 µM) and the plant extract were diluted tenfold by the respective DPPH methanol solution between the ranges of 0.1 to 2.0 mM. Before introducing a sample into the CE capillary or LC column, the aliquots of the reaction mixture were incubated with a stable radical until the steady state was reached.
The Fenton reaction was performed by using hydrogen peroxide in the range of 0.1-1.0 M and the ferrous (II) sulphate (1 mM) dissolved in water. An appropriate aliquot of the hydrogen peroxide solution (35 µl) was added to 40 µl of the radical scavenger (the mixture of phenolic compounds or plant extract, respectively). The reaction was started by the addition of 5 µl of the Fe^{2+} solution to the reaction media. The pH of the reaction mixture was around 6. The sample mixture was introduced into the CE capillary and the oxidation reaction was stopped in the CE capillary by applying high voltage.
All the oxidation experiments were conducted at 25° C and all the analyses were performed in triplicate.
The radical scavenging ability of each polyphenols was expressed by the value of a "half maximal effective concentration" – EC_{50}. In this study, a decrease in peak areas of the respective compound by the oxidation at different concentrations of the DPPH radical was established. The corresponding peak areas, or, in the case of LC, heights, were plotted against the concentrations of the DPPH radical. The value of EC_{50} was found by a linear regression equation.

3. RESULTS AND DISCUSSION

3.1. Determination of the content of bioactive phenolics in plant extracts (Papers I-III)

In this work, the phenolic content of one plant family, *Solanaceae* was determined. The plant comprises a number of important vegetables, such as *Solanum lycopersicum* (tomato), *Solanum melongena* (eggplant), *Solanum tuberosum* (potato) and *Capsicum annuum* (pepper), just to name a few of them. Due to their high content of several healthy constituents, the plants were well suited for research[37,90-93]. Throughout the study, an ultrasonic liquid extraction method with ultrasonic based on the study in [I] was used. Besides being simple, there is no need any other modification of extracts. After filtration the extract could be directly introduced into CE or LC. The phenolic compounds were separated and identified by both CZE and LC-MS was performed.

3.1.1. Profiling of phenolic compounds

Phenolic compounds present in crude plant extracts of the family *Solanaceae* were separated and the fingerprints of their content were determined by CZE.
As the pKa value of polyphenols varies in the range of 8-12[1,6], alkaline buffers are usually used as a background electrolyte in CE. It has been proposed by the studies[I,94,95] that the most efficient buffer for the separation of phenolic compounds is sodium tetraborate with pH 8-11 and concentration of 25-200 mM. The effectiveness of the separation of polyphenols may be explained by the formation of a complex between sodium tetraborate and *o*-dihydroxyl groups in the flavonoid nucleus and vicinal *cis*-hydroxyl groups of sugars[31,43,48,56].
The precision of the method was evaluated by reproducibility of the migration time and peak areas of each compound, which was determined run-to-run and day-to-day for each phenolic compound (0.01 mM of each analyte). The relative standard deviations (RSD) of the migration times and peak areas are summarized in Table 1. LOD were in the range of 0.1 – 0.4 μg/mL.

Table 1. Reproducibility of the method by CZE

Analyte	RSD (%)			
	Peak area		Migration time	
	Run-to-run	Day-to-day	Run-to-run	Day-to-day
trans-Resveratrol	3.89	3.94	2.28	2.35
Astringin	4.45	2.94	1.51	3.14
Naringenin	2.12	1.74	2.32	2.72
Rutin	1.97	2.41	0.69	2.53
Chlorogenic acid	3.01	0.95	0.61	6.22
Quercetin	1.03	4.13	0.28	1.89
Gallic acid	4.17	1.05	1.89	4.87
Caffeic acid	5.62	3.40	1.53	0.56

Figure 3 depicts the comparison of the electropherograms of crude plant extracts and the compounds identified by CZE. Table 2 presents overview the compounds identified in the extracts investigated. The results are also presented in [I-III, V].

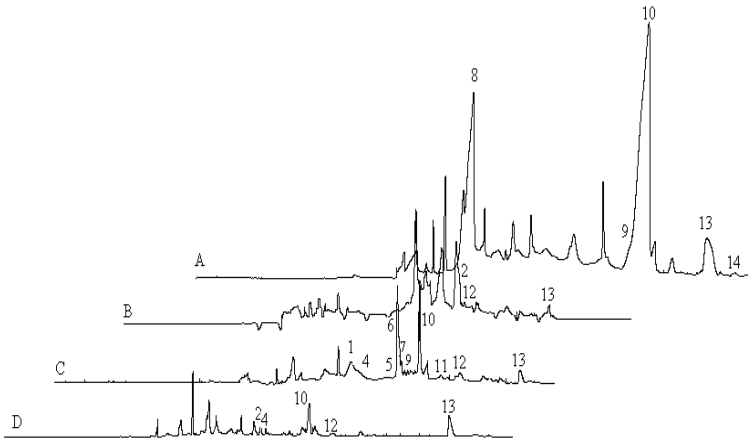


Figure 3. The electropherograms of the profiles of phenolic compounds in plant extracts: **A** – the extract of *S. melongena*; **B** – the extracts of *C. annuum*; **C** – the extract of *S. lycopersicum*; **D** – the extract of *S. tuberosum*. ***Peaks***: 1 – genistein, 2 – catechin, 3 – luteolin, 4 – rutin, 5 – naringenin, 6 – naringenin chalcone, 7 – ascorbic acid, 8 – dihydroxycinnamoyl amide and cinnamic acid derivatives, 9 – cinnamic acid, 10 – chlorogenic acid, 11 – myricetin, 12 – quercetin, 13 – caffeic acid, 14 – ferulic acid.
CZE separation conditions: background electrolyte 25 mM of sodium tetraborate (pH 9.3), the effective length of the capillary 50 cm, applied voltage +25 kV, UV detection at 214 nm, the injections were performed hydrodynamically over 15 s.

Table 2. The compounds identified in various plant extracts by CE

[1]Peak / [2]Extract	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A								+	+	+			+	+
B		+										+	+	
C	+			+	+	+	+		+	+	+	+	+	
D		+		+						+		+	+	

[1] **Peaks:** 1 – genistein, 2 – catechin, 3 - luteolin, 4 - rutin, 5 - naringenin, 6 – naringenin chalcone, 7 – L-ascorbic acid, 8 – dihydroxycinnamoyl amide and cinnamic acid derivatives, 9 – cinnamic acid, 10 – chlorogenic acid, 11 – myricetin, 12 – quercetin, 13 – caffeic acid, 14 – ferulic acid.
[2] **The extracts**: **A** – the extract of *S. melongena*; **B** – the extracts of *C. annuum*; **C** – the extract of *S. lycopersicum*; **D** – the extract of *S. tuberosum*.

All the phenolic compounds were identified by a spiking procedure, which included the addition of the respective commercial standard resulting in an increase of the respective analyte peak. Additionally, the phenolic compounds found in the plant extracts were identified by comparing their UV spectra with those of standard compounds obtained in a wavelength range between 190-600 nm by DAD. The compounds corresponding to peak 6 (Figure 3 C) and peaks 8 and 10 (Figure 3 A) were isolated as fractions and identified by HPLC-MS [III, IV].

As seen from Figure 3, mainly flavonoids were identified in the extract of *S. lycopersicum*, while *S. melongena* was rich in phenolic acids. The same tendency is supported by the value obtained by the measurement of total phenolic content [II, III]. The extract of *S. melongena* possessed the highest value of total phenolics, 900±0.09 mg of tannic acid equivalent in 100 g of dry weight, followed *S. lycopersicum* with the value of 360±0.02 mg of tannic acid equivalent to 100 g of dry weight. Also, the highest proportion of total flavonoid contents of *S. melongena* and *S. lycopersicum*, were the highest, being approximately 73% and 67%, respectively.

A few phenolic compounds were determined in the extract of *C. annuum* and *S. tuberosum* under the conditions applied. Hence, these plant extracts were not subjected for further investigation.

