UNIVERZITA KARLOVA V PRAZE
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APLIKACE KAPALINOVÉ CHROMATOGRAFIE ZA ULTRAVYSOKÉHO TLAKU A HMOTNOSTNÍ SPEKTROMETRIE V ANALÝZE POLYFENOLICKÝCH LÁTEK

Disertační práce

2010 Mgr. Zdeněk Spáčil

CHARLES UNIVERSITY IN PRAGUE
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DEPARTMENT OF ANALYTICAL CHEMISTRY

APPLICATION OF ULTRA-HIGH PRESSURE LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY DETECTION IN ANALYSIS OF POLYPHENOLS

Doctoral Thesis

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Zdeněk Spáčil

PROHLÁŠENÍ

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány.

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ABSTRAKT

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Název disertační práce: Aplikace kapalinové chromatografie za ultravysokého tlaku a hmotnostní spektrometrie v analýze polyfenolických látek

Hlavním cílem této disertační práce zaměřené na využití kapalinové chromatografie (LC) a hmotnostní spektrometrie (MS) je vývoj moderních separačních metod určených k analýze polyfenolických látek. Obecné aspekty těchto technik jsou diskutovány v teoretické části a jejich praktický dopad je pak dokumentován čtyřmi originálními pracemi uvedenými v příloze (supplement I-IV). Stručný komentář k publikovaným článkům je dále uveden v kapitole „Results and discussion“. Kapalinová chromatografie byla použita v každé z publikovaných prací a poslední dvě práce (supplement III-IV) navíc kombinují jak přednosti LC, tak MS. Takto byla například semi-kvantitativní informace získaná za použití techniky laserem indukované desorpce/ionizace (LDI-MS) ověřena technikou kapalinové chromatografie za ultravysokého tlaku (UHPLC) s MS detekcí (supplement IV). Mnohostranné využití, jednoduchá obsluha a dobrá znalost fundamentálních principů dělají z LC „metodu volby“ v celé řadě vědeckých odvětví, při analýze různých analytů. Snadno proveditelné a robustní spojení LC-MS přináší vysoce efektivní separaci analytu na dvou úrovních: i) separaci a zakoncentrování analytu do úzké zóny pomocí kapalinové chromatografie a ii) separaci založenou na struktuře analytu, na poměru molekulové hmotnosti a náboje získaného při ionizaci (*m/z*), která se odehrává v hmotovém analyzátoru. Technika MS poskytuje vysoce selektivní detekci a navíc strukturní informaci, čímž vyniká nad kteroukoliv jinou LC „on-line“ detekční techniku. Od roku 2004 je UHPLC trendem v oblasti kapalinové chromatografie a její výhody oproti konvenční HPLC dokumentované na příkladu

polyfenolických látek v čaji a červeném hroznovém víně jsou shrnuty v první publikované práci (supplement I). Metody založené na UHPLC nabízejí vyšší separační účinnost ve srovnání s běžnou HPLC, vzhledem k použití sorbentů o velikosti částic menší než dva mikrometry (sub-2-micron). Dále přinášejí úsporu v čase analýzy doprovázenou nižší spotřebou rozpouštědel. Výhodou použití UHPLC při analýze polyfenolických látek může být také rychlá úprava vzorku, většinou pouze filtrace (supplement I-III). Vzorky červeného hroznového vína analyzované v poslední práci (supplement IV) technikou LDI/MS byly upraveny pomocí extrakce na pevné fázi (SPE). Tato práce demonstruje na příkladu fenolických kyselin využití techniky LDI/MS k záznamu vysoce přesné molekulové hmotnosti a dostatečného hmotnostního rozlišení, které postačují k identifikaci analytů ve složité přírodní směsi bez nutnosti předchozí chromatografické separace. Nicméně citlivost, selektivita a všestrannost analýzy je významně navýšena za použití kombinované techniky LC/MS. Získané výsledky mají předpoklad vysoké reprodukovatelnosti a přinášejí přesné kvantitativní informace, což je dobře dokumentováno analýzou katechinů obsažených v zeleném čaji (supplement III).

Tato disertační práce ukazuje využití obecných zákonitostí analytické a fyzikální chemie k vývoji nových analytických metod a aplikací v analýze polyfenolických látek.

ABSTRACT

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Title of Doctoral Thesis: Application of Ultra-High Pressure Liquid Chromatography with Mass Spectrometry Detection in Analysis of Polyphenols

The major focus of presented doctoral thesis is development of advanced separation methods utilizing liquid chromatography (LC) and mass spectrometry (MS) for the analysis of polyphenols. The general aspects of techniques are discussed in theoretical part and the actual practical impact is documented by the results published in four original articles appended in the supplement I-IV. LC was used in each published study, while combined with MS in the last two works (supplement III-IV). Thus, the semi-quantitative data acquired by emerging laser desorption/ionization (LDI) MS technique were confirmed by UHPLC-MS. The broad availability, versatile configuration, convenient handling and well understood principles make LC the “method of choice” for variety of scientific applications to analyze diverse analytes. LC-MS coupling is convenient and robust offering two-staged separation: i) chromatographic separation and pre-concentration into narrow bands using LC and ii) separation based on mass-to-charge ratio (*m/z)* performed in the MS instrument. Additional selectivity and/or structural information, which can be obtained through MS detection, exceed any other on-line LC detection technique. The advantages of UHPLC over conventional HPLC for the analysis of polyphenols (PPs) in tea and wine were evaluated (supplement I), while UHPLC methods typically offered higher separation efficiency, together with substantial reductions in run time and solvent consumption compared to HPLC. The red wine samples were also analyzed using LDI-MS technique with previous solid phase extraction (SPE) sample work-up (supplement IV). LDI-MS application

shows an example of highly accurate molecular weight and mass resolution measurements sufficient for identification of analytes in complex mixture purely based on MS data, without prior LC separation. However, the sensitivity, selectivity and versatility of assay are greatly improved with prior LC separation step also giving more reproducible and quantitative results. Thus, UHPLC system was coupled with tandem MS detection for the analysis of catehins contained in green tea (supplement III).

In general, studies overlaying this doctoral thesis demonstrates that careful attention to the fundamentals of physical chemistry provide solid insights that can greatly facilitate the development of novel analytical methods and emerging applications in the analysis of polyphenols.

ABBREVIATIONS

α	Selectivity
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
BEH	Ethylene bridged hybrid
C_8, C_{18}	Octyl, octadecyl silica
CI	Chemical ionization
CID	Collision-induced dissociation
CV	Coefficient of variation; the ratio of the standard deviation to the mean
Da	Dalton
DC	Direct-current
d_p	Particle diameter
EM	Electron multiplier
ESI	Electrospray ionization
FD	Fluorescent detection
FWHM	Full width at half maximum
ICR	Ion cyclotron resonance mass spectrometer
H	Plate height
HPLC	High performance liquid chromatography
HTLC	High temperature liquid chromatography
k'	Capacity factor
l	Column length
LC	Liquid chromatography
LDI	Laser desorption/ionization
MCP	Microchannel plate
MP	Mobile phase
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
m/z	Mass-to-charge ratio; the dimensionless quantity, where m is the mass of the ion in unified atomic mass units (u) and z is the number of charges on the ion
N	Number of theoretical plates
p_c	Peak capacity
ppm	Part per million
Q	Quadrupole mass analyzer
QIT	Quadrupole ion trap mass analyzer
R	Retention ratio
R_s	Chromatographic resolution
RF	Radio frequency
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

RP	Reversed phase
σ	Standard deviation
SIM	Selected-ion monitoring
SP	Stationary phase
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TOF	Time-of-flight mass spectrometer
t_R	Retention time
υ	Zone velocity
UHPLC	Ultra-high pressure liquid chromatography
UPLC™	Ultra performance liquid chromatography
v	Flow velocity
w	Peak width

CONTENTS

1. INTRODUCTION

The instrumental analytical methods based on principles of liquid chromatography (LC) are widely used in the pharmaceutical analysis. Nowadays high performance liquid chromatography (HPLC) and ultra-high pressure liquid chromatography (UHPLC) are methods of choice for pharmaceutical companies and regulatory authorities for quality control, bioequivalence assays or research and development. Although number of detection techniques, such as ultraviolet-visible spectrophotometric (UV/VIS), fluorescent (FD), evaporative light scattering (ELSD), refractive index (RI) or electrochemical (EC) is available, the mass spectrometry (MS) coupling through electrospray ionization (ESI) is used increasingly with respect to numerous advantages. LC/MS is featured by superior selectivity and sensitivity supplemented with structural information about analyte. Highly reproducible and rapid LC/MS methods are suitable for routine measurements with automated data acquisition and processing. They are universal for wide range of analytes, usually with no need for derivatization. The improving accessibility of MS instruments together with cost effective analysis predestines LC/MS for even broader future use in many scientific and commercial fields.

2. AIM

The general aim of the experimental work presented in this doctoral thesis was to build on the previous research in the field of separation methods conducted at Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Králové and to develop novel analytical methods for the identification and quantification of important biologically active molecules present in natural samples. The polyphenolic compounds from the groups of flavonoids, phenolic acids and coumarins are in the scope of pharmaceutical and nutraceutical industry, due to their potent antioxidant activity. They minimize oxidative stress damage of cells and have protective effects against age-related degenerative diseases, such as cancer and cardiovascular diseases. The polyphenols are abundant in fresh fruits and fruit products (e.g. juices, wines), in teas and herbal infusions, coffee, chocolate, beer and to a lesser extent vegetables. Tea and wine samples were chosen for the analysis such as typical examples of widely consumed natural products and rich sources of polyphenols. The sample workup strategies presented here reflect requirements for rapid workflow and at the same time are sufficient to address complexity of natural samples. Subsequently the advanced methods of LC and MS were developed in order to provide accurate, precise and reproducible results, while analyzing wide range of heath-promoting polyphenolic compounds. The common denominator for the novel analytical techniques was high throughput without compromised detection selectivity or sensitivity. Thus, the separation and detection conditions were optimized based on detailed understanding of theory and practical experiences with instrumentation. Neither economical nor environmental aspects were omitted. This doctoral thesis is largely based on four original publications (supplement I-IV) well documenting the potential of the novel LC/MS approaches for pharmaceutical, nutraceutical and food processing industries.

3. THEORETICAL PART

3.1. General introduction

Liquid chromatography (LC) is the most widely used of all separation methods, because of its outstanding separation power and versatility. The LC instrument is intrinsically simple in operation, despite of the complicated mechanistic fundamentals governing LC. A column typically packed with a granular sorptive material and carrying a stream of liquid through its length will convert a droplet of a complex mixture, injected at one end, into number of molecular constituents, which emerge individually from the other end. The directness and simplicity of LC serves the life scientist to reveal the complex chemical processes in living organisms; the chemist in pharmaceutical industry to determine the composition of drugs; the environmental chemist to trace contaminants through air and water; the organic chemist in studies of reaction mechanisms and rates as revealed by the analysis of reaction products; as well as scientists from literally dozens of other disciplines requiring the analysis of complex multicomponent mixtures. The basic level of LC separation can be realized fairly easily, but the complex mixtures confronting many scientists require higher level of sophistication [1]. The analysis of small organic molecules might be hampered by structurally related compounds (e.g. isomers) and also by the compounds with widely different physicochemical properties contained in the complex mixture and potentially interfering with the analysis. The ballast compounds typically exceed the concentration of analyte by orders of magnitude. Such conditions require highly efficient separation of analyte from the sample matrix and/or its selective and sensitive detection. Though, the challenge in LC is not simply in making it work, but in making it work exceptionally well to deal with truly complex mixtures [1].

The MS techniques are capable to detect attomole quantities of molecule based on molecular weight and structure. However, the intolerance to contaminants, particularly surfactants and charged non-volatile species is considered as main drawback. Thus, the analyte response may be suppressed through the competition with other species during desorption/ionisation process. To reduce this risk, the

analyst should always follow the basic rule of using the minimal amounts of additives/reagents, which applies to MS technique practice in general. On the other hand the composition of natural sample matrix is mostly beyond the analyst's control setting up challenge, which drives the development of techniques. The analytical chemistry is an essential multidisciplinary science able to produce reliable results, needed in other fields of scientific research. The sound fundamental knowledge of chemistry and physics is required to understand analytical processes and instrumentation, which together with a rational systematic approach ranks the analytical chemistry on the borderline between fundamental and applied science.

3.2. Liquid chromatography

The birth year of chromatography is the year 1903, when the Russian botanist Mikhail Semyonovich Tswett firstly spoke about his chromatographic analysis at a session of the biology, Division of the Warsaw Society of Naturalists [2]. He used a column packed with powdered calcium carbonate and petroleum ether mobile phase to study chemistry of important plant pigments (chlorophyll and carotenoides). The components were separated into individual zones, each located at different point along the column's length and each visible because of its color. The colored bands led Tswett to employ the name chromatography (Greek: "color writing") [2]. Although, Tswett's report caused a lively discussion between all those present and him, the invention of chromatography was ignored for several decades. The technique was reintroduced after 44 years and the principles were described by the Nobel Prize laureates Martin and Synge [3-6], awarded for their invention of partition chromatography in 1952. The development in the instrumentation allowed the switch from reliance on atmospheric pressure and gravity flow to the use of pressurized pumping systems [7], which was indicated by former term high-pressure liquid chromatography. However, since early 1970s the term high-performance liquid chromatography established by Prof. Horváth is counted for the acronym HPLC [8]. Typical HPLC instrument is operated at back-

pressures up to 6 000 psi (41 370 kPa or 401 bars). Although very small particle sobents with enhanced chromatographic performance can be used at ultra-high pressures (>400 bar), as suggested 40 years ago by Knox [9]. Therefore, tremendous leap in LC began since 2004 when Waters Corp. introduced the first commercial ultra-performance liquid chromatography instrument (UPLC™) [10]. "UPLC", such as the trademark registered by the manufacturer is not recommended to be used in scientific texts and the term UHPLC will be used here instead. Presently UHPLC systems capable to handle backpressures up to 15 000 psi (~103 MPa or 1034 bars) are available from several manufacturers and their superior efficiency established new higher standards for LC separations [11]. The underlying processes governing chromatographic performance are among the most complicated of those controlling any separation method. They therefore require a considerable depth of understanding on the part of anyone seeking to maximize and extend chromatographic capabilities [1].

3.2.1. Column and planar techniques

In LC, analytes are dissolved in mobile phase (MP) and then the separation of the solutes is accomplished by continuously passing a MP over a stationary phase (SP). The solutes flow with the MP and they are partitioned between the MP and SP, while the stronger the interaction with the SP, the longer the time is required for a solute to pass through the chromatographic system. The physicochemical interactions between a solute and both the MP and SP are determined by liquid-solid adsorption, liquid-liquid partitioning, ion exchange and size exclusion [12]. The basic types of LC arrangement are column chromatography and planar chromatography [13]. The sorptive medium is ordinarily contained in a column to give it the proper structure (length and diameter), to allow the application of pressure to drive flow, and to confine the MP along the desirable path. This form is categorized as column chromatography, where the SP is held in a narrow tube, and the MP is forced through the tube simply by gravity or under pressure [1]. In contrast, planar methods operate without the confinement of a column wall. These

methods employ either an open granular bed or a simple sheet of paper, whilst the MP moves through the SP by capillary action or under the influence of gravity. Typical examples of planar chromatography are represented by the thin-layer chromatography (TLC) on silica gel and paper chromatography. The noncolumn techniques are simple and convenient, but their flexibility lack in the flow control and extending bed length to optimize separation. Thus, only the column chromatography methods will be considered in the following text.

3.2.2. Molecular equilibrium

The key element in the description of LC separation processes is an open system, which might be one phase of an LC system. An open system is the one which can undergo all changes allowed for closed system and in addition it can lose and gain matter across its boundaries, which allow for the transport of matter both in and out [1]. For example, if dn_A moles of component A enter the system, and there are no changes in temperature (T) and pressure (p) and no other components (n_B) is crossing in or out, Gibbs free energy (G) will change by a small increment proportional to dn_A

$$dG = \left(\frac{\partial G}{\partial n_A} \right)_{T,p,n_B} dn_A \qquad \textbf{Equat. 1.}$$

The magnitude of the increment is of such importance that the rate of change, or partial derivative, is given a special symbol μ_A, called the chemical potential

$$\mu_A = \left(\frac{\partial G}{\partial n_A} \right)_{T,p,n_B} \qquad \textbf{Equat. 2.}$$

It is essentially the amount of G brought into a system per mole of added constituent A at constant T and p or simply energy per mole [1]. SP and MP represent two open systems, which may exchange matter only between them and thus at constant T and p they constitute together a closed system. Then the equilibrium for solute distributed between interconnected regions is defined by

$$\mu_A^{MP} = \mu_A^{SP} \qquad \textbf{Equat. 3.}$$

In this equality the μ_A value for solute A in a given phase depends on the intrinsic thermodynamic affinity of the solute to the phase and the dilution of the solute. The μ_A in the form appropriate for the study of dilute solutions, which corresponds to the conditions of LC separation, is represented by

$$\mu_A = \mu_A^0 + \Re T \ln c_A \qquad \textbf{Equat. 4.}$$

where $\Re$ is the gas constant, c_A is the concentration of component A and μ_A^0 is the standard-state chemical potential [1]. The latter is a reference value of the chemical potential in a hypothetical standard state with component A at unit concentration but with each molecule of A surrounded by solvent as found at infinite dilution. The value of μ_A^0 is generally lowest in phases where intermolecular interactions are strongest (i.e. solute-solvent affinity is greatest). If **Equat. 4.** is substituted into **Equat. 3.** and the resulting expression solved for the ratio of two concentrations c_A^{SP} and c_A^{MP} the resulting equilibrium expression is

$$\left(\frac{c_A^{SP}}{c_A^{MP}}\right)_{eq} = \exp\left(\frac{-\Delta\mu_A^0}{\Re T}\right) \qquad \textbf{Equat. 5.}$$

where the equilibrium ratio $(c_A^{SP}/c_A^{MP})_{eq}$ is the distribution coefficient K, giving

$$K = \exp\left(\frac{-\Delta\mu_A^0}{\Re T}\right) \qquad \textbf{Equat. 6.}$$

Then the amount of solute accumulating in the SP relative to that in the MP is the relative measure of solute affinity to the SP termed the capacity factor (k'):

$$k' = \frac{c_s^* V_s}{c_m^* V_m} \qquad \textbf{Equat. 7.}$$

where V_s ; V_m are the volumes of SP and MP in the column with the equilibrium values c_s^*; c_m^* of a solute concentrations in SP and MP. The same reasoning can be used to define equilibrium the fraction R' of the total solute found in the MP [1]

$$R' = \left(\frac{V_m c_m}{V_m c_m + V_s c_s}\right)_{eq} = \frac{V_m}{V_m + KV_s} = \frac{1}{1+k'} \qquad \textbf{Equat. 8.}$$

3.2.3. Terms and definitions

The basic migration process has to be well defined in order to understand chromatographic separation, which is a result of the differential migration of components. In LC the solutes are distributed over the separation path as discrete zones and the success of separation hinges on keeping the zones reasonably narrow to avoid overlap and cross contamination with neighboring zones [1]. The retention time (t_R) is defined as the time between the introduction of the solute and the time it appears at highest concentration (peak maximum) in the zone passing through the detector. The zone velocity (υ) is slow for strongly sorbed solutes (low R' value) since only the fraction R' of the total solute in the zone is carried along with the MP at average flow velocity (v). The remaining fraction ($1-R'$) is held stationary at zero velocity. This can be quantitatively expressed as: $R' \times v + (1-R') \times 0 = R'v$. Thus the zone velocity is directly proportional to R'

$$\upsilon = R'v \qquad \textbf{Equat. 9.}$$

In fact the thermodynamic parameter R' is equivalent to the relative velocity term called the retention ratio (R),

$$R = \frac{\upsilon}{v} \qquad \textbf{Equat. 10.}$$

which is a measure of the retardation of the chromatographic zone with the respect to the MP velocity, thus $R'v = Rv$ [1]. A peak that experiences no retardation because its solute does not partition into the SP ($R = 1$) is termed a void peak, such a peak travels at MP velocity ($\upsilon = v$). On the other hand solute retained to some extent by the SP migrates as a retained peak ($R < 1$), whilst the smaller R, the greater the retention. The retention volume (V_r) is the volume of the MP, measured as it emerges at the outlet, necessary to flush the peak center to the end of the column. The containment of zone spreading is the main goal of separation sciences and can be measured in LC by peak width (w). For Gaussian peaks, standard deviation (σ) is the distance from the peak center to the point of inflection, while 95.6% of a Gaussian peak area lies between -2σ and $+2\sigma$ and total of 4σ is termed as “effective zone”. Thus, the 4σ can be arbitrarily taken as

peak width assuming that the zone acquire a Gaussian form [1]. Inasmuch as the basic diffusion equations lead to a Gaussian profile, in most cases the Gaussian spreading can be described as an apparent diffusion process, with an effective diffusion coefficient (D) related to σ and time (t):

$$D = \frac{\sigma^2}{2t}$$ **Equat. 11.**

However a Gaussian zone is the model and there are frequent departures from the Gaussian profile because of various perturbations or nonidealities. For a true Gaussian shape, an infinite number of random steps is needed, each with infinitely short duration and displacement. Thus any random step of finite displacement will perturb the Gaussian by a finite degree. Such processes include adsorption-desorption steps, partitioning into a MP of finite depth, chemical interconversions, trapping in void volumes or simply winding around a support particle. When these processes are encountered with long time constants e.g. slow adsorption-desorption cycles or the trapping the of solute in large dead volumes (inside or outside of the column), the distortion can be serious [14]. The following text is intended to establish the basic laws governing the structure and evolution of zones and to relate zone broadening to indices measuring the effectiveness of chromatographic separation [1]. The most useful criterion for the effectiveness of LC separation of two components is chromatographic resolution (R_s). However the R_s, as the separation factor, differs for each specific component pair and therefore fails as a global criterion of separation. Thus, more universal indices were evolved such as height equivalent to a theoretical plate (H), number of plates (N) and peak capacity (p_c).

3.2.3.1 Plate height

The parameter H, simply called plate height, is commonly used to assess column efficiency in isocratic LC. The concept of theoretical plates evolved from studies of distillation and countercurrent distribution. However, its wide adoption in

chromatography can be traced to the seminar work of Martin and Synge [15]. The concept of theoretical plates assumes the separation path to be divided into a series of linked stages or plates, where partitioning between MP and SP occurs, which is neither very appropriate nor very useful in describing the continuous transport processes in LC. However, since continuous zonal systems such as LC share the key proportionality with staged systems, the name “plate height” for the constant of proportionality can be retained whether the system is staged or continuous [1]. The parameter H is defined as:

$$H = \frac{2D_T}{v} \qquad \textbf{Equat. 12.}$$

where D_T corresponds to the total effective diffusion coefficient and v to the average flow velocity. In order to use H broadly, the number of theoretical plates has to be defined. Quantity of parameter N is given by $N = l / H$, which is the number of units (plates) encountered in migration distance, the column length (l). The parameter N is dimensionless and depends on both the properties of the column and the solute [1].

3.2.3.2 Chromatographic resolution

The chromatographic resolution is the ratio of the distance between centers of the two component zones to the average zone width and thus categorizes the overlap (or lack of it) of two specified component zones. If the distance is expressed by retention time, the quantitative measure of separation between two chromatographic peaks A and B relative to the zone dispersion ($2\sigma_A$ and $2\sigma_B$) is,

$$R_s = \frac{t_{R,B} - t_{R,A}}{2(\sigma_A + \sigma_B)} = \frac{\Delta t_R}{2(\sigma_A + \sigma_B)} = \frac{\Delta t_R}{0.5(w_B + w_A)} \qquad \textbf{Equat. 13.}$$

where $t_{R,A}$; $t_{R,B}$ are the respective retention times and w_A; w_B are the respective zone dispersions expressed by peak width. Inasmuch as close-lying components generally have similar properties reflected in comparable w values, w_A and w_B may be replaced by an average value w

$$R_s = \frac{\Delta t_R}{0.5(w_A + w_B)} = \frac{\Delta t_R}{w} = \frac{\Delta t_R}{4\sigma}$$ **Equat. 14.**

which means that R_s is unity when two peak centers are separated by Δt_R equal to the distance $w = 4\sigma$. Similarly two distinct maxima of equally height peaks are found only when $R_s > 0.5$ and the "baseline" resolution is characterized by $R_s > 1.5$. Although unit resolution ($R_s = 1$) is adequate for most analytical purposes, the Gaussian zones do not disengage from one another completely as they move further apart because the concentration at the edges, while dropping off dramatically, does not reach zero. For example, two equal Gaussian zones having $R_s = 1$ still penetrate one another to the extent that 2.2% of each zone's content [1].

The apparent diffusion coefficients defined in **Equat. 11.** are additive and their total (D_T) can be used to describe Gaussian spreading. Thus, each zone acquires a variance σ^2 proportional to time (t) providing the constant D_T:

$$\sigma^2 = 2D_T t$$ **Equat. 15.**

The distance to center of gravity zone (l) traversed by the zone in given time t at the constant velocity of component zone (υ) is simply

$$l = \upsilon t$$ **Equat. 16.**

The substitution of **Equat. 16.** and **Equat. 15.** results in

$$\sigma^2 = \left(\frac{2D_T}{\upsilon}\right) l$$ **Equat. 17.**

which includes the proportionality of σ^2 to zone migration distance and **Equat. 12.** can be substituted into this equation, yielding more simple expression [1]

$$\sigma^2 = Hl$$ **Equat. 18.**

The separation between two chromatographic peaks A and B occurs because they are traveling at different velocities υ_A and υ_B at given t. When **Equat. 18** is solved for σ and substituted into **Equat. 14.**, where Δt_R is replaced by the difference in separation distance (Δl) the resulting expression is

$$R_s = \frac{\Delta l}{4(Hl)^{1/2}}$$ **Equat. 19.**

showing that resolution improves with decreasing plate height H. The separation distance of chromatographic zones can be expressed as

$$\Delta l = (\upsilon_B - \upsilon_A)t = \Delta \upsilon t$$ **Equat. 20.**

which demonstrates that t represents mean distance over mean velocity and can be expressed as l/υ, therefore

$$\Delta l = \frac{\Delta \upsilon l}{\upsilon}$$ **Equat. 21.**

Finally, the general expression for resolution, where separation parameters are included is obtained after the substitution of **Equat. 21.** into **Equat. 19.**:

$$R_s = \frac{\Delta \upsilon}{\upsilon}\left(\frac{l}{16H}\right)^{1/2} = \frac{\Delta \upsilon}{\upsilon}\left(\frac{N}{16}\right)^{1/2}$$ **Equat. 22.**

where $N = l/H$ was used to obtain the latter expression. The mean velocity of component υ in chromatography is Rv; the difference $\Delta\upsilon$ in the velocity of two zones is therefore $(\Delta R)v$. Thus, the latter expression of **Equat. 22** becomes

$$R_s = \frac{\Delta R}{R}\left(\frac{N}{16}\right)^{1/2}$$ **Equat. 23.**

when R is substituted by equilibrium parameters ($R = V_m/V_m + KV_s$), the R_s can be expressed in another way [16]

$$R_s = \left(\frac{N}{16}\right)^{1/2}\frac{\Delta K}{K}\left(\frac{k'}{1+k'}\right)$$ **Equat. 24.**

which includes parameters corresponding to column efficiency (N), column relative selectivity ($\Delta K/K$) and capacity factor (k'). These parameters contain wealth of information on how to improve R_s or to do faster separation without compromising R_s. For example R_s increases with the relative selectivity $\Delta K/K$, which can be altered by changing chemical (e.g. MP/SP composition and pH) and physical (e.g. temperature) parameters [1]. This is particularly effective in those cases where the solute is weakly retained by the SP, so the original k' is small or

nearly equal to 1. The chromatographic conditions have to be altered in a way, so they are more selective for one of the solutes. In the LC separations k' is easily modified either by changing concentration of the organic constituent in MP or by the temperature [17]. However, these changes must not to drive the solutes excessively into the MP causing k' to approach zero and thus erode R_s. It is desirable to keep $k' > 1$, which is maintained by a reasonable level of retention. On the other hand increased k' value is result of solute's low affinity to the MP, thus it spends proportionally more time in the SP. Also increased volume of SP (e.g. using a wider or longer column) will lead to an increase of k' value, as shown in **Equat. 7.** Clearly, any improvement in R_s obtained by increasing k' generally comes at the expense of a longer analysis time. In some cases k' value is insufficient to resolve early eluting solutes, but increasing k' leads to an unacceptably long retention time for later eluting solutes [1]. This so called "general elution problem" can be solved by incremental adjustments of k' over time during gradient elution [18], while the composition of MP is gradually changed to improve the range of separation. The gradient elution has favourable effects on the zone spreading (σ^2), hence a zone migrates in conditions, where k' continually decreases, therefore the zone tail always moves faster than the band front. The resulting focusation of the zone is referred to as gradient compression [19]. In situations where the value of k' is given, the larger parameter N can be achieved either decreasing plate height H or increasing column length l.