Since flavonoids are mainly present in plant extracts as glycosides, references compounds for their identification are rarely available. This also complicates the determination of all phenolic compounds in the extracts by CZE. Thus, to obtain detailed information especially about the glycosides present in plant extracts, their parallel identification was performed by rp-HPLC-MS/MS, which data are reported in [III, IV]. As separation by rp-LC is based on the interaction of an analyte with the non-polar stationary phase, the eluation order of the compounds under study is reversed order compared to that in CE. So, in the former, more polar phenolic acids eluate first, followed by flavonoids. The LOD was in the range of 1 – 100 μg/mL.

However, it must be considered that the overlapping of individual phenolics is inevitable because of the diversity of polyphenols. Therefore, to identifying a compound in a plant extract, fraction collection of the extract component migrating out of the CE capillary may be applied and further analysis of the collected fractions by various spectral instruments performed.

3.2. Fraction collection in capillary electrophoresis (Paper IV)

As indicated above, the complex composition of crude plant extracts results in unresolved peaks. To overcome this problem, the fraction collection method was utilised in CE for the separation and analysis of small molecules, like polyphenols. In the analysis, the plant extract of *Sophora japonica* (the Japanese pagoda tree) was served as a model due to its high content of several phenolic compounds[96-100]. Its extract is of interest in traditional medicine as well[96-100].
The off-line mode of CE-MS helps to overcome the problem with background electrolyte in an on-line coupling of CE to MS. In the current study, sodium tetraborate as a background electrolyte in CE and acetic acid as a sheath liquid configuration were applied (*paragraph 2.1.2.*). To ascertain that acetic acid does not interfere with the separation of analytes in the capillary, the speed of migration of acetic acid ions must be smaller than EOF on the anionic side. A comparison of the experimental mobilities (μ) of acetic acid ions ($\mu = -2.6 \times 10^{-8}$ m^2 s^{-1} V^{-1}) and the EOF ($\mu = 6.4 \times 10^{-8}$ m^2 s^{-1} V^{-1}) showed that sheath liquid ions probably do not migrate into the CE capillary and thus not interfere with the separation of analytes.
The fraction time was roughly estimated based on the migration time of the analyte peak observed at the UV detector. Assuming that the mobility of the analyte stayed constant during electrophoresis, the time during which the analyte band reaches the capillary outlet (t_o) was computed from the migration time corresponding to the top of the analyte peak as its zone passed through the detector window (t_d). The computation was performed as follows: $t_o = (l_o / l_d)t_d$, where l_o is the full length of the capillary and l_d, the effective length of the capillary to the detector.

3.2.1. Design of the fraction collection system

The fractions collected by CE were further analysed by rp-micro-HPLC-ESI-MS. First, the validation of the fraction collection system was performed using a mixture of the standard solution consisting of flavone, naringin, rutin and quercitrin. The electropherograms of these four polyphenol standards are shown in Figure 4. The stripes on the electropherogram (Figure 4 A) stand for an electrical impulse of each exiting droplet in the anionic side of the capillary.
The precision of the method is characterized by the analytical parameters given in Table 3.

Table 3. The analytical parameters for the off-line mode of CE-MS

Analyte	Q-TOF		
	RSD[1] %	LOD[2] μM	LOQ[3] μM
Flavone	2.1	100	300
Naringin	3.9	35	105
Rutin	4.7	200	600
Quercitrin	3.9	250	650

[1] Relative standard deviation
[2] LOD for Q-TOF
[3] Limit of quantification in the extract introduced to CE (estimated)

The estimated of LOD of the initial sample [IV] introduced into the capillary for fraction collection was found to be 50 μM by CE-rp-HPLC-ESI-MS. The reproducibility of 4% was achieved.

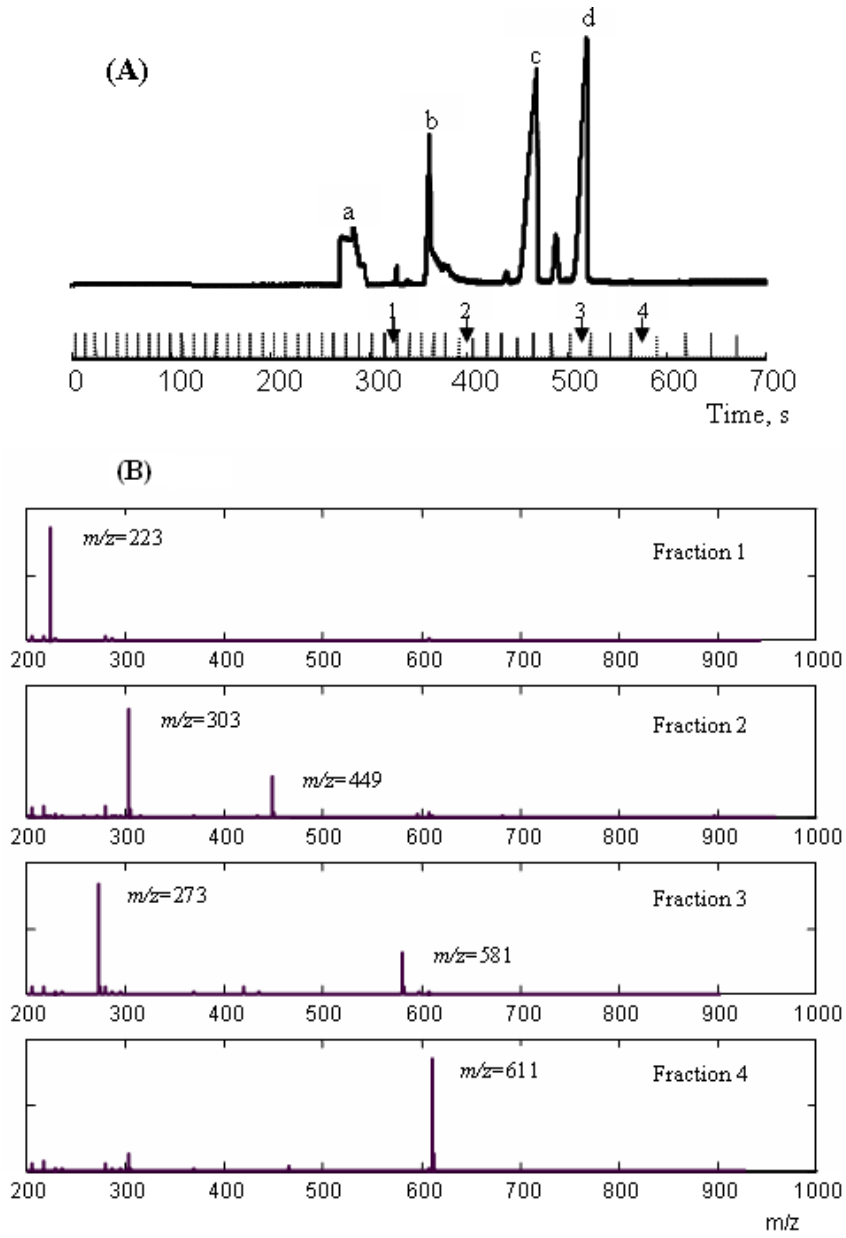


Figure 4. The analysis of a standard mixture of flavonoids by CE-ESI-MS. (**A**) The electropherogram of the solution of standards (each of 1 mM). ***Peaks***: ***a*** – flavone; ***b*** – naringin; ***c*** – rutin; ***d*** – quercitrin. (**B**) Mass spectra of the corresponding fractions.
CZE separation conditions: background electrolyte 25 mM sodium tetraborate (pH 9.3), sheath liquid 1 M acetic acid; the effective length of the capillary 57 cm, applied voltage +15 kV, UV detection at 210 nm, the injections were performed hydrodynamically over 15 s.

The shape of the peak of flavone (Figure 4 A) is explained by co-migration with the EOF. Below the electropherogram (Figure 4 A) a “comb-like” signal from the droplet counter is seen. The time interval between two peaks of this signal corresponds to the time during which a droplet was formed. As the sheath liquid was delivered by gravity, which caused the decrease of the level of the sheath liquid in the vessel, the slight increase in time interval between two consecutive droplets was observed. The arrows marked with numbers in the Figure 4 A indicate fractions for which an HPLC-MS signal was detected. An apparent shift of the fractions compared to the CE detection signal is due to the detector position at 13 cm from the cathode end of the capillary.