3.2.3.3 Peak capacity

The total number of zones or peaks separable during LC analysis rather than resolution of specific pairs is needed in the separation of complex mixtures having many components (>100). The peak capacity (p_c) is the maximum number of separated peaks that can be fit (with adjacent peaks at given R_s value) into the column length (l) or elution volume range ($V_{\max} - V_{\min}$) [20,21]. When R_s is defined to be 1, the peak capacity for adjacent peaks can be expressed as:

$$p_c = \frac{l}{w} = \frac{l}{4\sigma}$$ **Equat. 25.**

This equation also shows that $R_s < 1$ allows more peaks to be separated on given separation length (l) and p_c increases in accordance with term $p_c = l/4\sigma R_S$. Now if the square root of σ^2 from **Equat. 18.,** is used, **Equat. 25.** reduces to

$$p_c \approx \frac{N^{1/2}}{4}$$ **Equat. 26.**

However, the value of p_c can be significantly higher than the value indicated in **Equat. 26.** since each successive elution volume bring forth and resolve a new group of peaks, then p_c (at $R_s = 1$) is approximately

$$p_c = 1 + \frac{N^{1/2}}{4} \ln \frac{V_{\max}}{V_{\min}}$$ **Equat. 27.**

where V_{min} and V_{max} are the smallest and largest volumes of MP in which a solute can be eluted and detected [1]. Thus p_c increases not only with N, but also with the workable elution volume range $V_{\max}/V_{\min}$, which may reach a value of 50 and more in LC. The presented terms indicates that p_c is inversely proportional to w or σ (reflected in H and N), which emphasizes the concern with zone spreading phenomena and the fundamental transport processes that underline them. Since p_c is a measure of the maximum number of peaks that can be accommodated in a chromatogram, it may help to exclude from consideration columns that do not have sufficient theoretical plates to separate a complex mixture. In practice, random jumble of peaks rather than even distribution is observed together with deviations from ideal Gaussian profile of chromatographic peaks. Therefore, considerably more space for peaks measured by p_c has to be provided to assure that most (rarely all) component peaks separate cleanly. The non-Gaussian zones can arise out of system nonlinearities caused by overloading, in which component molecules are so numerous that they interfere with one another's migration. This interference makes local zone velocity υ (along with diffusion coefficient D) dependent on

concentration, which voids the theory leading to Gaussian zone. The way in which overloading causes zone asymmetry is that at high concentrations the surface sites of SP become almost saturated with solute and will adsorb very little more, even when additional amounts are added to the mobile phase. Therefore, the MP solute fraction (R or R') increases with solute concentration and the local velocity ($\upsilon = Rv$) of a given component can no longer be counted on as a constant characteristic of that component. Since R and Rv increase with concentration, the solute migrates fastest at the center of the zone where the overall concentration is greatest. The zone center consequently overtakes the leading edge while leaving the trailing edge behind. This tailing phenomenal is undesirable, because the tail often reaches back far enough to mix with and obscure slower components. Thus, the mean peak velocity $\langle \upsilon \rangle$ changes with sample size and component can no longer be by a constant well-defined t_R. However, modest tailing at the working sample loads is common in chromatography. Sometimes tailing has a kinetic origin, rather than equilibrium, stemming from slow desorption processes. Thus the observation of tailing does not prove that the column is overloaded and the reduction of solute load will not eliminate tailing [1].

3.2.4. Molecular basis of migration

Each molecule is moving along with an erratic motion and its path is determined by several independent statistical processes, which controls zone spreading. The processes relevant to chromatography are: i) longitudinal molecular diffusion, ii) sorption-desorption in the SP and in the MP and iii) flow and diffusion in the MP.

3.2.4.1 Longitudinal diffusion

The longitudinal diffusion factor is the result of ceaseless Brownian motion of solute molecules along the column axis. The erratic motion of molecules will cause them to move in series of random steps up and down the flow axis. The contribution of longitudinal diffusion to zone broadening refers to diffusion coefficient (D), so the variance can be obtained directly from **Equat. 15.** in which

overall diffusion coefficient (D_T) is replaced by the solute's specific diffusion coefficient in the MP D_m:

$$\sigma^2 = 2D_m t \qquad \textbf{Equat. 28.}$$

In LC, the diffusion time can be replaced by the time spend in the MP during the process of migrating through the column. Since this time is determined by traveled distance l at the MP velocity v, the variance becomes

$$\sigma^2 = \frac{2D_m l}{v} \qquad \textbf{Equat. 29.}$$

The numerous obstructions in the flow path of LC columns prevent diffusion along a straight line. Therefore the diffusion process is retarded as measured by obstructive factor γ (≈0.6) and D_m is replaced with γD_m. From **Equat. 18.** the plate height can be expressed as $H = \sigma^2 / l$, thus the contribution of longitudinal diffusion to plate height yields

$$H_{Ds} = \frac{2\gamma D_m}{v} \qquad \textbf{Equat. 30.}$$

where γ is a constant related to the column packing. Clearly, the H_{Ds} term is inversely proportional to flow velocity v, so the effect on total H is minimized by a high MP velocity [1]. The H_{Ds} is often referred as

$$H_{Ds} = \frac{B}{v} \qquad \textbf{Equat. 31.}$$

where B is a constant [22].

3.2.4.2 Sorption and desorption

The sorption-desorption in the SP and MP causes another important irregularity in molecular migration. Every time a molecule is sorbed by the SP, its downstream motion ceases and proceeds again, when it is desorbed. A SP term designated as H_s is called mass-transfer term, because it accounts for the transport speed of molecule through one phase to reach another. The sorption/desorption of molecule to the SP require certain finite time, when the solute molecule has to diffuse

through the MP to reach the equilibrium [1]. The band broadening occurs whenever the solute's movement is insufficient to quickly reach the equilibrium. In LC desorption involves getting the solute molecules from the interior of the small units of absorbing liquid to the surface where they can escape, which is a diffusion process again. If the SP is characterized by thickness (d), the time needed for solute to diffuse out will be roughly $d^2/2D_s$, where D_s is the diffusion coefficient for solute in the SP. Then a configuration factor q accounting for the shape of the SP [23] defines the contributions of mass transfer as:

$$H_s = qR(1-R)\frac{d^2 v}{D_s} = C_s v \qquad \textbf{Equat. 32.}$$

where C_s is called the SP plate height coefficient [1]. The parameters of this equation suggest that the H_s contribution to the H is reduced by dispersing the SP into extremely fine units or as very thin film so that the average depth d is small. It also shows that the H is least affected by H_s when the MP flow velocities are low.

3.2.4.3 Flow and diffusion in the mobile phase

The last process referred as the flow and diffusion in the MP (H_c) depends on the size of the interstitial channel, proximity to a particle surface, obstacles blocking the flow path, etc. Although, the interstitial pore space is of great complexity, a general relationship can be used to describe how the H_c depends upon particle size, flow velocity and diffusivity. Furthermore theoretical models combined with empirical data can provide criteria for the inherent quality of the column packing. The distance between adjacent channels or across channels can be scaled in proportion to the particle diameter d_p and all velocities can be scaled in proportion to the flow velocity v in the packed medium as long as the flow remains laminar [1]. The chromatographic peak spreading occurs due to different velocity states that molecules can occupy, which allows one molecule to get ahead of or behind another. The molecules of solute pass various paths through the chromatographic

column, due to the complicated structure of a bed of packed particles, which arises these velocity increments: i) molecules travel faster at the center of narrow flow channels than at the outside edge; ii) molecules travel faster in some channels than in others close by because of differences in shape, openness, obstruction etc.; iii) molecules travel faster in the flow channels between particles than in the narrow pores within porous support particles; iv) molecules generally travel faster near the column wall than at the column center [1]. All of these increments contribute to a velocity bias resulting into zone spreading. For example in the case of the short-range interchannel effect (ii) [16] the molecule can be removed from a velocity bias by two mechanisms: i) flow will eventually slow down a molecule carried in a fast stream path encountering constrictions or obstructions that hinder fluid motion; ii) diffusion will cause a molecule to move from a fast stream path to a slow one and vice versa. In case that flow is dominant mechanism, the average channel length of the channel having a consistently higher or lower permeability than average permeability will be responsible for a velocity bias. Seeing that distances in the packed bed are scaled to d_p, the principal contribution to H, caused by non homogeneous column packing (e.g. variations in particle size and packing structure) is expressed as:

$$H_f = 2\lambda d_p = A \qquad \textbf{Equat. 33.}$$

where λ is a constant related to packing-structure. The term H_f as referred above, is sometimes called the eddy diffusion term, accounting for the consistency of the packing-structure. Typical values of λ for well-packed columns are $0.8-1.0$ [24]. A narrow distribution in particle size and more even packing results in a smaller value of λ, while H_f is eliminated completely for capillary columns [1]. If diffusion terminates the velocity bias, then a molecule is carried the distance (d) downstream in average time necessary to diffuse into a nearby flow channel having a different velocity. Since the distance is scaled to d_p it can be written as $d = \omega_\alpha d_p$, where ω_α is a constant. Then the plate height is

$$H_{Dm} = \omega \frac{d_p^2 v}{D_m} = C_m v$$ **Equat. 34.**

where ω is the combined constant $\omega_\alpha^2 \omega_\beta^2 / 2$ [1]. Thus, the two distinct kinds of plate height are recognized for MP; a term H_{Dm} proportional to v, valid when diffusion terminates a molecule's velocity bias and term H_f, which is velocity-independent and valid when flow terminates the bias. The additivity does not apply to H_{Dm} and H_f, because they do not have independent random walks. Therefore, in the case of extremely high flow velocities, when diffusional exchanges are negligible H_f accounts for MP plate height ($H_c = H_f$). Similarly in the low velocity extreme, when flow exchanges become so slow that all velocity exchanges are caused by diffusion ($H_c = H_{Dm}$). The coupling equation [16] gives complete expression for the MP random walk:

$$H_c = \sum \left(\frac{1}{2\lambda d_p} + \frac{D_m}{\omega d_p^2 v} \right)^{-1}$$ **Equat. 35.**

where H_c is a function of d_p, D_m and v, while the ω and λ constants are unspecified, but same for equally well packed columns, for all MPs and for all solutes. The later expression can be added to the longitudinal diffusion **Equat. 30.** and SP terms **Equat. 32.** resulting into overall height expression

$$H = H_{Ds} + H_c + H_s = \frac{2\gamma D_m}{v} + \sum \left(\frac{1}{2\lambda d_p} + \frac{D_m}{\omega d_p^2 v} \right)^{-1} + qR(1-R)\frac{d^2 v}{D_s}$$ **Equat. 36.**

which can be condensed to highlight the velocity dependence

$$H = \frac{B}{v} + \sum \left(\frac{1}{A} + \frac{1}{C_m v} \right)^{-1} + C_s v$$ **Equat. 37.**

This equation provide summary of all the constants ($\gamma, q, \omega, \lambda$) and the nature of the dependence of H on the controllable system parameters v, d_p, d, R, D_m and D_s important for practical chromatography.

3.2.5. Plate height equations

The overall height plate expression **Equat. 37.** for packed column can be approximated as,

$$H = \frac{B}{v} + C_m v + C_s v \qquad \textbf{Equat. 38.}$$

at low and medium velocities, where diffusion controls the MP random walk and $C_m = \omega d_p^2 / D_m$; ω. On the other hand at higher velocities, when flow dominates diffusion in the central term on the right of **Equat. 37.**, giving the limiting form

$$H = A + \frac{B}{v} + C_s v \qquad \textbf{Equat. 39.}$$

which is equivalent to van Deemter equation [25] and can be empirically fit to most H versus v data over limited range. Since it does not accurately reflect column dynamics it must be used with caution, when interpreting experimental results. Typical values for well-packed columns are roughly 1 for A, 2 for B and C value varies from $0.1-0.2$ [26,27]. Taking the basic components of plate height in packed columns (H_f and H_D), they are equal at specific velocity v_c, which is transition point between a flow-controlled and diffusion-controlled random walk. The velocity v_c is fundamental parameter characterizing every packed system and can be expressed by

$$v_c = \frac{A}{C_m} = \left(\frac{2\lambda}{\omega}\right)\frac{D_m}{d_p} \qquad \textbf{Equat. 40.}$$

Seeing that λ and ω are constants and their exact values are difficult to determine, v_c can be simplified into expression

$$v_c = \frac{D_m}{d_p} \qquad \textbf{Equat. 41.}$$

The state of dynamic balance between MP processes in every LC column is governed by the flow velocity relative to the fundamental velocity v_c

$$v_{red} = \frac{v}{v_c} = \frac{d_p v}{D_m} \qquad \textbf{Equat. 42.}$$

where dimensionless v_{red} is termed the reduced velocity [28]. The particle diameter d_p is a fundamental unit of length, to which most distances in the column are scaled. Therefore, it can be used to reduce H to dimensionless form. Also if v equals the fundamental velocity v_c, then the MP H terms are close to d_p in value. Thus $C_m v$ becomes $C_m v_c$, which equals

$$C_m v_c = \frac{\omega d_p^2 v_c}{D_m} = \omega d_p \qquad \textbf{Equat. 43.}$$

which shows that H is scaled to d_p. The H can be expressed in multiples of d_p for universal representation, that is in terms of the reduced plate height defined by

$$h = \frac{H}{d_p} \qquad \textbf{Equat. 44.}$$

Now v_{red} and h can be used to replace v and H in **Equat. 37.** resulting in a reduced plate height equation,

$$h = \frac{2\gamma}{v_{red}} + \sum \frac{1}{(1/2\lambda) + (1/\omega v_{red})} + \Omega v_{red} \qquad \textbf{Equat. 45.}$$

where Ω is coefficient ($\Omega = C_s D_m / d_p^2$). The equation explicitly show that as far as the MP is concerned, h is a function only of v_{red} and the packing-structure constants (γ, λ and ω) without dependence on d_p and D_m. Thus, the h versus v_{red} curves representing MP effects will be about the same for similarly packed columns with similar packing constants. This can be used to determine relative efficiency of any column with any d_p or D_m. It has been found empirically that the coupling term of in **Equat. 45** can be replaced by ωv_{red}^n, where ω is a constant and n is an exponent of order $1/3$. Then **Equat. 45.** becomes

$$h = \frac{2\gamma}{v_{red}} + \omega v_{red}^n + \Omega v_{red} \qquad \textbf{Equat. 46.}$$

which is known as the Knox equation [29].

3.2.6. Efficient and faster separations

The plate height is affected by many factors, including flow velocity, particle diameter, packing uniformity, diffusivities, degree of retention, SP structure, temperature and pressure, whereas some of them are interdependent. To find a minimum with the respect of all these parameters is extremely complicated, but some simple rules for optimizing a few major parameters will be summarized in following text. For example **Equat. 38.** can be approximated by

$$H = \frac{B}{v} + C_m v + C_s v = \frac{B}{v} + Cv \qquad \textbf{Equat. 47.}$$

The H term can be simply adjusted by v, thus the longitudinal diffusion (B/v) term becomes very large at low v due to the inverse proportionality to v and the nonequilibrium (Cv) is large at high v by the direct proportionality. Therefore column efficiency will always be limited at low v by longitudinal diffusion and at high v by the mass transfer term. The minimum H at some intermediate velocity (v_{opt}) can be found by differentiating previous equation with respect to v and solving it which yields

$$v_{opt} = \left(\frac{B}{C}\right)^{1/2} \qquad \textbf{Equat. 48.}$$

and when this optimum v is substituted back into **Equat. 38.** the minimum H emerges

$$H_{\min} = 2(BC)^{1/2} \qquad \textbf{Equat. 49.}$$

The values obtained for v_{opt} and $H_{\min}$ clearly depends on the relative roles of MP (C_m) and SP (C_s) nonequilibrium terms [1]. In LC separations C_s can be modified more readily than C_m and in the most favorable case is reduced to negligible proportions ($C_s << C_m$). So former expressions $B = 2\gamma D_m$ and $C_m = \omega d_p^2 / D_m$ can be substituted into **Equat. 48.** and **Equat. 49.** resulting in

$$v_{opt} = \frac{D_m}{d_p}\left(\frac{2\gamma}{\omega}\right)^{1/2} \approx \frac{D_m}{d_p} \qquad \textbf{Equat. 50.}$$

and

$$H_{\min} = d_p(8\gamma\omega)^{1/2} \approx d_p \qquad \textbf{Equat. 51.}$$

the latter expressions can be written since γ and ω are of order unity. These equations show the order of magnitude of the optimum point for the case in which SP effects are negligible. There the $H_{\min}$ differs only by numerical constant from the d_p. Thus, the use of uniformly packed columns with minimal ω is important. Furthermore, d_p of the packing should be as small as possible [30]. Therefore the sub-2-µm particles are used in modern chromatography with advantages for both low plate height and high separation speed (increased v_{opt}). However, decreasing d_p has other implications related to need of increased pressure. The smaller particles the greater column back-pressure is induced according to Darcy's law, which shows the latter inversely proportional to the square of d_p:

$$\Delta P = \phi \cdot \frac{\eta \cdot l \cdot v_{opt}}{d_p^2} \qquad \textbf{Equat. 52.}$$

where η is the viscosity of the MP and ϕ the flow resistance [31,32]. Thus, the pressure drop applicable to a column is constrained to some maximum value by mechanical limitation. When SP nonequilibrium terms are dominant so that $C_s >> C_m$ and therefore $C \cong C_s$, the increased magnitude of C shifts v_{opt} to lower values and $H_{\min}$ to higher levels than found above in **Equat. 48.** and **Equat. 49.** This compromises the chromatographic performance and thus gives good reasons to decrease the SP effects. **Equat. 32** suggests that it can be done by reduction in the SP film thickness (d) or increasing diffusivity (D_s). The latter is accomplished by increasing temperature or choosing SP of lower viscosity, while these changes have to be consistent with other requirements of the separation (e.g. selectivity). HPLC and UHPLC systems are usually operated close to v_{opt} or above with assuming that somewhat "over-speeded" column will perform at lower H than the one "under-speeded" by the same difference. This is with regards to plate height

versus velocity plot. It has to be considered that additional peak spreading collectively called extracolumn effects can origin outside the column. These are results of finite sample volumes, spreading within the injection chamber, connecting tubing and detector. Clearly, extracolumn effects are reduced by using small sample sizes and keeping external volumes small compared to the volume of the chromatographic column, while the external volumes should be narrow and streamlined in configuration [33,34].

As pointed out earlier the effective separation requires large plate number N. This parameter can be simply increased, while a longer column is used, which is accompanied with proportional increase of the separation time. However, the time required to achieve separation rises dramatically, soon reaching impractical levels. The time required to get through the N plates is defined as

$$t = \frac{l}{Rv} = \frac{N}{R}\frac{H}{v} \qquad \textbf{Equat. 53.}$$

whereas t increases with N, any reduction of plate number will reduce t. For example, the use of a column with high relative selectivity $\Delta K / K$ for a critical pair leads to reduced N. Similarly, the utilization of MS detection allows to extract the analytical information from overlapping peaks, thus reducing the required R_s and N. The separation can be done faster if v is increased, which sets off a chain reaction of necessary adjustments. Firstly the pressure drop increases proportionally to v along with the plate height (for $v > v_{opt}$), which requires the column length to be increased to keep the needed N plates. The increased l means further increase in pressure drop. Due to the practical limits such as pump limitations and column bursting strength, the maximum separation speed is limited by the maximum pressure drop available [35]. The effect of particle size on the separation time can be shown, when writing **Equat. 53.** in reduced form

$$t = \frac{N}{R}\frac{h}{v_{red}}\frac{d_p^2}{D_m} \qquad \textbf{Equat. 54.}$$

where t is quadratically linked to d_p. Thus, the separation speed can be effectively increased, while decreasing of d_p. The well-packed columns with a minimal SP contribution to the H are needed, so that h versus v_{red} curves are as low-lying as possible. An optional way to decrease separation time involves the use of increased MP temperature above ambient temperature. This significantly affects all the parameters of a separation except the mass of the packing material in the column. Consequently changes in the column efficiency, selectivity and v_{opt} are observed. The dependence of H on the column temperature can be explained by its effects on the molecular diffusivities and the rate constants of the mass transfer kinetics [36]. Thus, the overall result depends on whether the effects of axial dispersion or those of the mass transfer resistances are the main contributors to zone spreading. However, the experimental practice shows that the v_{opt} value increases with temperature, while the $H_{\min}$ is affected insignificantly. Although controversial results concerning the effects on $H_{\min}$ have been reported [37], high-temperature LC (HTLC) is no longer recognized as a technique that can significantly improve column efficiency [38,39]. Nevertheless, HTLC leads to faster separations for a given $H_{\min}$ and thus, together with UHPLC, fits into the concept of efficient and rapid LC separations [38].

3.2.7. Stationary phases

SP has different effects on equilibrium according to intramolecular forces involved. The modern SP media generally consist of porous silica particles providing a large surface area. The particle sorbents used in traditional HPLC are typically ranging between 2.5-5 µm in diameter. However, silica-organic hybrid materials with particle sizes bellow 2 µm (sub-2-micron or sub-2-µm) are used increasingly, following the development of UHPLC instrumentation. For example porous ethylene bridged hybrid (BEH) material was recently developed by the group of professor Jorgenson [40]. In silica-organic hybrid materials the inorganic structure of silica gel is reinforced by organic polymeric material, resulting in higher physical

and chemical stability. Apart from columns packed with particulate sorbents there are special types of SPs packed in capillary and monolithic columns. In capillary columns the SP is arranged in a thin layer around the column's inner wall, analogously to planar chromatographic systems. Therefore, their inner diameter is narrower than in particulate columns, but they are much longer in order to provide sufficient surface area. This type of column is commonly used in gas chromatography and, at smaller scale in LC applications. The SP medium used in a monolithic column is prepared by polymerization of organic or inorganic monomers, resulting in a single piece of porous cross-linked polymer or silica. In such material the MP flows through macropores with lower back pressure than through particulate sorbents, while mesopores provide sufficient surface area for efficient separation. However, the benefit of monolithic columns for LC/MS applications is limited due to the use of relatively high flow-rates.

3.2.8. Reversed-phase liquid chromatography

Tswett's MP was nonpolar liquid, hence his method is called normal-phase LC. While, the nonpolar liquid is exchanged for more polar one the technique is denoted as reversed phase (RP) LC instead [1]. It is the most versatile type of LC separation, which utilizes a polar MP (e.g. water) and a nonpolar SP, such as *n*-octyl (C_8) or *n*-octadecyl (C_{18}) hydrocarbon chains chemically-bonded to the solid silica surface [41]. Partitioning into such a one molecule thick layer is affected by the nearby surface, especially since the configuration and motion of each attached molecule is restricted by its fixed anchor to the surface. The separation mechanism is based on hydrophobic interactions between the analytes and non-polar functional groups of the SP. The bonded SP is attached via reaction with an organochlorosilane, thus some of the silanol groups are derivatized [42]. However a great number of silanol groups remain unreacted and available for interactions with analytes. This causes distorted peak shapes and excessive tailing, particularly for basic compounds. The majority of modern RPLC SP media are formed by a reaction with trimethylchlorsilane to reduce the number of free silanol groups. This

procedure, so-called “endcapping” [43], also improves column chemical stability [44]. The activity of silanol groups can also be modified by adjusting the MP composition and pH. Therefore, organic amines such as triethylamine, trioctylamine and cetyltrimethylammonium salt are used as MP additives, due to their high affinity to silanols. The number of ionized silanols is also decreased by using an MP with low pH, providing another way to mitigate their undesirable effects. Indeed, acidic aqueous MP is frequently used in LC-MS separations to improve ionization of analytes in the ion source.

3.3. Mass spectrometry

Mass spectrometry (MS) is an analytical technique in which atoms or molecules from a sample are ionized, separated according their mass-to-chage ratio (*m/z)*, and consequently recorded into mass spectra [45]. It originates in the works of English physicist Joseph John Thomson awarded the Nobel Prize in physics (1906) for discovery of the electron. His student Francis William Aston, also Nobel Price laureate (1922), build the first mass spectrometer based on experimental instrumentation developed by Thompson [46]. The use of MS has greatly increased due to the association with gas chromatography since the 1960s when volatile organic mixtures were first analyzed by GC-MS. Since the mid-1970s, HPLC had been coupled with MS to analyze involatile organic mixtures. The great effort invested in the development of ionization techniques for MS instruments culminated in the 2002 Nobel Prize in chemistry awarded to John B. Fenn [47,48] and Koichi Tanaka [49] for the development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules [47,48,50]. This was in consequence of widely used “hyphenated” techniques enabling routine use of MS, in conjunction with separation techniques such as liquid chromatography and electrophoresis. Particularly LC/MS become an important technique in the analysis of biological macromolecules such as nucleic acids [51,52], proteins [53-55], carbohydrates [56,57] or lipids [58-60]. A mass spectrometer typically consists of the ion source, the mass analyzer and the detector. Since many ion sources, the

analyzer and the detector operate at low pressure (typically 10^{-3}-10^{-10} Pa), an efficient pumping provided by a rotary vacuum pump combined with a turbo-molecular pump is required. Modern MS systems are controlled by a computer, which is also used to record and process acquired MS data [50].

3.3.1. Ion source

The process of ionization takes place in the ion source, where molecules of analyte are changed into the molecular ions and transferred into gas phase, whereas only the gas phase ions can be introduced into the mass analyzer. The ion sources can be simply sorted according to i) operating pressure or ii) energy applied to the molecule. The typical ion source operated at atmospheric is electrospray ionisation (ESI), desorption electrospray ionisation (DESI), atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI). On the other hand chemical ionization (CI), electron ionization (EI), laser desorption/ionization (LDI) and matrix-assisted laser desorption/ionization (MALDI) sources requires vacuum. Second criterion reflects the degree of analyte fragmentation during the ionization and gas phase transfer. The more energy is applied to the molecule the more extensive fragmentation is observed. Thus “soft” ion sources (APCI, APPI, CI, ESI, DESI, LDI and MALDI) mainly produce intact molecular ions and some of them can be used for the analysis of large fragile biomolecules [61] or even noncovalently bound complexes [62]. Conversely, the intense fragment ions along with the molecular ion are produced in EI. Since ESI was used during the studies this doctoral thesis is based upon (supplement III-IV), it will be discussed in more details.