To verify the presence of the standard in the respective fraction, the corresponding mass spectrum (Figure 4 B) of fraction 1 revealed that it contained a peak of the molecular ion of flavone (($M_{flavone}$+1)/z = 223) and that of fraction 4 had a molecular ion peak of rutin ((M_{rutin}+1)/z = 611). The mass spectrum of fraction 2 possessed two peaks. A peak at ($M_{naringin}$+1)/z = 581 corresponded to a molecular ion of naringin and a peak at m/z = 273 is apparently due to its partial fragmentation at the ionization source because of the breaking of a weak glycoside bond. The same may be observed in the case of the mass spectrum of fragment 3. A peak at ($M_{quercitrin}$+1)/z = 449 belongs to molecular ion of quercitrin and at m/z = 303 there is a peak of another fragment of the compound.

Subsequently the fraction collection system was applied to a model object - the extract of *S. japonica*, whose electropherogram (Figure 5 A) has a few well separated peaks and intense peaks.

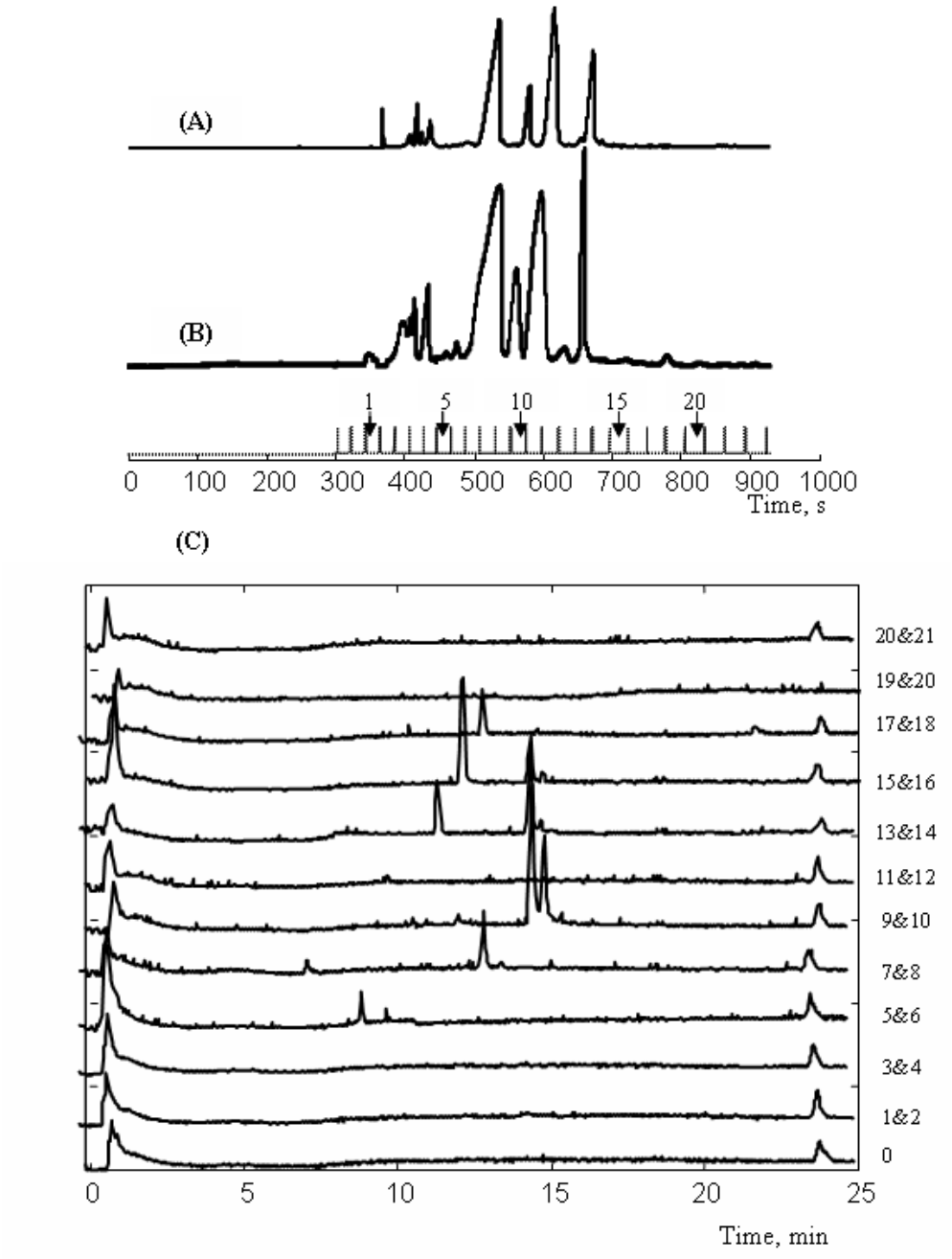


Figure 5. The analysis of the extract of *S. japonica*. **(A)** – the electropherogram of the extract of *S. japonica*; **(B)** – the electropherogram of the concentrated extract; **(C)** – the corresponding total ion chromatograms of the fractions. The CZE separation conditions are the same as in Figure 4.

The numbers on the *y*-scale (Figure 5 C) designate the number of droplets merged into one fraction. By this the number of the fractions to be analyzed by HPLC-MS was reduced. In all chromatograms, a peak at the retention time of 24 min (Figure 5 B) appeared which is probably due to the fraction collection system used. The majority of fractions, however, had just one peak, except a fraction from the droplets 11 and 12. The traces of the two compounds can also be found in the next fractions. Besides identification of the main components in the extract reported earlier also in[96,100] compounds, such as metoxykaempferol glucoside, kaempferol rutinoside glucoside, cyanidin rutinoside, methylquercetin rutinoside and quercetin diglucoside rhamnoside were determined in the extract of *S. japonica* for the first time. Appendix I list the compounds identified.

Although the fractions could have been introduced into the MS directly, the purpose was to use HPLC as a second dimension if CE alone was not able to separate analytes. Moreover, the HPLC column acted as a concentrator medium for the analytes diluted in the fraction. Thus, in fact, using the fraction collection system a two-dimensional separation took place. Another advantage of the system is that it can be implemented for various stand-alone instruments. So, as reported in **IV**, preliminary studies of the applications of inductively coupled plasma mass spectrometry (ICP-MS) and MALDI were carried out.

3.3. Evaluating of radical scavenging capability (Papers I, III,V)

In order to assess the most potent antioxidants, which was the purpose of the third part of the study, a further insight was taken into the analysis of antioxidative capability. An on-line method for the evaluating of antioxidative capability of phenolic compounds with different structural and substitution patterns was developed by CZE **I,III,V**. Commonly, antioxidative capability is mainly determined by batch-type reactions measured spectro(photo/fluoro)metrically. The colorimetric assay by DPPH is a simple, yet highly sensitive method and is not able to determine the antioxidative capability of individual phenolics in the sample, but estimates only overall antioxidative capability. As a result, the role of individual phenolic compounds as antioxidants is neglected. In the following approach a simultaneous monitoring of the oxidation reaction of several phenolic compounds over time was carried out. This allowed the estimation of their individual contribution to antioxidative capability was allowed.

Antioxidative capability was investigated by using both a stable free radical DPPH and a highly reactive hydroxyl radical. At first the oxidation reaction was conducted with a model mixture of standard polyphenols. Then the same reaction was carried out with the plant sample by using the respective radical. To compare the results obtained by CZE, the method was adjusted to LC-MS. The data obtained are presented in **I,III,V**.

3.3.1. DPPH radical scavenging

In the colorimetric assay using DPPH to determine antioxidative capability, phenolic compounds (PhOH) act as a hydrogen donor and the proposed reaction by which DPPH accepts hydrogen from the antioxidant is as follows[7,13,101,102]:

$$DPPH\bullet + PhOH \rightarrow DPPH - H + PheO\bullet$$

The advantages of using a stable free radical DPPH are its commercial availability, there is no need to generate it by another oxidant and the exact quantity of the radical required for study is easy to monitor. DPPH has a strong absorption band at the wavelength of 517 nm in methanol, which does not overlap with the spectra of flavonoids and phenolic acids. The absorbance of DPPH decreases as the colour changes from purple to yellow due to the scavenging of the free radical by antioxidants through the donation of hydrogen to form a stable DPPH–H molecule.
The model mixture of standard polyphenols consisted of *trans*-resveratrol, astringin, rutin, 3-chlorogenic acid, quercetin and gallic acid. The model solution (each compound of 50 μM) was mixed with a stable free radical DPPH at different concentrations in the range of 0.1 up to 2.0 mM, although at the DPPH concentration of the 2 mM majority of the phenolic compounds under investigation, except chlorogenic acid and resveratrol, were oxidized to a high extent. The radical scavenging capability of polyphenols determined by using DPPH at different concentrations is depicted in Figure 6. During the radical scavenging reaction new components were identified by comparing the UV spectra of the standard mixture of polyphenols with those of the oxidized mixture obtained in the wavelength range between 190-600 nm registered by DAD.