3.3.1.1 Electrospray

The development of electrospray ionization (ESI) for MS is credited to John Fenn at Yale University in 1984 [63]. Since Fenn reported the detection of multiple-charge ions in 1988 [64], ESI become a breakthrough in the LC-MS coupling, replacing other contemporary interfaces and ionization techniques such as thermospray [65] and particle beam interfaces [66]. Nowadays the hyphenated

techniques based on ESI-MS are used in a number of research and routine applications, e.g. chemistry, biology, biochemistry, medicine and pharmacy [67,68]. ESI-MS produce ions by atomizing a solution of the analyte in electric field, when the solution is nebulized and the droplets that contain the analyte are desolvated by mild heat and a flown of drying gas, so called desolvation or auxiliary gas (typically high-purity nitrogen). A fine spray is generated by flown of nebulizer gas through a narrow-bore needle (typically 0.1-0.3 mm) to which a potential of 3-5 kV has been applied. Then the charged droplets evaporate to a point, where the number of electrostatic charges on the surface become so large relative to the droplet size that an explosion occurs to produce a number of smaller droplets (Coulombic explosions). The process repeats until the quasi-molecular ions are released from the droplet (ion desorption) or all the solvent has evaporated to leave the ions in the gas phase (ion evaporation). The generated ions are accelerated into a conventional mass analyzer (mostly a quadrupole) [46]. ESI-MS interface will also have a curtain gas, usually a flow of high-purity nitrogen across the orifice ensures a stable, clean environment for the sample ions entering the mass spectrometer. The gas curtain prevents air or solvent from entering the analyzer region of the instrument while permitting the sample ions to be directed into the vacuum chamber by the electrical fields. The presence of the solvent vapor or moisture in the analyzer region of the mass spectrometer contaminates the Q_0 rod set causing a reduction in Q_0 resolution, stability and sensitivity, and an increase in chemical background noise [46]. Thus, in order to prevent instrument contamination the curtain gas flow should be optimized at the highest possible setting that does not cause a significant reduction of signal intensity.

The charge in the positive ion detection mode results from the presence of the protons that are normally attached in solution. The macromolecular ions are usually highly charged, meaning that conventional quadrupole mass analyzer with *m/z* range up to 2000 is sufficient to detect ions of masses up to at least 50-100 kDa. In this case a series of peaks is observed, corresponding to the same molecule with different numbers of attached charges. ESI is useful for compounds

ranging from medium to very high hydrophilicity with molecular weights up to 100 kDa, though ionization of large (glyco)proteins and noncovalent biocomplexes up to 2 MDa has been reported [69].

3.3.2. Mass analyzer

Gas phase ions are separated in the mass analyzer according to their *m/z* rations by applying electromagnetic forces. In magnetic-sector mass spectrometers, ions are accelerated from the ion source at a fixed value of 1-10 kV into a tube of a fixed radius of curvature. The tube is subjected to a magnetic field with the direction of magnetic flux at a right angle to the path of the ions. Only ions of a single narrow *m/z* range will have the same radius of curvature as the tube and reach a detector at the end of the tube. The accelerated ion trajectory depends on the magnetic field strength, *m/z* and ion velocity [46]. Quadrupole (Q) and quadrupole ion trap (QIT) mass analyzers separate ions based on oscillations in an electric field created with a combination of radio frequency (RF) and direct-current (DC) potentials. For a given amplitude of a fixed ratio of RF (at fixed frequency) to DC, ions of narrow *m/z* range will oscillate in a stable trajectory and pass through a set of four poles positioned to form a hyperbolic cross section [46,50]. The fundamentals of Time-of-flight mass spectrometer (TOF) were describe in 1946 [70,71]. It uses no external force to separate ions of different *m/z* values. Ions are accelerated into a flight tube at a few hundreds to several thousand volts (as high as 30 kV). The ions have different velocities based on their *m/z*, thus the time difference between the signal start (e.g. acceleration of the ion) and the pulse generated when the ion hits the detector is registered. TOF based instruments provide very high scanning speeds (500 Hz over 500 *m/z* range), high sensitivity, which decreases with increasing molecular weight and fair dynamic range. Their *m/z* range is theoretically unlimited and detection of macromolecules up to 2.2 MDa has been reported [72]. The ion cyclotron resonance (ICR) and the Orbitrap mass spectrometer measure specific frequency corresponding to certain *m/z* ratios and the transient is Fourier transformed to create the mass spectrum.

The ions trapped in ICR by a strong magnetic field move in circular orbits (cyclotron motion) in a plane perpendicular to the magnetic field. They are held in the cell by electric potentials applied to the trapping plates that are perpendicular to the magnetic field. Mass analysis is accomplished by the application of a radio frequency electric potential to the transmitter plates to cause trapped ions to be excited into large circular orbits. When the excited ions pass near the receiver plates, the frequency is detected as an induced current. The frequency of the motion of an ion is inversely proportional to its mass. ICR instruments are capable of extremely high resolving power [46]. The Orbitrap analyzer developed by Alexander Makarov is the latest in ion trapping devices [73,74]. It has been commercialized recently as part of a tandem-in-space mass spectrometer using a linear QIT developed by Thermo Electron (San Jose, California) and introduced at the 2005 ASMS Meeting on Mass Spectrometry and Allied Topics (San Antonio, Texas June 5-9). The Orbitrap uses static electric fields, whereas Q and QIT use a dynamic electric field typically oscillating at ~1 MHz. The Orbitrap's axially symmetric electrodes create a combined "quadrologarithmic" electrostatic potential [75]. The radial, angular and axial frequencies are all *m/z* dependent, but the axial frequency is completely independent of the energy and spatial spread of the ions in the trap, therefore suitable for accurate *m/z* determination. The high resolving power is due to ability to accurately define the trapping field. Ions are accumulated, cooled and pulsed into the trap, where they start coherent axial oscillations without any additional excitation. All ions have exactly the same amplitude, but ion packets of different *m/z* will execute their axial oscillations at their respective frequencies, while a current induced due to motion along the *z* axis is detected [46].

The brief overview presented in this section pointed out some of the key parameters of contemporary mass analyzers, such as mass resolution and accuracy, *m/z* range, sensitivity, dynamic range or scanning speed. However, only the triple-quadrupole (QqQ) mass analyzer will be further discussed since intimate understanding of its principles and advantages is important for studies underlying this dissertation (supplement III-IV).

3.3.3. Triple-quadrupole

The QqQ mass analyzer was developed at Michigan State University by Rick Yost and Chris Enke based on discussions with Jim Morrison's group at La Trobe University in Bundoora, Victoria, Australia [76]. However, the term "triple-quadrupole" is misleading since it implies that there are three transmission quadrupole mass spectrometers in tandem. In fact, there are only two quadrupoles (Q_1 and Q_3) separated by a collision cell (q_2). Q_1 and Q_3 perform as mass filters, since at given values of DC and RF potentials, only ions within a certain narrow *m/z* window will have stable trajectories through the Q rod set. The other ions will collide with the rods and thus never reach the detector. The collision cell is usually an octopole or a hexapole and contains a collision gas at pressure of 10^{-1} Pa. The collision gas is an inert gas (He, Ar, Xe or N_2) causing inelastic collisions with the precursor ion to bring about collision activation or collision induced dissociation (CID) [46]. QqQ instruments are generally characterized by high sensitivity (femtomole/attomole), high dynamic range (linear responses commonly across five orders of magnitude) and high scanning speeds (around 10 000 Da/sec) making this instrument excellently suitable for quantification. Weak spots of QqQ are low resolution (typically 3000-5000 FWHM), low mass accuracy (±0.2 Da) and a relatively limited *m/z* range (typically 25-2000 *m/z*) [50].

3.3.4. Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is a mass spectral technique in which ions are cause to change mass via decomposition, but can also form a heavier product from collision with a "reactive" neutral. Thus, may be produced an information structurally related to analyte and not obtainable from an initial precursor ion. This is carried out either using multiple *m/z* analyzers (tandem-in-space) or using ion trap mass spectrometers (tandem-in-time). QqQ mass spectrometer is typical example of tandem-in-space instrument capable to provide four basic types of MS/MS analyses: i) product-ion analysis, ii) precursor-ion analysis, iii) common-neutral-loss analysis and iv) selected reaction monitoring (SRM). The Q_1 and Q_3 tandem mass analyzers connected through a q_2 can either scan within a

defined *m/z* range or are fixed at a certain *m/z*. For example, during product-ion analysis the Q_1 is fixed at one certain *m/z* to isolate precursor ion and a full mass spectrum of the fragment ions is recorded after fragmentation in q_2 and passing through the scanning Q_3. When precursor-ion analysis is performed the Q_1 scans all precursor ions and Q_3 is set to allow only a single *m/z* value to reach detector, after fragmentation in q_2. This scan mode is useful for finding compounds that produce a common fragment. The common-neutral-loss analysis means that the Q_1 and Q_3 both scan at a fixed *m/z* difference, which is used to find compounds that lose a common neutral species. SRM is similar to selected-ion monitoring (SIM), but allows for much higher degree of specificity due to two-step mass filtering. In SIM only the ion current at one or few selected *m/z* values is allowed to pass though Q_1 and Q_3 without any CID occurring in q_2. Similarly, in SRM Q_1 is fixed to allow only ions of given *m/z* value to pass into q_2, but then CID with the collision energy ranging from 10-100 eV is applied and Q_3 is set to allow only product ions of specific *m/z* value to reach the detector. This means that the precursor ion selected by Q_1 must undergo a selected transformation or reaction to result in a response from the detector. The absence of scanning provides relatively long time periods to focus on the precursor and product ions, thus increasing sensitivity. Recently published comparison showed that QqQ in SRM gives 20-fold higher sensitivity for most compounds then Q-TOF or QIT. It also had a linear dynamic range of at least three orders of magnitude, opposed to one and two orders of magnitude achieved by Q-TOF and QIT, respectively [77]. However, the QqQ as a scanning device achieves lower sensitivity in full mass scan as compared to the Q-TOF and QIT. The term SRM applies regardless of whether there is more than one precursor ion and multiple product ions from a single precursor ion, just as SIM applies when there is more than one ion monitored. The term multiple reaction monitoring (MRM) was created by an instrument manufacturer to distinguish their instruments from instruments of other manufactures. Since this term is misleading as to what is actually being monitored in the mass spectrometer and it should not be used in the context of QqQ [46].

3.3.5. Ion detection

After the ions are *m/z* analyzed by mass spectrometer, their energy is converted into a current signal by a detector. For example, the energy from the impact induces the emission of secondary particles such as electrons or protons. The current signal is then registered by electronic devices and transferred to the computer. The dynode provides secondary emission to amplify the signal, when positive or negative ions, as well as fast-moving neutrals impacts on its surface. The dynode is an element in an electron tube called electron multiplier (EM). In Q and QIT mass spectrometer, higher-mass ions strike EM with less velocity than lower-mass ions, which can result in mass discrimination. Thus, high-energy dynode is used to accelerate the ions, so that the secondary emission caused by the impact of the ion on the EM's first dynode is more equal regardless of its mass. A discrete-dynode EM consists of a series of dynodes connected via resistors, so that the process of secondary emission is repeated on 10-20 stages through several generations of emitted electrons. The continuous-dynode EM uses a continuous surface of lead-doped glass that produces the same effect at lower operating voltage (<2 kV). The microchannel plate (MCP) is an array of 10^4 to 10^7 continuous-dynode EM (10-100 μm diam), each acting as an independent emitter to form a spatially resolved array detector. The advantage of an MCP is that it provides short transit times, but on the other hand an electron cascade in one channel drains the neighbouring channels for several microseconds, causing nonlinear responses due to saturation effects. The detectors are often coupled together in order to increase the gain or dynamic range of the instrument [46,50].

3.3.6. Hyphenated techniques

The term "hyphenated methods" was first coined by Hirschfeld [78], when the hyphen was the interface between two instruments, one being ideally suited for quantitation and the other being ideally suited for qualification [46]. Since the advent of coupling through ESI interface, the analytical technique of LC/MS was accomplished with an instrument that is both a liquid chromatograph and a mass spectrometer (LC-MS). This hyphenated technique is increasingly used in various

scientific fields. Next milestone for the LC/MS was the introduction of UHPLC, especially due to UHPLC-QqQ coupling. The contemporary QqQ mass spectrometers are capable of SRM analysis with extremely rapid duty cycles (<5 ms). Thus, the sufficient number of data points over single chromatographic peaks (7-12 points), can be recorded even for multi-component monitoring, while UHPLC is coupled. ESI is concentration-dependent and although can tolerate a flow-rate of >1.0 $mL.min^{-1}$, high flow-rates are likely to reduce signal/noise ratios. The optimum sensitivity is generally achieved at a few hundreds of microlitres per minute. Therefore UHPLC system, which employs relatively low flow-rates (typically 0.2-0.8 $mL.min^{-1}$) provides better conditions for atmospheric pressure operated ion sources, then conventional HPLC system. Nowadays, UHPLC/MS become a "method of choice" in pharmaceutical industry overwhelming conventional HPLC/MS, due to abovementioned advantages and higher separation efficiency.

3.4. Sample work-up strategy

Straightforward and convenient sample work-up is preferred with reference to the wide routine use. This can be afforded due to efficient LC separation and/or highly selective/sensitive MS detection. Additionally, analyzed polyphenols were present at appreciable levels ($mg.g^{-1}$/$g.L^{-1}$) in the natural samples (supplement I-III) or selectively ionized/desorbed prior the MS detection (supplement IV). Thus, mostly simple filtration through a membrane filter with 0.22 µm pores was used prior to LC-MS analysis (supplement I-III). Similarly straightforward, one-step sample pre-concetration/clean-up represented by solid phase extraction (SPE) was used in the last study (supplement IV). More complex analytical protocols has to be applied, when low concentrations of the target compounds are expected in the sample (<1.0 $\mu g.g^{-1}$ of dry weight) and/or the sample matrices is extremely complex. This is illustrated in some studies published by author, but not subjected to this doctoral thesis [79,80].

3.5. Polyphenols

Polyphenols (PPs) are intensively studied through decades mainly regarding their ability to scavenge excessive free radicals, such as reactive oxygen species (ROS, e.g. superoxide, hydroxyl and peroxyl radicals) and reactive nitrogen species (RNS, e.g. nitric oxide and peroxynitrous acid) [81,82]. The potent antioxidant activity of PPs minimizes oxidative stress damage of cellular proteins, lipids and DNA, thus protecting their biological functions [83]. The clinical studies, which have been summarized in recent review articles [84-88] brought the solid evidence that increased oxidative damage is associated with the development of most age-related degenerative diseases, such as cancer [84-86] and cardiovascular diseases [86-88]. The PPs were also reported to be acting protectively against inflammation [85,86]. The PPs are plant secondary metabolites falling into sub-classes of flavonoids, phenolic acids, coumarins, anthocyanins and stilbenes [89], which are important micronutrients with the total intake about 1 g/day [90]. Although it is strongly dependent on the individual's dietary habits and can be enhanced using food supplements [88]. The main polyphenol dietary sources are fruit and beverages (fruit juice, wine, tea, coffee, chocolate and beer) and, to a lesser extent vegetables, dry legumes and cereals. Up to 200-300 mg of PPs per 100 g fresh weight are contained in fruits such as apples, grapes, pears and cherries, while a typical glass of red wine or cup of tea contains about 100 mg of PPs [88].

3.5.1. Flavonoids

Flavonoids are the largest group of PPs, since more than 6500 flavonoid products of secondary plant metabolism have been characterized in various plant species to date, but their total number is estimated at 8000 [91]. They are structurally related to a chroman-type skeleton linked to variously derivatized phenyl substituent at the C_2 or C_3 position. Thus, the basic skeleton contains three rings typically hydroxylated at positions 3, 5, 7, 3´, 4´ or 5´. The hydroxylic groups are frequently modified (methylated, acetylated, prenylated or sulphated) and the flavonoid

aglycones are in nature frequently associated with sugar moiety forming O- or C-glycosides [92]. Six flavonoid families are classified according to substitution pattern and oxidation degree of the central pyran ring embracing flavanols, flavonols, flavones, flavanones, isoflavones and anthocyanins (**Figure 1**) [89]. These natural compounds are considered a plant pigments, largely responsible for the colour of flowers, fruits and sometimes leaves.

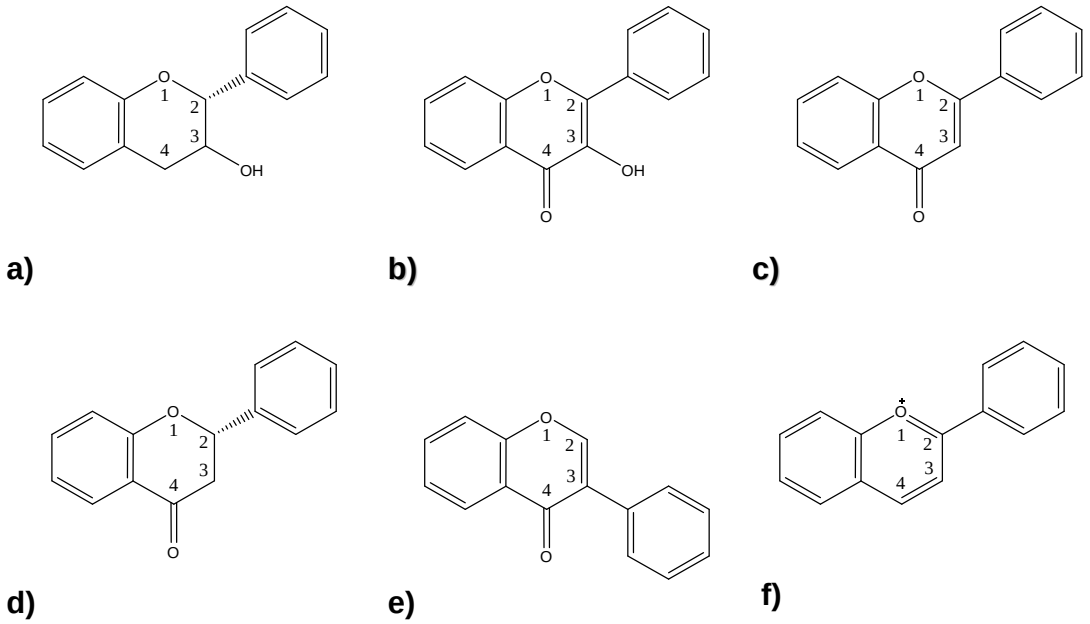

Figure 1. *Structural types of the most common flavonoid aglycones a) flavan-3-ol; b) flavonol; c) flavone; d) flavanone; e) isoflavone; f) anthocyanin.*

3.5.2. Phenolic acids

The phenolic acids (PHAs) are secondary plant metabolites, but they are structurally classified as either hydroxybenzoic or cinnamic acid derivates. Examples (frequently found in tea and herbal infusions) include gallic, caffeic, vanillic, ferulic and chlorogenic acids and their esters [93], **Figure 2**.

derivatives of benzoic acid	R1	R2	
gallic acid	OH	OH	
protocatechuic acid	H	OH	
vanillic acid	H	OH_3	
syringic acid	OCH_3	OCH_3	
derivatives of cinnamic acid	**R1**	**R2**	
caffeic acid	OH	OH	
chlorogenic acid	OH	OH	quinate
ferulic acid	OH	OCH_3	
4-coumaric acid	OH	H	
cinnamic acid	H	H	
coumarins	**R1**	**R2**	**R3**
aesculin	OH	glucose	H
scopoletin	OH	OCH_3	H
4-hydroxycoumarin	H	H	OH
6-methylcoumarin	H	CH_3	H

Figure 2. *Typical examples of phenolic acids and coumarins.*

3.5.3. Coumarins

Coumarins are also often found in phenolic fractions of plant extracts, and more than 1000 have been described in the plant family Fabaceae (which includes sweet clover and tonka bean). Although they share some of the structural characteristics with flavonoids, these 2H-1-benzopyran-2-ones (**Figure 2**) have substantially lower antioxidant activity [94,95].

3.6. Analysis of natural products

The PPs content in raw materials is determined by the cultivar type, growing conditions and climate. Accordingly substantial differences in PPs content can be found even among the cultivars of the same type, but grown under different conditions such as soil type or harvested in different time within the year [96]. Considering the complex role of many factors the great differences in PPs content are expectable. Talking about natural products the final PPs pattern is also strongly influenced by secondary factors including processing technology and storage conditions. Seeing that the PPs content is determined by external and internal factors, the knowledge in PPs content in the raw material and final product can be used to provide valuable qualitative information, e.g. about geographical origin, production technology or about the storage conditions. Such data are of great

importance for both consumers and regulatory authorities, therefore an appropriate analytical approach for their acquisition has to be established and validated. Since food production has become a tightly regulated field, cost-efficient and high-throughput analyses are highly desirable for profiling, process optimization and quality control. Tea and wine are typical examples of widely consumed natural products and are known to be rich sources of polyphenols.

3.6.1. Tea samples

Tea is technically aqueous infusion, simply made by steeping processed leaves, buds, or twigs of *Camellia sinensis* (L.) Kuntze (Theaceae) in hot water for a few minutes. It is, after water the most frequently consumed beverage worldwide. The tea infusions are rich natural sources of alkaloids (caffeine, theobromine, theophylline, theacrine, adenine and xanthine), phenolic compounds (phenolic acids, flavanols and flavonols), amino acids (L theamine), metals and vitamins (ascorbic acid) [97]. The polyphenolic fraction of tea contains flavanols (catechins), flavonols (myricetin, quercetin and kaempferol) and condensation products (proanthocyanidins). The tea leaves contain mainly (+)-catechin, (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECg) and (-)-epigallocatechin gallate (EGCg), constituting up to 30% of their dry mass [98]. The respective contents of catechins in tea infusions dependents on the processing methods applied to the raw material. Thus EGCg is preferential component of unfermented green tea [99], accounting for ca. 10-50% of the total green tea catechins. The antioxidant potential of EGCg greatly exceeds ascorbic acid (100-fold) and tocopherols (25-fold) [100]. After fermentation process (-)-catechin gallate (Cg) becomes the most abundant catechin, while the content of EGCg is approximately 10-fold decreased [101]. Beside catechins the small quantities of flavonols 3-O-glycosides are also present in tea leaves.

3.6.2. Wine samples

White and red wines are produced through the ethanol fermentation performed by yeast from grapes of *Vitis vinifera* (L.) Vitaceae. Red wine is made from the pulp of

red grapes that undergo fermentation together with the grape skins. White wine is made by fermenting juice prepared by pressing crushed grapes, while the skins are removed not playing further role during the process. Thus, white wine can be made from red grapes, as long as the minimal contact between the juice and the grapes' skins is accomplished during the juice extraction. Rosé wines are made from red grapes in case that the juice is in contact with the red skins for short time sufficient to cause pink/light red color, when the most of the tannins remains in the skins. This gives an example how the PPs content in the wine can be altered significantly during wine-processing. The wide range of more than 200 PPs classified as phenolic acids, flavonoids, stilbenes or anthocyanins have been identified in red wine, and grape seed extract [88]. Thus, red wine is the remarkable source of the natural phytoalexin *trans*-resveratrol, quercetin, delphinidin or (+)-catechin. These compounds has shown to have pharmacological properties, perhaps partially explaining the beneficial effects of moderate red wine consumption against coronary diseases [102]. Importantly PPs feature organoleptic properties of wine, for instance monomeric PPs, such as (+)-catechin, contribute to bitter taste, while polymeric tannins are highly astringent, or anthocyanins predominantly present in the red grape skin are responsible for the color of red wine [103]. The PPs and tannins comprise a significant proportion of the nonvolatile matrix of wine and affect the aroma volatility and perception. For instance the volatility of 2-methylpyrazine (which has a nutty odour) is significantly decreased by gallic acid, probably through specific π-π stacking stabilized by hydrogen bonds between the galloyl ring of phenolic acid and the aromatic ring of the odorant [104].

4. RESULTS AND DISCUSSION

(ANNOTATION OF PUBLICATIONS)

4.1. Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography

The main goal of this study (supplement I) was to assess advantages of UHPLC over conventional HPLC, while both systems were equipped with sub-2-micron particle columns. Thus, two novel chromatographic methods (UHPLC and HPLC) were developed for the analysis of representative groups of phenolic compounds (phenolic acids, flavonoids, catechins and coumarins) and these were compared in terms of system suitability test, retention time and peak area repeatability, peak symmetry, resolution, peak capacity and the column plate height. Although, the analytical columns with very similar particle diameters were used: C_{18} BEH (100mm×2.1mm, 1.7µm) for UHPLC and C_{18} silica-based (50mm×4.6mm, 1.8 µm) for HPLC, the same column length and internal diameter was not used with regards to the generated back-pressure. Therefore, this work should be understood as the comparison of two different LC platforms operated near the optimal conditions, rather than the comparison of two LC columns. The reason for this assumption is that well-packed sub-2-micron particle columns have its optimum performance ($H_{\min}$) at the linear velocities >2 mm.sec^{-1} [105], while here the UHPLC column is operated at 2.17 mm.sec^{-1} and the HPLC column at 1.00 mm.sec^{-1}. Obviously, it is unfair to compare the column performance, while the optimum velocity aspect is not addressed. However, the comparable linear velocities would cause substantial pressure drop, so the conventional HPLC system will no longer be capable to handle it. The results generally showed superior performance of UHPLC specifically: greatly enhanced retention time repeatability (factor 1.74-5.67); typically enhanced peak area repeatability (factor 0.64-2.81); greatly decreased the analysis duration (factor 2.6-7.5), associated with even greater drop of solvent consumption (factor 5.5-15) and slightly enhanced peak symmetry. Thus, while the similar chromatographic resolution for the groups

of phenolic acids, flavonoids and coumarins was provided by both LC methods, the peak capacity was enhanced by the factor 1.93-3.52 in the favour of UHPLC. On the other hand, when lower chromatographic resolution was accomplished for catechins in case of UHPLC, the peak capacity remained similar and the analysis duration was dramatically cut down. These results show two possible ways to go, if UHPLC is employed. First one is to maintain given chromatographic resolution and peak capacity, which leads to savings in the analysis duration. The second option is to reduce the analysis time maximally, while still being able to obtain good chromatographic parameters. The practical application of developed methods was demonstrated on four samples including a white grape wine, a red grape wine, a black tea and a green tea. Higher sensitivity and improved resolution of some critical pairs among phenolic acids, catechins and flavonoids allowed identification of more analytes in the UHPLC runs compared to HPLC.

4.2. Rapid qualitative and quantitative ultra high performance liquid chromatography method for simultaneous analysis of twenty nine common phenolic compounds of various structures

In the previous work (supplement I) the different groups of phenolic compounds were analyzed in separate chromatographic runs, however in this work (supplement II) twenty nine phenolic compounds comprising nine phenolic acids, sixteen flavonoids (including eight tea catechins, glycosides and aglycones), four coumarins and caffeine were analysed during single chromatographic run within 20 min. UHPLC system with PDA detection was equipped with C_{18} BEH analytical column (100 mm×2.1 mm, 1.7 µm) and the 0.1% formic acid:methanol mobile phase was eluted in the gradient mode. The profile of gradient elution was based on the experiences from previously published work (supplement I), ensuring good resolution between peaks of chlorogenic, vanillic and caffeic acid as well as epigallocatechin and catechin. These critical pairs are hardly separated during conventional HPLC analysis, due to very similar retention properties. The basic chromatographic parameters for all investigated phenolic compounds were assessed including resolution (>1.5), peak symmetry factor (0.84-1.17), peak capacity (typically 65.8), retention time repeatability (CV 0.09-0.52%) and peak area repeatability (CV 0.17-0.91%). Furthermore, the complete method validation was performed in the terms of standard calibration curve linearity (r^2 > 0.9990), calibration range (typically 1.0-100 $mg.L^{-1}$), LOD (typically 0.06 $mg.L^{-1}$), LOQ (typically 0.20 $mg.L^{-1}$), inter/intra-day precision (typically <3%/<9%) and inter/intra-day accuracy (typically 100±10%). Subsequently the method was employed for the identification and quantification of polyphenolic analytes and caffeine present in eight tea infusions and extracts. These included the non-fermented teas Kokaicha, Vietnam tea with jasmine and Formosa Gunpowder, the fermented teas Pu-Erh and Nepal SFTGFOP-Maloom, the mixture of non-fermented and fermented tea

leaves “Festival”, the fruit teas “Messina” and “Cinnamon-Apple” and the green tea extracts “GT ex. p.” (min. 80% catechins, max 8% caffeine) and “Ethyl Acetate Free Green Tea extract” (80% catechins/50% EGCg). It was found that non-fermented teas typically contained 7-9% (w/w) of catechins, while the highest total quantity was found in Formosa Gunpowder (93 $mg.g^{-1}$). The caffeine content in non-fermented teas was around 2% (w/w). Other phenolic compounds such as phenolic acids (gallic, chlorogenic, protocatechuic, vanillic, caffeic and ferulic) were present at concentrations around 1 $mg.g^{-1}$ and trace amounts of quercetin and rutin were found in some samples. The fermented teas and infusions from fruit mixtures contained much lower quantities of monitored phenolic compounds. The tea extracts with certified quantities of phenolic compounds were assayed to verify quantitative capability of developed method, while good agreement between both experimental and certified values was found.