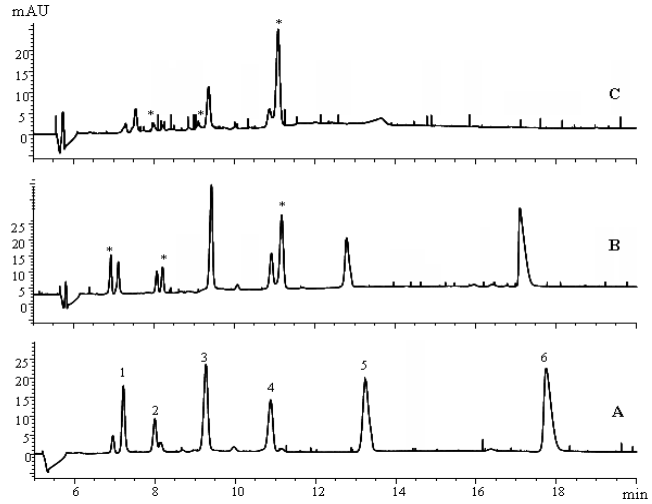


Figure 6. The electropherograms of the monitoring of the oxidation reaction by a stable free radical DPPH. **A** – the model solution of phenolic compounds (50 μM); **B** – the model solution of the phenolic compound treated with a 0.2 mM DPPH; **C** – the model solution of the phenolic compound treated with a 0.8 mM DPPH. ***Peaks***: 1 – *trans*-resveratrol; 2 – astringin; 3 – rutin; 4 – 3-chlorogenic acid; 5 – quercetin; 6 – gallic acid; * – a product of the oxidation reaction.
CZE separation conditions: background electrolyte 25 mM sodium tetraborate (pH 9.3), an effective length of the capillary 50 cm, voltage applied +20 kV, UV detection at 210 nm, the injections were performed hydrodynamically over 10 s.

As seen in Figure 6, quercetin and gallic acid were oxidized to the highest extent and thus only traces of these compounds were observed after the reaction conducted with the DPPH solution concentration of 0.8 mM. Rutin as quercetin glycoside was oxidized to the lowest extent. Besides, several by-products of the oxidation reaction were formed.

The oxidized part of each polyphenol (%) was calculated using the original peak areas as reference. The larger the oxidized part of a polyphenols, the better radical scavenger the compound is. The oxidized parts (calculated at a concentration of DPPH of 0.6 mM) of quercetin and gallic acid were the largest, constituting 98.5±0.7% and 93.1±1.2%, respectively. In the case of chlorogenic acid and astringin, the respective values were 63.2±1.2% and 67.6±4.2%, respectively. The oxidized parts of resveratrol and rutin were the smallest, 49.2±3.5% and 38.4±5.4%, respectively.

The same oxidation reaction was applied to selected plant extracts [III,V], which results are shown in Figure 7. The oxidation of the plant extract of *S. lycopersicum* was conducted using a 0.5 mM DPPH solution. On the electropherogram (Figure 7) the main decreasing peaks are shown. The UV-spectra of the peaks of the extract were analysed and compared with the initial ones. Changes in the peak areas of rutin, naringenin chalcone, chlorogenic and caffeic acids were mainly observed. By the unoxidized part (%), peaks 3 (57.6±3.7%) and 5 (28.2±3.2%) disappeared

immediately, while the largest unoxidized part belonged to peak 1 (78.6±5.5%) and 2 (81.8±10.6%). However, the unknown compounds labelled as X (22.0±2.0%), Y (41.7±4.9%) and Z (34.3±3.7%) shown in Figure 7 were oxidized most. The unknown compounds are probably phenolic acids, judging by the comparison of their UV spectra with those of other phenolic compounds.

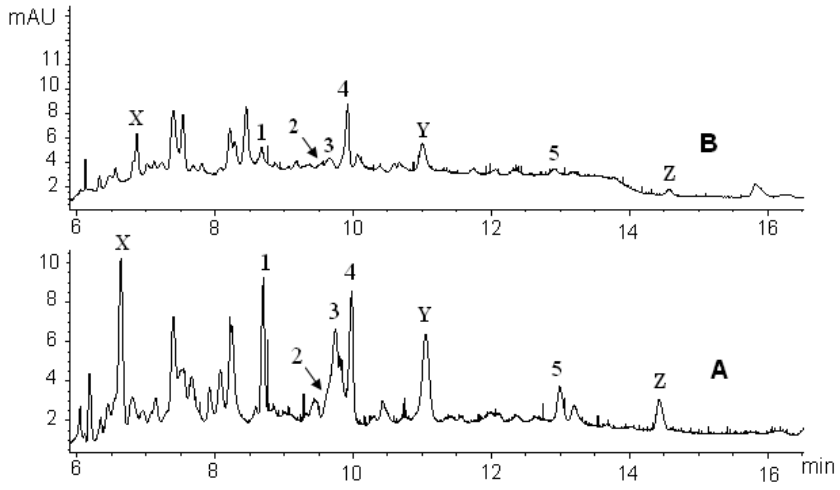


Figure 7. The electropherograms of monitoring of the antioxidative capability of the extract of *S. lycopersicum* using DPPH by CE: **A** – 10 µl of the extract mixed with 90 µl of a 80:20 methanol/water. ***Peaks***: 1– rutin, 2 – naringenin, 3 – naringenin chalcone, 4 – chlorogenic acid, 5 – caffeic acid; **B** – the reaction after 5 min. The CZE separation conditions are the same as in Figure 6.

As expected, due to the complex composition of plant extracts and unavailability of reference compounds the identification of polyphenols by CZE was incomplete, and, thus, the compounds were further identified by LC-MS. The list of identified compounds and the value of unoxidized part is given in **III**.

3.3.2. Oxidation reaction by a hydroxyl radical

For the oxidation of polyphenols, a hydroxyl radical (HO•) was used as it exhibits the strongest oxidative activity and can non-specifically oxidize all groups of biological macromolecules including lipids, proteins, and nucleic acids. If HO• is generated in excess, the cellular antioxidant defence is impaired.
As described in the previous paragraph, a similar monitoring of the oxidation reaction of phenolic compounds by a HO• generated via the Fenton reaction was carried out. The main source of hydroxyl radicals *in vitro* is the metal-catalyzed decomposition of hydrogen peroxide known as the Fenton reaction, which may be described by the following simple equation[7,11,103]:

$$M^{n+} + H_2O_2 \rightarrow M^{n+1} + {}^{\bullet}OH + OH^-$$

In the present study, Fe^{2+} was used in the Fenton reaction system as a transition metal. The hydroxyl radical was produced by mixing the phenolic solution (0.5 mM) with 0.1 M hydrogen peroxide and finally 1 mM of the ferrous (II) sulfate solution was added to the reaction mixture.

As described in the previous paragraph (*3.3.1.*), the oxidation reaction was let run to completion, but in the case of the HO• radical, the oxidation reaction was monitored over time to demonstrate possibilities of CE for such measurements. After the injection of the reaction mixture into the CE capillary and applying high voltage, the components in the mixture were rapidly separated from one another and the reaction was stopped. The time during which the zones of the reactants and products stay overlapped in the capillary depends on the difference in their migration times. The time necessary for the complete separation of hydrogen peroxide from the reactants can be estimated by the formula given in[95]. From the above equation it can be concluded that the reaction in the capillary was stopped 5-8 s after the injection.

In Figure 8 the electropherograms of the monitoring of the oxidation reaction over time *in vitro* by the HO• radical are depicted. Considering that the HO• is very unstable and the most reactive oxygen species and is highly unstable, the electropherogram B (Figure 8) was registered immediately after preparing the oxidation mixture (*paragraph 2.5.*).

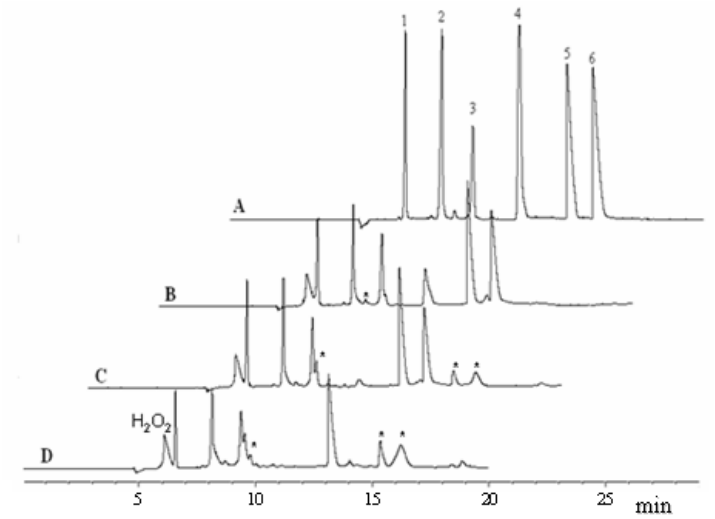


Figure 8. The electropherograms of the monitoring of oxidation reaction over time by the hydroxyl radical generated via the Fenton reaction. **A –** an artificial solution of phenolic compounds (each of 0.25 mM); **B** – the oxidation reaction registered immediately after the initiation of the oxidation; **C** – the oxidation reaction after 45 min; **D** – oxidation reaction after 90 min. ***Peaks*:** 1 – *trans*-resveratrol, 2 – rutin, 3 – chlorogenic acid, 4 – quercetin, 5 – caffeic acid, 6 – gallic acid, * – by-products of the oxidation reaction. The CZE separation conditions are the same as in Figure 6.

The disappearance of phenolic compounds and the simultaneous appearance of by-products during the oxidation are clearly seen in Figure 8. The rate of the oxidation of quercetin was the highest, while caffeic acid as the weakest antioxidant among the phenolic compounds in the mixture was oxidized last, although the amount of the radical during the reaction was, contrary to its initial amount, decreased oxidized by more active phenolic compounds. Methanol was used as a solvent to prepare the standard solution. The interaction between methanol and hydrogen peroxide has to be considered[104], because hydroxyl radical may react with methanol in some extent.
A more detailed description of the oxidation reaction by HO• to crude plant extracts are reported in [I,V].

3.3.3. Monitoring of the free radical scavenging of phenolic compounds by LC-DAD-MS

To improve the oxidation method for the determination of antioxidative capability, the approach used in CE was adjusted to LC-MS. The latter enabled to monitor the oxidation of the solution of standard phenolics compounds at different concentrations (0.1 - 2.0 mM) of the DPPH solution. The UV-chromatograms registered at the wavelength of 280 nm obtained in the oxidation reaction at different concentrations of DPPH are depicted in Figure 9.

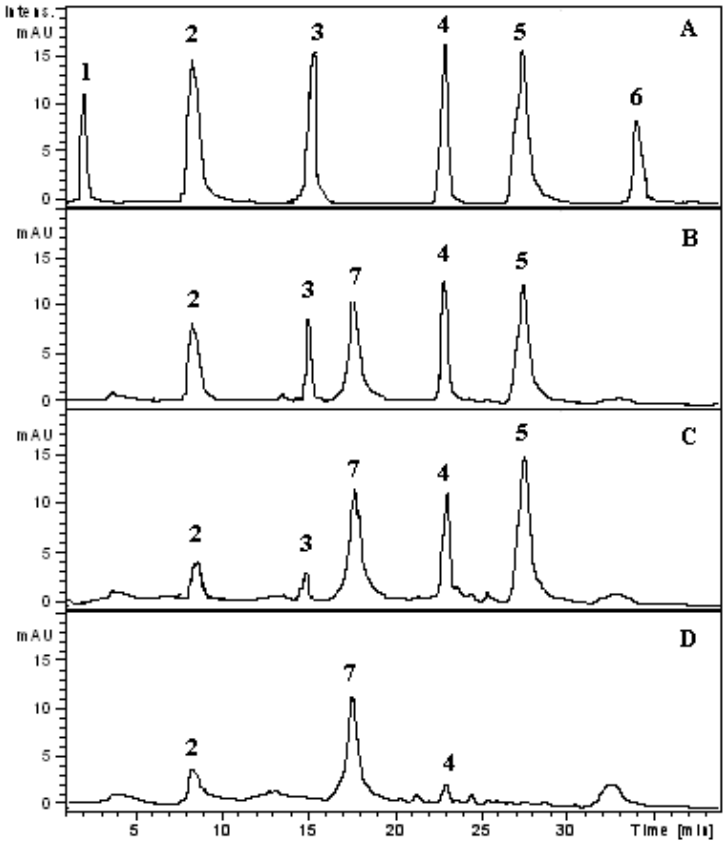


Figure 9. The UV-chromatograms of the standard mixture of polyphenols and the oxidation of the mixture of phenolic compounds by the DPPH radical with the concentration of 0 mM (**A**), 0.5 mM (**B**), 1.0 mM (**C**) and 2.0 mM (**D**). ***Peaks:*** 1 – gallic acid (t_r=2.0 min), 2 – chlorogenic acid (t_r=8.3 min), 3 – astringin (t_r=14.6 min), 4 – rutin (t_r=22.7 min), 5 – *trans*-resveratrol (t_r=27.2 min), 6 – quercetin (t_r=34.0 min), 7 – a mixture of the products of quercetin oxidation and methoxylation products.

When comparing the results obtained by CZE with those obtained by LC-MS it became clear that the number of oxidation compounds identified by the latter was higher. But comparing the electropherograms in Figure 6 with HPLC chromatograms in Figure 9 it is clear that the CE separation time in CE is twice shorter. Another evident is complete reversal of the eluation order of the compounds compared LC to CE result. Rp-HPLC is indeed "reversed phase" to CE here. However, the oxidation reaction showed the same trend as observed by CZE (Figure 6).

The ability to scavenge free radical was expressed as a "half maximal effective concentration" – EC_{50}, which is commonly defined as the amount of an antioxidant necessary for decreasing the initial DPPH radical concentration by 50%[101,102,105]. Thus, the lower the value of EC_{50}, the higher the antioxidant power of a compound is. In the present study, an opposite approach was taken. Hence, EC_{50} is defined as the concentration of the radical necessary to oxidize 50% of a compound in competition

with all the other oxidizable compounds in the mixture. It follows that a compound is a better antioxidant if its value of the EC_{50} is lower. The EC_{50} values calculated by the decrease of the height of the UV peak at an optimal wavelength on the chromatogram of a particular compound are given in Table 4. The EC_{50} values (*paragraph 2.5.*) calculated by the decrease of the corresponding peak area of the compound oxidized at different concentrations of the DPPH radical obtained by CZE (*paragraph 3.3.1.*) are compared.

Table 4. The values of EC_{50} of the phenolic compounds determined by LC-MS, LC-UV/Vis and CZE

Compound	EC_{50} (mM DPPH)		
	LC-MS	LC-UV/Vis (280 nm)	CE-DAD
Gallic acid	0.30 ± 0.09	0.30 ± 0.08	0.25 ± 0.07
Astringin	-	-	0.30 ± 0.05
Caffeic acid	10.0 ± 2.00	-	10.0 ± 0.30
Chlorogenic acid	0.25 ± 0.07	0.25 ± 0.07	0.20 ± 0.19
Rutin	1.50 ± 0.40	1.50 ± 0.50	1.00 ± 0.30
trans-Resveratrol	0.50 ± 0.15	0.60 ± 0.18	0.20 ± 0.08
Quercetin	0.25 ± 0.07	0.30 ± 0.09	0.25 ± 0.09

The relative antioxidative capability was calculated as a ratio of EC_{50} of gallic acid (GA) to that of the other antioxidants present in the mixture. Gallic acid was used as a standard antioxidant in the corresponding system, but in order to estimate the dependence of EC_{50} on the concentration of each phenolic compound in the mixture, the concentration of quercetin, was increased. Table 5 presents the ratios of $EC_{50\ GA}/EC_{50}$ obtained by LC. These were rather constant at different concentrations of quercetin in the reaction system. The ratio is given to compare the EC_{50} values obtained under different experimental conditions or using various extracts.