4.3. Comparison of positive and negative ion detection of tea catechins using tandem mass spectrometry and ultra high pressure liquid chromatography

This work (supplement III) was focused exclusively on tea catechins, as an important commodity for the growing market of dietary supplements and functional foods. Notably, quantitative analysis of tea catechins is essential for frequent human intervention studies, manufacturers of dietary supplements and quality control authorities. UHPLC-ESI-MS/MS method partially based on the gradient elution profile published previously (supplement I) featured extremely high throughput. Thus, the chromatographic separation of eight tea catechins was accomplished within 2.5 min on C_{18} BEH analytical column (100 mm×2.1 mm, 1.7 μm), whilst the water:methanol mobile phase with addition of volatile organic acid was used. The concentration of organic acids in the mobile phase was optimized within the range of 0.01–0.1% (v/v). The lowest detectable on-column amounts were 10.2-16.8 fmol/inj. in positive and 102.1-168.1 fmol/inj. in negative ion detection mode by virtue of particular and detailed tuning of all important ESI-MS parameters. Some of them had minute effects on the signal intensity (e.g. capillary, extractor and RF lens voltage; cone gas), whilst the others were fundamental; e.g. cone voltage, desolvation temperature and nebulizing gas flow rate. Firstly, selected-ion monitoring (SIM) was optimized to obtain the intense response of precursor ion. Then the product-ion analysis was performed in both ion detection modes applying energies between 10-40 eV to the collision gas. The specific product ions for each monitored precursor were used to set up SRM transitions. Subsequently the collision energies were closely optimized and the transitions showing the highest ion counts number were utilized for the quantification. Observed fragmentation pattern was compared to literature and some interesting structural aspects of gas chemistry ions were briefly concluded in Figures 3 and 4. The optimized UHPLC-ESI-MS/MS method was validated in terms of standard calibration curve linearity (>0.9997; >0.9990), calibration range

(0.02-2.40 mg.L^{-1}; 0.15-24.00 mg.L^{-1}), LOD (3.0-4.8 µg.L^{-1}; 30.1-48.0 µg.L^{-1}), LOQ (9.9–15.8 µg.L^{-1}; 150.5-240.0 µg.L^{-1}), intra-day precision (CV 4.4-7.1%; 3.3-5.1%), accuracy (94.06-113.7%; 89.5-108.4%), retention time repeatability (0.00-0.54% RSD; 0.00-0.58% RSD), and peak area repeatability (1.16-3.98% RSD; 2.36-3.53% RSD) both for positive and negative ion detection modes, respectively. The quantitative measurements of positive and negative ion detection mode agreed within an experimental error, which was confirmed by *t*-test at 95% confidence level.

4.4. Matrix-less laser desorption/ionisation of polyphenols in red wine

The last published work (supplement IV) surpasses the borders of LC, although the UHPLC method previously published by the author (supplement I) was coupled to ESI-MS detection and used for confirmation of obtained results. The phenolic acids, such as ferulic acid are common matrixes in matrix-assisted laser desorption/ionisation (MALDI). Therefore, the author assumed that the shared phenolic structure is related to the propensity as an active MALDI matrix and it follows that direct laser desorption/ionisation (LDI) should be possible for polyphenols. Indeed LDI-MS was achieved whereby the analyte functions as a matrix and was used to monitor phenolic acids and *trans*-resveratrol in wine samples. The sufficient sensitivities (0.12–87 pmol/spoted on LDI-MS plate) were observed for eight phenolic acids (4-coumaric, 4-hydroxybenzoic, caffeic, ferulic, gallic, protocatechuic, syringic, vanillic) and 0.02 pmol/spoted for *trans*-resveratrol. The polyamide resin SPE cartridge was used as suitable clean-up, after conditioning with 3 mL of methanol followed by 3 mL of water. Then, two milliliters of each wine sample were passed through and the cartridges and washed with 3 mL of 0.1 M TFA. Finally, the elution step was performed using 2 mL of methanol:0.1 M TFA (80:20 w/w). In this way, 4-coumaric, 4-hydroxybenzoic, caffeic, ferulic, gallic, syringic, vanillic acids and *trans*-resveratrol were identified in four red grape wine samples (Cabernet Sauvignon and Merlot vintage 2007, two pairs from two different wineries). The wine samples were cross-compared using signal-to-noise (S/N) ratios of analytes. The conclusion was that there is generally higher content of PHAs in wines from Chile (factor 2-3), when geographical regions are compared and higher content of the most PHAs in Merlot wines, comparing varieties. Interestingly, the content of *trans*-resveratrol is significantly higher in Merlot compared to Cabernet Sauvignon wines regardless of geographical. Fast analysis together with facile sample pre-treatment makes the LDI-MS technique potentially appropriate for fingerprinting, screening and quality control purposes.

Furthermore, it can be used as complementary technique to LC-MS quantitative analysis. Thus, LC-MS separation may be employed or omitted with dependence on the LDI-MS results.

5. SUPPLEMENTS

5.1. Publications

This doctoral thesis is largely based on the following publications:

5.1.1. Supplement I (page 70)

Zdeněk Spáčil, Lucie Nováková, Petr Solich: Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography, Talanta 78 (2008) 189-199

5.1.2. Supplement II (page 82)

Lucie Nováková, Zdeněk Spáčil, Marcela Seifrtová, Lubomír Opletal, Petr Solich: Rapid qualitative and quantitative ultra high performance liquid chromatography method for simultaneous analysis of twenty nine common phenolic compounds of various structures, Talanta 80 (2010) 1970-1979

5.1.3. Supplement III (page 93)

Zdeněk Spáčil, Lucie Nováková, Petr Solich: Comparison of positive and negative ion detection of tea catechins using tandem mass spectrometry and ultra high pressure liquid chromatography, Food Chemistry 123 (2010) 535-541

5.1.4. Supplement IV (page 101)

Zdeněk Spáčil, Mohammadreza Shariatgorji, Nahid Amini, Petr Solich, Leopold L. Ilag: Matrix-less laser desorption/ionisation of polyphenols in red wine, Rapid Communications in Mass Spectrometry 23 (2009) 1834-1840

5.2. Publications not included in this doctoral thesis

1. Zdeněk Spáčil, Jana Folbrová, Nikolaos Megoulas, Petr Solich, Michael A. Koupparis: Simultaneous liquid chromatographic determination of metals and organic compounds in pharmaceutical and food-supplement formulations using evaporative light scattering detection, Analytica Chimica Acta 583 (2007) 239-245

2. Pavel Jáč, Petr Los, Zdeněk Spáčil, Marie Pospíšilová, Miroslav Polášek: Fast assay of glucosamine in pharmaceuticals and nutraceuticals by capillary zone electrophoresis with contactless conductivity detection, Electrophoresis 29 (2008) 3511-3518

3. Mohammadreza Shariatgorji, Zdeněk Spáčil, Gianluca Maddalo, Leopold L. Ilag: Matrix free TLC-LDI mass spectrometry for facile separation and identification of medicinal alkaloids, Rapid Communications in Mass Spectrometry 23 (2009) 3655-3660

4. Zdeněk Spáčil, Johan Eriksson, Sara Jonasson, Ulla Rasmussen, Leopold L. Ilag, Birgitta Bergman: Analytical protocol for identification of BMAA and DAB in biological samples, Analyst, 135 (2010) 127-132

5. Sara Jonasson, Johan Eriksson, Lotta Berntzon, Zdeněk Spáčil, Leopold L. Ilag, Lars-Olof Ronnevi, Ulla Rasmussen, Birgitta Bergman: Transfer of a cyanobacterial neurotoxin within a temperate aquatic ecosystem suggests pathways for human exposure, PNAS, 107 (2010) 9252–9257

6. Sandra A. Banack, Tim G. Downing, Zdeněk Spáčil, Esme L. Purdie, James S. Metcalf, Simone Searle, Maranda Esterhuizen, Geoffrey A. Codd, Paul A. Cox: Distinguishing the Cyanobacterial Neurotoxin β-N-methylamino-L-alanine (BMAA) from its Structural Isomer 2,4-Diaminobutyric acid (2,4-DAB), Toxicon (in press)

5.3. Oral presentations

1. Zdeněk Spáčil, Jana Folbrová, Nikolaos Megoulas, Petr Solich, Michael A. Koupparis: Stanovení hořčíku, vápníku a hliníku ve farmaceutických formulacích s využitím iontové chromatografie a ELS detekce, Students' Scientific Conference 2006, April 19th 2006, Faculty of Pharmacy, Charles University in Prague, Czech Republic, 15 min. (Czech)

2. Zdeněk Spáčil, Lucie Nováková, Petr Solich: Hodnocení polyfenolických látek metodou UPLC-UV-MS, Students' Scientific Conference 2007, April 4th 2007, Faculty of Pharmacy, Charles University in Prague, Czech Republic, 15 min. (Czech)

3. Zdeněk Spáčil, Mohammadreza Shariatgorji, Lucie Nováková, Leopold L. Ilag, Petr Solich: Health Promoting Polyphenols in the Sight of Mass Spectrometry, 18th International Mass Spectrometry Conference 2009, August 31st 2009, Bremen, Germany, 20 min. (English)

4. Zdeněk Spáčil, Lucie Nováková, Leopold L. Ilag, Petr Solich: UPLC-MS/MS Analysis of Health Promoting Polyphenols, 1st Nordic MS Summit 2009,Waters corporation, October 22nd 2009, Varberg, Sweden 30 min. (English)

5. Zdeněk Spáčil, Johan Eriksson, Sara Jonasson, Ulla Rasmussen, Leopold L. Ilag, Birgitta Bergman: LC-MS/MS Identification of BMAA in Cyanobacteria, Sixth International BMAA Conference 2009, Institute for Ethnomedicine, November 3rd 2009, Miami, FL, USA, 30 min. (English)

5.4. Poster presentations

1. Zdeněk Spáčil, Lucie Nováková, Petr Solich: Application of Ultra Performance Liquid Chromatography in Analysis of Phenolic Compounds, HPLC 2007, Ghent, Belgium

2. Lucie Nováková, Zdeněk Spáčil, Petr Solich: Advantages of UPLC in method development, HPLC 2007, Ghent, Belgium

3. Zdeněk Spáčil, Lucie Nováková, Petr Solich: Analysis of Polyphenolic Compounds using Ultra Performance Liquid Chromatography coupled with Tandem Mass Spectrometric Detection, Chiranal 2007, Olomouc, Czech Republic

4. Zdeněk Spáčil, Lucie Nováková, Petr Solich: Rapid Analysis of Tea Catechins using Ultra Performance Liquid Chromatography with Tandem Mass Spectrometric Detection, 19th International Symposium on Pharmaceutical and Biomedical Analysis 2008, Gdansk, Poland

5. Zdeněk Spáčil, Mohammadreza Shariatgorji, Nahid Amini, Ralf Torgrip, Petr Solich, Leopold Ilag: Analysis of Phenolic Compounds in Red Wine using LDI-ToF, Analysdagarna 2008, Goteborg, Sweden

6. Zdeněk Spáčil, Lucie Nováková, Petr Solich: Rapid Evaluation of Polyphenols in Tea Infusions Using Ultra High Pressure Liquid Chromatography and Tandem Mass Spectrometry 14th International Symposium on Separation Science 2008, Primosten, Croatia

7. Zdeněk Spáčil; Johan Eriksson; Leopold L. Ilag: LC-MS/MS identification of β-N-methylamino-L-alanine in Cyanobacteria Chromatography and Tandem Mass Spectrometry, 57th ASMS Conference on Mass Spectrometry 2009, Philadelphia, USA

8. Zdeněk Spáčil; Johan Eriksson; Sara Jonasson; Birgitta Bergman; Leopold L. Ilag: Identification of β-N-methylamino-L-alanine (BMAA) in Cyanobacteria, 12th EuCheMS International Conference on Chemistry and the Environment 2009, Stockholm, Sweden

9. Zdeněk Spáčil; Ly Jiang; Pawel Dziedzic; František Tureček; Armando Cordova: The SALDI-MS screening and LC-MS/MS identification of amino acids and amines from the Miller volcanic spark discharge experiment, 58th ASMS Conference on Mass Spectrometry 2010, Salt Lake City, USA

5.5. Supplement I

Zdeněk Spáčil, Lucie Nováková, Petr Solich:

Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography, Talanta 78 (2008) 189-199

Talanta 76 (2008) 189–199

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Analysis of phenolic compounds by high performance liquid chromatography and ultra performance liquid chromatography

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ABSTRACT

Two novel chromatographic methods both based on utilization of sub-2-micron particle columns were developed for the analysis of phenolic compounds in this work. An HPLC system was equipped with C_{18} silica-based analytical column (50 mm × 4.6 mm, 1.8 μm) and a UPLC system with ethylene-bridged hybrid C_{18} analytical column (100 mm × 2.1 mm, 1.7 μm).