Table 5. The ratio of $EC_{50\,GA}/EC_{50}$ of phenolic compounds at different concentrations of quercetin

Compound	Initial concentration of quercetin (mM)			Mean value
	0.5	1.0	2.0	
Quercetin	1.50	1.32	1.50	1.44 ± 0.07
Astringin	1.14	1.32	1.00	1.10 ± 0.11
Gallic acid (GA)	1.00	1.00	1.00	1.00
Chlorogenic acid	0.41	0.37	0.50	0.42 ± 0.08
Rutin	0.33	0.32	0.47	0.37 ± 0.09
trans-Resveratrol	0.22	0.27	<0.45	0.25 ± 0.03*

* - The results obtained at two lower concentrations of quercetin

As follows from Table 4 the results obtained by CE and HPLC are in excellent agreement. Therefore the method was used for the estimation of the antioxidative of a more complex sample, *S. melongena,* by LC-MS [V]. The data are presented in Figure 10.

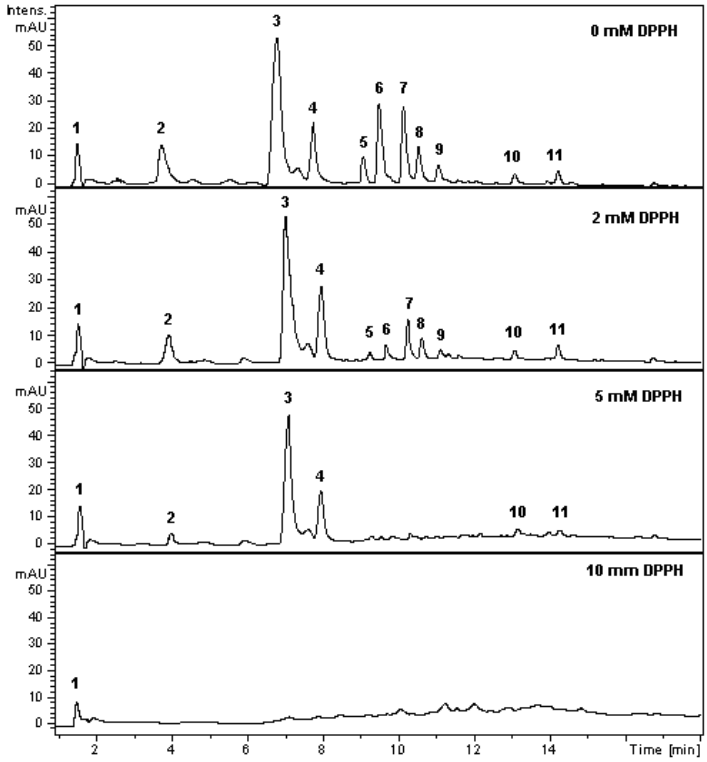


Figure 10. The chromatograms of the *S. melongena* extract registered at λ= 280 nm at different concentrations of DPPH. ***Peaks***: 1 – quinic acid derivative, 2 – unknown compound, 3 – isomer 1 of chlorogenic acid, 4 – isomer 2 of chlorogenic acid, 5 – isomer 3 of chlorogenic acid, 6 – cinnamic acid derivative, 7 – isomer 1 of dihydroxycinnamoyl amide,
8 – isomer 2 of dihydroxycinnamoyl amide, 9 – *N,N*-dicaffeoylspermidine, 10 – kaempferol rutinoside, 11 – kaempferol glucoside.

Among the phenolic compounds identified in the extract of *S. melongena*, cinnamic acid derivatives and isomers of dihydroxycinnamoyl exhibited the highest antioxidative capability with the values of EC_{50} of up to 2 mM. The respective value for the other compounds identified ranged from 3 mM to 10 mM. So, it may be concluded that among the compounds identified, cinnamic acid derivatives and isomers of chlorogenic acids are more responsible for the antioxidative capability. Appendix II lists the compounds identified and their values of EC_{50} determined by LC-MS.

3.4. Structure-radical scavenging capability relationship

As is known, the antioxidative capability depends on the structure of the respective phenolic compound and therefore, it is mainly determined by the accessibility of the radical centre of DPPH to each polyphenols. There exists a correlation between

structural pattern and antioxidative capability. In general, the structural criteria for the efficiency of a maximum radical quenching are rendered by the easiness with which an H atom from the aromatic OH group of the phenol is donated to a free radical. It has been noted that small molecules that have better access to the radical site have a relatively higher antioxidant capability[106].

First, the *ortho*-dihydroxy configuration conveys a high stability to the radical formed[21]. However, this is hindered in case of binding, for example, a glycosidic group to one of vicinal OH groups. The OH group at the position C_3 and C=O configuration in the C-ring, like in the molecule of quercetin, has the same ability, but somewhat weaker.

Secondly, the conjugated π-electron structure delocalizes the unpaired electron[17,107]. The stability of the flavonoid radical is, thus, designated by the conjugation between the A- and the B-rings, which allows the resonance effect of the aromatic nucleus[17]. For instance, the conjugation of the B-ring to the 4-oxo group via the C_2-C_3 double bond designates the electron delocalization from the B-ring. But the OH group at the positions C_5 and C_3 with the 4-oxo group between allows the electron delocalization from the 4-oxo group to both substituents. It follows that the longer the conjugated system, the better antioxidant the compound is. In this conjugation, appropriate chains outside the aromatic cycle, such as in caffeic or *p*-coumaric acids can also be involved.

These statements were confirmed by the results obtained in the present study, for arranging polyphenols by their ability to scavenge free radical **[III,V]** described by the unoxidized part (%) (*paragraph 3.3.1.*) and the value of EC_{50} (*paragraph 3.3.3.*). On the basis of the data obtained by the oxidation of DPPH (*paragraph 3.3.1.*) and the hydroxyl radical (*paragraph 3.3.2.*) using a standard mixture of polyphenols, the compounds could be arranged by antioxidative power as follows: quercetin, gallic acid, resveratrol, astringin, rutin, chlorogenic acid and caffeic acid. The most potent antioxidant, quercetin, had a high ability to scavenge free radical. This is an agreement with the fact that it owns the *ortho*-dihydroxy configuration and preferable conjugation for electron delocalization in the molecule. The radical scavenging ability of gallic acid, which has more hydroxyl groups in its structure, scavenged radicals to a greater extent than chlorogenic and caffeic acids. Resveratrol possessed a lower antioxidative capability than quercetin, because it lacks a ring with an oxo-group and also dihydroxylation in the B-ring. The antioxidant capability of rutin and astringin was weaker than that of their aglycones, quercetin and resveratrol. This may be explained by fact that the glycosylated form of a compound diminishes antioxidative capability.

The EC_{50} values obtained by analytical methods independently are well comparable with the results given in Table 4. By the same approach, similarly, the potent antioxidants found in crude plant extracts were determined **[III,V]**.

CONCLUSIONS

The principal aim of the present work was to elaborate a suitable and rapid CE method for the separation and identification in profiling the compounds in crude plant extracts. To determine the overlapping of the compounds the fractionation of the respective peak is desirable. The antioxidative capability of phenolic compounds was valued. To estimate the antioxidative capability of individual polyphenols, CE was used. The assay was easily adapted to LC.

The results of the study can be summarized as follows:

● CE enables a rapid and precise analysis of crude plant extracts. When applicable, the results were confirmed by a more common HPLC-MS. Both the methods complement each other as demonstrated in the present work. The ability of MS to determine both free and conjugated forms of phenolic compounds was shown. Although it is believed that HPLC-MS is a universally preferable method for the analysis of plant extracts, it appeared that several compounds could not be determined by HPLC-MS because of peak overlapping. But CE alone was able to determine the compounds as demonstrated in the case of phenolic acids.

● The fraction collection implemented in CE was a convenient method to identify compounds on the electropherograms. Several drawbacks, such as interfacing, which occurs due to the on-line coupling of CE to MS, were eliminated. Due to the flexibility of the fraction collection system, the experimental conditions for CE and MS instruments can be optimized separately. The effectiveness of the fraction collection was demonstrated by the analysis of a crude extract sample.

● CE proved to be a potent method for the determination of antioxidative capability. The method for the evaluation of antioxidative capability, using any free radical, such as DPPH and a hydroxyl radical in a chemical system was fast and efficient. It enabled monitoring the radical scavenging capability of both standard mixture of phenolic compounds and crude plant extracts. The assay enabled the evaluation of the ability of any single phenolic compound to act as antioxidant in the mixture separately.

● The radical scavenging capability of each compound was assessed by EC_{50}, which indicates the molar concentration of the radical causing a 50% of reduction of the area or height of the peak of an antioxidant. The concentration of the radical may be chosen to cover the interval of EC_{50} values of individual mixture components. The results obtained by CE were validated against those obtained by HPLC with good precision. However, CE is more advantageous than HPLC, enabling measurement of

oxidation kinetics, since the speed of the former allows a more precise determination of the rate of with the radical.

● The protocols developed for the determination of antioxidative capability enabled the correlation to be established between the structural pattern and antioxidative capability of a compound under study. The results obtained demonstrated that the ability of polyphenolics to scavenge free radicals greatly depends on chemical structure and relative orientations of various moieties, particularly the substitution pattern of free hydroxyl groups on the structure. In these terms especially important are the *ortho*-dihydroxy structure in the B-ring, the 3-hydroxyl group in the C-ring, and the 2,3-double bond in conjugation with an oxo function in the C-ring.

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APPENDIX I

The compounds determined in the extract of *S. japonica*

Fraction	Retention time, [min]	m/z data		Compound
		M+1	MS/MS	
7&8	9.00	595	595→433, 271	Genistein glucoside
	9.50	741	741→595,433,271	Genistein rutinoside glucoside
9&10	6.20	*		Unknown
	13.5	433	433→271	Genistin
	13.7	463	463→301, 286	Methoxykaempferol glucoside
11&12	9.90	757	757→595,433,287	Kaempferol diglucoside rhamnoside
13&14	10.8	*		Unknown
	14.5	865	865→433→271	Genistin dimer
	14.7	579	579→433, 271	Sophorabioside
15&16	12.3	757	757→595,449,287	Kaempferol rutinoside glucoside
	14.5	433	865→433→271	Genistin dimer
	14.7	579	579→433, 271	Sophorabioside
17&18	12.0	611	611→287	Kaempferol sophoroside
	14.2	595	595→449,287	Cyanidin rutinoside
	14.6	625	625→479, 317, 302	Methylquercetin rutinoside
19&20	10.6	773	773→611,449,303	Quercetin diglucoside rhamnoside
	13.0	611	611→449,465,303	Rutin
	22.3	271	271→243,215,153	Genistein

* Compound not identified

APPENDIX II

The values of EC_{50} of the compounds determined by LC-MS and LC-DAD

Peak (Figure 10)	t_r, min	$[M-H]^-$	Compound	EC_{50} (mM DPPH)	
				MS/MS	UV/Vis (λ)
-	7.90	611*	Delphinidine rutinoside	<1.00	<1.00 (520)
6	9.50	472	Cinnamic acid derivative	1.50	1.50 (280)
8	10.5	470	Isomer 2 of Dihydroxycinnamoyl amide	1.50	2.50 (280)
7	10.1	470	Isomer 1 of dihydroxycinnamoyl amide	2.00	2.00 (280)
9	11.1	468	*N,N*-dicaffeoylspermidine	2.20	2.00 (280)
-	14.2	515	Dicaffeoylquinic acid	3.00	-
-	12.5	609	Quercetin rutinoside (rutin)	4.00	-
-	7.20	355	Caffeic acid conjugate	4.50	-
2	4.00	249	Unknown compound	5.00	5.00 (280)
5	9.10	353	Isomer 3 of chlorogenic acid	5.00	3.00 (280)
3	7.10	353	Isomer 1 of chlorogenic acid	6.00	2.50 (280)
4	7.80	353	Isomer 2 of chlorogenic acid	7.50	6.00 (280)
10	13.3	593	Kaempferol rutinoside	7.50	7.50 (280)
-	13.0	609	Kaempferol diglucoside	10.0	-
11	14.2	447	Kaempferol glucoside	10.0	10.0 (280)
1	1.40	533	Quinic acid derivative	>10.0	10.0 (280)

* - $[M+H]^+$

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ABSTRACT

Naturally occurring polyphenols aroused interest of scientists because of their beneficial biological activities, the capability to scavenge free radicals being the most notable property of the compounds. The reactive oxygen species produced in excess in the organism attack biomolecules including proteins, nucleic acids and polyunsaturated fatty acids, which as a result, destroy cells and impair their functioning. At present, mostly chromatographic techniques are used to analyse polyphenols in plant extracts. However, due to a number of advantages, such as high efficiency, speed of analysis, possibility to separate both neutral and charged compounds in the same run, low sample/buffer consumption, also capillary electrophoresis (CE) has found application in these analyses for this.

In this study, first, a suitable method by CE for profiling phenolic compounds was developed. Due to the complex composition of crude plant extracts, additional analyses by high-performance liquid chromatography tandem diode array detection-mass spectrometry (HPLC-DAD-MS/MS) were performed. The latter enabled a further investigation of the structure of unknown compounds. But as not all commercial reference compounds are available, MS as a detector is indispensable. The coupling of CE to MS has been demonstrated to be effective and selective as well. However, the successful on-line coupling of CE to MS is restricted to that CE requires a running buffer, which is not always appropriate for MS. This may be overcome by an off-line coupling of CE to MS, which was effectively utilized in the current study. After the efficient separation of the sample by CZE by the stream of a 5% acetic acid each droplet at the cathode end of the capillary was fractionated. The fractions collected were sequentially analysed by MS.

In general, various *in vitro* methods have been proposed for the determination of total antioxidative capability, using any stable free radicals, for instance, 2,2-diphenyl-1-picrylhydrazyl (DPPH). Although antioxidative capability is simple and easy to measure by conventional methods, the main disadvantage is that the contribution of every single phenolic compound is neglected. Hence, in the current study, the possibility of applying CE and HPLC-MS/MS to determine the radical scavenging capability of individual phenolic compounds was demonstrated. The methods developed enabled a simultaneous evaluation of antioxidative capability of each compound separately in the mixture of polyphenols. The oxidation process was carried out using two radicals, viz. the 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radicals generated via the Fenton reaction. To evaluate relative antioxidative capability, “half maximal effective concentration” – EC_{50} was used. In the present study, EC_{50} (mM) is defined as the concentration of the radical necessary to oxidize 50% of a compound in competition with all the other oxidizable compounds in the mixture. It follows that a compound is a better antioxidant if its value of the EC_{50} is lower.

KOKKUVÕTE

Teaduslik huvi fenoolsete ühendite vastu on tingitud nende omadusest siduda reaktiivseid hapnikuühendeid, mis tekivad organismis ainevahetusprotsesside tulemusel. Olukorras aga, kus reaktiivseid hapnikuühendeid tekib liias, põhjustavad nad rakkude koostisesse kuuluvate lipiidide ja nukleiinhapete kahjustusi. Antud töös käsitleti eelkõige taimedes sünteesitavaid sekundaarseid metaboliite - fenoolseid ühendeid, mille põhieesmärk on kaitsta taimi erinevate stressorite eest. Taimeekstraktide antioksüdatiivsed omadused on põhjustatud neis sisalduvate polüfenoolide poolt, mille omadus käituda antioksüdandina on otseses sõltuvuses nende keemilisest struktuurist.

Käesoleva töö eesmärgiks oli välja töötada sobiv ja kiire lahutusprotokoll taimeekstraktides sisalduvate fenoolsete ühendite (flavonoidid ja fenoolsed happed) mitmekülgseks analüüsiks kapillaarelektroforeetiliselt, mis oma märkimisväärsete eelistega, nagu minimaalne proovi (nanoliitrites) ja solvendi kogus, lühikesed analüüsiajad, kõrge efektiivsus ja ka aparatuuri lihtsus, on leidnud järjest enam poolehoidu nende ühendite analüüsil. Looduslikult esinevate polüfenoolide mitmekesisus on tingitud nende esinemisest peamiselt glükosiididena, mis põhjustab nõudluse efektiivsemate analüüsimeetodite järele. Tänapäevaks on välja töötatud mitmeid lahendusi eelkõige kromatograafiliste meetodite ühendamiseks mass-spektromeetriaga, mille rakendamise olulisus on välja toodud ka antud töös. Ühendatud meetodid annavad suurepärase võimaluse lisaks ainete lahutamisele ka identifitseerida tundmatuid ühendeid vastavalt nende struktuurile. Samas kapillaarelektroforeesi ühendamisel mass-spektromeetriaga on esile kerkinud mõned takistused. Lisaks tehnilistele lahendustele on piiratud sobiva lahutuspuhvri valik, mis võimaldaks efektiivse analüütide lahutamise elektroforeetiliselt ja vastaks samas mass-spektromeetria tingimustele. Eespool nimetatud probleemide lahendamiseks töötati välja käesoleva töö käigus vastav süsteem fraktsioonide kogumise jaoks kapillaarelektroforeetiliselt, mis võimaldas kogutud fraktsioone ilma spetsiaalse eeltöötluseta kohe analüüsida mass-spektromeetriliselt.

Teades polüfenoolide sisaldust taimeekstraktides, on oluline järgnevalt teada iga ühendi antioksüdatiivsuse osakaalu antud süsteemis. Traditsioonilised meetodid annavad tulemuseks üldise antioksüdatiivsuse vastava segu kohta, kuid käesoleva tööga väljapakutud lihtne kapillaarelektroforeetiline meetod demonstreeris võimalust anda hinnangut iga fenoolse ühendi antioksüdatiivsuse võime kohta eraldi antud segus. Võrdlemaks saadud tulemusi kapillaarelektroforeesiga, oli väljatöötatud meetod kergesti rakendatav ka vedelikkromatograafias. Suhtelise antioksüdatiivsuse hindamiseks kasutatakse parameetrit „efektiivne kontsentratsioon" – EC_{50}, mis on defineeritud kui vastava antioksüdandi kogus, mis on vajalik radikaali algkontsentratsiooni vähendamiseks 50%. Antud töös aga kasutati vastupidist lähenemist – EC_{50} (mM), mille väärtusele vastab radikaali kontsentratsioon, mis on vajalik poole aine koguse oksüdeerimiseks.

PUBLICATION I

K. Helmja, M. Vaher, T. Püssa, K. Kamsol, A. Orav, M. Kaljurand. Bioactive components of the hop strobilus: Comparison of different extraction methods by capillary electrophoretic and chromatographic methods. *J. Chromatogr. A* 2007, 1155, 222- 229.

PUBLICATION II

K. Helmja, M. Vaher, J. Gorbatšova, M. Kaljurand. Characterization of bioactive compounds contained in vegetables of the Solanaceae family by capillary electrophoresis. *Proc. Estonian Acad. Sci. Chem*. 2007, 4, 172-186.

PUBLICATION III

K. Helmja, M. Vaher, T. Püssa, P. Raudsepp, M. Kaljurand. Evaluation of antioxidative capability of the tomato (*Solanum lycopersicum*) skin constituents by capillary electrophoresis and high-performance liquid chromatography. *Electrophoresis* 2008, 19, 3980-3988.

PUBLICATION IV

K. Helmja, M. Borissova, T. Knjazeva, M. Jaanus, U. Muinasmaa, M. Kaljurand, M. Vaher. Fraction collection in capillary electrophoresis for various stand-alone mass spectrometers. *J. Chromatogr. A* 2009, 1216, 3666-3673.

PUBLICATION V

K. Helmja, M. Vaher, T. Püssa, M. Kaljurand. Analysis of the stable free radical scavenging capability of artificial polyphenol mixtures and plant extracts by capillary electrophoresis and liquid chromatography-diode array detection-tandem mass spectrometry. *J. Chromatogr. A* 2009, 1216, 2417-2423.

CURRICULUM VITAE

1. Personal data

Name	Kati Helmja
Date and place of birth	29.03.1980, Pärnu, Estonia

2. Contact information

Address	Department of Chemistry, TUT, Akadeemia tee 15, 12618 Tallinn
Phone	+372 6204359
E-mail	helmja@staff.ttu.ee

3. Education

1998, Pärnu Co-education Gymnasium, secondary education
2003, Tallinn University of Technology, B. Sc., chemical and material science
2005, Tallinn University of Technology, M. Sc., chemical and material science

4. Special Courses

June–July, 2001 – Fluka Production, Switzerland
May–June, 2009 – University of Bern, Switzerland

5. Professional Employment

May 2006–May 2007 – cheaf specialist, Agricultural Research Center, Saku, Harju county, Estonia
Since September 2008 – assistant, Department of Chemistry, TUT

6. Theses defended

2003, bachelor thesis – "Monitoring of biodegradation of phenols from sewage sludge capillary electrophoretically"
2005, master thesis – "Optimization of the conditions of the bioprocess analysis using on-line capillary electrophoresis"

7. Main areas of scientific work/current research topics

Bioactive compounds, antioxidativity, capillary electrophoresis, liquid chromatography

8. List of original publications

1) M. Kudrjashova, H. Tahkoniemi, K. Helmja, M. Kaljurand. Capillary electrophoretic monitoring of microbial growth: determination of organic acids. *Proc. Estonian Acad. Sci. Chem*. 2004, 2, 51-64.

2) H. Tahkoniemi, K. Helmja, A. Menert, M. Kaljurand. Fermentation reactor coupled with capillary electrophoresis for on-line bioprocess monitoring. *J. Pharm. Biomed. Anal*. 2006, 41, 1585-1591.
3) K. Helmja**,** M. Vaher, T. Püssa, K. Kamsol, A. Orav, M. Kaljurand. Bioactive components of the hop strobilus: Comparison different extraction methods by capillary electrophoresis and chromatographic methods. *J. Chromatogr. A* 2007, 1155, 1155-229.
4) K. Helmja, M. Vaher, J. Gorbatšova, M. Kaljurand. Characterization of bioactive compounds contained in vegetables of the Solanaceae family by capillary electrophoresis. *Proc. Estonian Acad. Sci. Chem*. 2007, 4, 172-186.
5) K. Helmja, M. Vaher, T. Püssa, P. Raudsepp, M. Kaljurand. Evaluation of antioxidative capability of the tomato (*Solanum lycopersicum)* skin constituents by capillary electrophoresis and high-performance liquid chromatography. *Electrophoresis* 2008, 29, 3980-3988.
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7) K. Helmja, M. Vaher, T. Püssa, M. Kaljurand. Analysis of the stable free radical scavenging capability of artificial polyphenol mixtures and plant extracts by capillary electrophoresis and liquid chromatography-diode array detection-tandem mass spectrometry. *J. Chromatogr. A* 2009, 1216, 2417-2423.
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ELULOOKIRJELDUS

1. Isikuandmed

Ees- ja perekonnanimi	Kati Helmja
Sünniaeg ja -koht	29.03.1980, Pärnu, Eesti

2. Kontaktandmed

Aadress	TTÜ Keemiainstituut, Akadeemia tee 15, 12618 Tallinn
Telefon	+372 6204359
E-posti aadress	helmja@staff.ttu.ee

3. Hariduskäik

1998, Pärnu Ühisgümnaasium, keskharidus
2003, Tallinna Tehnikaülikool, keemia- ja materjaliteaduse õppesuund, loodusteaduste bakalaureus
2005, Tallinna Tehnikaülikool, keemia- ja materjaliteaduse õppesuund, loodusteaduste magister

4. Täiendusõpe

Juuni–juuli 2001 Fluka Production, Šveits
Mai–juuni 2009 Berni Ülikool, Šveits

5. Teenistuskäik

Mai 2006–mai 2007 Põllumajandusuuringute Keskus, Saku, Harju maakond, Eesti
September 2008– assistent, TTÜ Keemiainstituut

6. Teadustegevus

Kapillaarelektroforees, bioaktiivsed ühendid, vedelikkromatograafia

7. Kaitstud lõputööd

2003, bakalaureusetöö – „Kohtla-Järve tuhamägede nõrgvee fenoolide biodegradatsiooni kapillaarelektroforeetiline uurimine”
2005, magistritöö – „Bioprotsesside reaalajas toimuva kapillaarelektroforeetilise analüüsi tingimuste optimeerimine”

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

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