In total 34 phenolic substances were divided into groups of phenolic acids, flavonoids, catechins and coumarins and were analysed in sequence using different gradient methods. System suitability test data, including repeatability of retention time and peak area, mean values of asymmetry factor, resolution, peak capacity and the height equivalent of a theoretical plate were determined for each gradient method by 10 replicate injections. The developed methods were applied in the analysis of real samples (grape wines, teas).

1. Introduction

In recent years considerable attention has been paid to natural substances with antioxidant activity, due to increasing incidence of serious pathologies such as cancer, cardiovascular diseases or inflammation. These disorders are caused, amongst other things, by the harmful effects of free radicals [1]. Free radicals can participate in the development of atherosclerosis and thus increase the risk of blood clot formation [2]. They can also accelerate the process of body tissue aging and they quite possibly have some relationship to the occurrence of diabetes, rheumatoid arthritis, Alzheimerĭs disease and Parkinsonĭs disease. Antioxidants are both natural and synthetic compounds able to scavenge free radicals and to inhibit oxidation processes. Antioxidants are a very large and diverse group of substances. These include: vitamins (A, B_6, C, E), zinc, selenium and phenolic compounds (namely phenolic acids and flavonoids) [3].

The phenolic acids are hydroxylated derivatives of either benzoic or cinnamic acid, their structures are depicted in Fig. 1. They are substances widely distributed in the plant kingdom and occur in a free state as well as combined into esters or glycosides. Especially the derivates of cinnamic acid are very common, for instance caffeic acid with its esters, ferulic acid or sinapic acid are the most frequent. Ferulic acid is often associated with food fibre (ester bond to hemicelullose). Chlorogenic acid (ester of caffeic acid) is broadly present in many kinds of fruit, vegetables and coffee.

Flavonoid compounds (also known as flavonoids) are an extensive group of plant phenols. They possess a basic structural element, the flavan (2-phenylchromane) skeleton and fall into different classes, e.g. flavonols (kaempferol, quercetin), flavan-3-ols (catechin), flavones (luteolin) or flavanones (naringenin). Natural flavonoids occur most frequently in the form of *O*-glycosides, which means, that they contain a non-sugar group in their molecule (aglycone) and a sugar group (monosacharides or oligosacharides). Structures of flavonoids used for the purpose of this work are given in Fig. 1. Catechins, sometimes classified as condensed tannins, are abundant in the tea leaves. They are indicating strong antioxidant activity. Some studies have proven, that the antioxidant activity of (−)-epigallocatechin gallate exceeds the effect of vitamin C at least 100 times [4]. Coumarins are 2H-1-benzopyran-2-ones substituted by hydroxyl groups often either methylated or engaged in a glycosidic linkage [5].

The recent methods dealing with the analysis of phenolics are summarized in review articles [6–8]. It can be seen that the high performance liquid chromatography (HPLC) occupies a leading position in the analysis of phenolics. In general, HPLC separations are based on C_{18} reverse-phased columns and a binary solvent gradient. The mobile phase usually consists of an aqueous solution of acid and an organic solvent (acetonitrile or methanol). Traditional HPLC is most frequently coupled with simple ultraviolet (UV) [9] or photodiode array (DAD) [10] detection, but HPLC applying a

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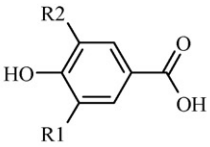
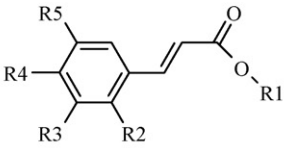
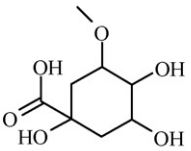
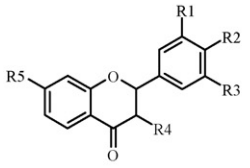


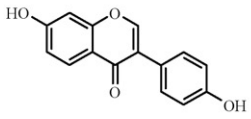
Benzoic type	R^1	R^2
Gallic acid	-OH	-OH
Protocatechuic acid	-OH	-H
Vanillic acid	$-OCH_3$	-H
Syringic acid	$-OCH_3$	$-OCH_3$



Cinnamic type	R^1	R^2	R^3	R^4	R^5
Chlorogenic acid		-H	-OH	-OH	-H
Caffeic acid	-H	-H	-OH	-OH	-H
p-Coumaric acid	-H	-H	-H	-OH	-H
Ferulic acid	-H	-H	$-OCH_3$	-OH	-H
Sinapic acid	-H	-H	$-OCH_3$	-OH	$-OCH_3$
o-Coumaric acid	-H	-OH	-H	-H	-H
Cinnamic acid	-H	-H	-H	-H	-H



Flavonoid	Bond type	R^1	R^2	R^3	R^4	R^5
Rutin	C_3=C_4	-H	-OH	-OH	-rhamnoglucoside	-OH
Naringin	C_3–C_4	-H	-OH	-H	-H	-rhamnoglucoside
Quercitrin	C_3=C_4	-H	-OH	-OH	-rhamnoside	-OH
Quercetin	C_3=C_4	-OH	-OH	-H	-OH	-OH
Hesperetin	C_3–C_4	-OH	$-OCH_3$	-H	-H	-OH
Kaempferol	C_3=C_4	-H	-OH	-H	-OH	-OH
Rhamnetin	C_3=C_4	-OH	-OH	-H	-OH	$-OCH_3$
Galangin	C_3=C_4	-H	-H	-H	-OH	-OH



Daidzein

Fig. 1. Structural types of analysed phenolic acids and flavonoids.

mass spectrometric (MS) detector has proven to be the method of choice, particularly in the identification of phenolics [11–14]. The electrochemical detection [15] provides in some cases additional selectivity, compared to the classical UV and DAD techniques. Apart from the HPLC methods, several electromigration methods such as capillary electrophoresis (CE) and micellar electrokinetic capillary chromatography (MEKC) have been also used [16–18]. These methods show higher efficiency, selectivity and speed compared to HPLC, but difficulties in sensitivity and reproducibility have been observed. Another possibility for the separation of phenolics is provided by gas chromatography (GC). The GC analysis can be performed with or without derivatization applying mainly MS detection. GC analysis without derivatization is suitable only for the identification of aglycones, but excellent selectivity and sensitivity was achieved using silylating agents.

There is a need for a method allowing simultaneous detection of a wide range of phenolics in a single analysis, in order to decrease the time necessary for the analysis of complex samples and reduce the analysis costs. This method should be able to characterize the occurrence of flavonoids and phenolic acids in various materials with sufficient selectivity and sensitivity during a short analysis time based on efficient separation. However recent methods often show weak points in one or more of these requirements. According to the table presented in a review article by Molnár-Perl et al. [7] the number of analytes detected simultaneously by most HPLC methods is usually around 10. Such number of phenolic compounds is usually satisfactorily separated in 45 or 50 min. The brief summary of more recent published separation methods in the field of simultaneous analysis of phenolics is presented in Table 1. On average, a similar figure was observed for the number of analytes (12), but the time of analysis was shorter (35 min).

In order to further increase the speed of analysis for phenolic compounds, a liquid chromatography system utilizing a sub-2-micron analytical column was used in this work. A conventional HPLC system and a ultra performance liquid chromatography (UPLC) system, equipped with columns containing similar stationary phases, were compared.

The conventional HPLC system was equipped with a Zorbax Stable Bond (SB) C_{18} analytical column packed with 1.8 μm silica-based particles containing diisobutyl groups in order to provide steric protection for the siloxane bonds. Acid labile end-capping reagents were not utilized. These adjustments improved column lifetime, as well as increased temperature and chemical stability in the pH 1–6 range. The use of this column in a conventional HPLC system was compared with a UPLC column containing hybrid sorbents based on BEH technology.

The main advantage of UPLC, in terms of separation efficiency, arises from the use of 1.7 μm, particles. This is in accordance with the Van Deemter theory [20]. As an LC technique, UPLC is still relatively new and has the potential for more pioneering research. It has enhanced sensitivity and separation power which results in decreased analysis time and solvent consumption. Using sub-2-micron particles provide maximum efficiency, leading to column back-pressures of more than 60 MPa, which are not achievable by conventional liquid chromatographic systems or columns [21]. UPLC systems allow work at extreme pressures, up to 100 MPa [22], due to the hardware adjustments.

The aim of this study was to compare HPLC and UPLC for the analysis of phenolic compounds. Both conventional HPLC, equipped with a sub-2-micron particle column, and a UPLC–UV method for determination of phenolic compounds was not published yet.

2. Experimental

2.1. Chemicals and reagents

The working standards of caffeic acid, cinnamic acid, ferulic acid, gallic acid, chlorogenic acid, *o*-coumaric acid, *p*-coumaric acid, protocatechuic acid, sinapic acid, syringic acid, vanillic acid, (+)-catechin (C), (−)-catechin gallate (Cg), (−)-epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCg), (−)-gallocatechin (GC),

Table 1
Recent methods for the simultaneous analysis of phenolic compounds

Method	Analytes	Run time (min)	Time per analyte (min)	Reference
HPLC–UV	Rutin; quercetrin; fisetin; myricetin; morin; luteolin; quercetin; apigenin; kaempferol; isorhamnetin; rhannetin; galangin	60	5	[9]
HPLC–DAD	Gallic acid; 3,5-dihydroxybenzoic acid; (−)-epigallocatechin; (−)-epigallocatechin gallate; (−)-epicatechin; (−)-epicatechin gallate; caffeine; (−)-catechin gallate; *p*-anisic acid; myricetin; 3,4,5-trimethoxycinnamic acid	25	2	[10]
HPLC–MS	6-*O*-Feruloylsucrose; 6-*O*-sinapoylsucrose; ferulic acid; sinapinic acid; *p*-coumaric acid; chlorogenic acid; caffeic acid; protocatechuic acid; hydroxybenzoic acid; vanillic acid; syringic acid	40	4	[11]
HPLC–MS/MS	Naringenin; genistein; kaempferol; apigenin; pinocembrin; galangin; acacetin; chrysin	35	4	[12]
HPLC–MS/MS	Gallic acid; protocatechuic acid; catechin; isorhoifolin; epicatechin; procyanidin; rutin; hesperidin; hyperoside; isoquercitrin; quercetin-*O*-pentose; naringenin-7-*O*-glucoside; rhamnetin-*O*-rutinoside; quercetin; luteolin; naringenin	20	1	[13]
HPLC–DAD–MS–FD–ED	Gallic acid; 5-HMF; protocatechuic acid; epigallocatechin; furfural; caftaric acid; *p*-hydroxybenzoic acid; *cis*-coutaric acid; catechin; caffeic acid; fertaric acid; epicatechin; vanillin; ferulic acid; *trans*-piceid; quercetin-3-glucuronide; quercetin-3-glucoside; *cis*-piceid; kaempferol-3-glucoside; quercetin-3-rutinoside; *trans*-resveratrol; *cis*-resveratrol; quercetin	80	3	[14]
HPLC–ECD	Gallic acid; protocatechuic acid; 4-hydroxybenzoic acid; 4-hydroxyphenylacetic acid; catechin; vanillic acid; chlorogenic acid; caffeic acid; syringic acid; *p*-coumaric acid; ferulic acid; sinapic acid; isoferulic acid; *o*-coumaric acid	50	4	[15]
CE–DAD	Protocatechuic acid; salicylic acid; *p*-hydroxybenzoic acid; vanillic acid; syringic acid; *p*-coumaric acid; ferulic acid; sinapic acid	4	1	[16]
CE–ECD	*Tert*butylhydroquinone; propyl gallate	7	4	[17]
MEKC–UV	Chlorogenic acid; syringic acid; ferulic acid; *p*-coumaric acid; vanillic acid; *p*-hydroxybenzoic acid; caffeic acid; cichoric acid; caftaric acid; 3,4-dihydroxybenzoic acid	35	4	[18]

(−)-gallocatechin gallate (GCg), daidzein, galangin, hesperetin, kaempferol, naringin, quercetin, quercitrin, rhamnetin, rutin, 4-hydroxycoumarin, 6-methylcoumarin, daphnoretin, aesculin, scopoletin, umbelliferone and caffeine used for the purposes of this study were purchased from Sigma–Aldrich (Prague, Czech Republic).

Mobile phase additive formic acid 98% p.a., LC–MS grade methanol (UPLC analysis) and HPLC grade methanol for the HLPC analyses were supplied by Sigma–Aldrich (Prague, Czech Republic).

HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and additionally filtered through a 0.22 μm membrane (UPLC), 0.45 μm for HPLC, respectively immediately before use.

The red wine, Blue Frankish 2005 vintage, was a product of Moravian Winery (Bzenec, Czech Republic) and the white wine, Kerner 2004 vintage, was manufactured in the winery Mikrosvín (Mikulov, Czech Republic). The green tea, Formosa Gunpowder and black tea, Nepal SFTGFOP-Maloom, were obtained from (OXALIS, Slusovice, Czech Republic).

2.2. Liquid chromatography instruments

A UPLC system Acquity (Waters, Prague, Czech Republic) consisting of a binary solvent manager and a sample manager was coupled to a tunable UV detector. All UPLC analyses were performed on a bridged ethylene hybrid (BEH) C_{18} analytical column (100 mm × 2.1 mm, 1.7 μm, Waters, Prague, Czech Republic). The analytical column was kept at 50 or 25 °C by column oven. The auto sampler stored run solutions at 4 °C. A partial loop injection mode with a needle overfill was set up, enabling 1.5 μL injection volumes when 5 μL injection loop was used. Mobile phase generated from 0.1% formic acid in water and methanol was mixed directly in the instrument. The flow rate was 0.45 mL min^{-1}. The UV detection wavelength was set at 280 nm, with a data acquisition rate of 40 Hz. Empower 2 software was used for chromatographic data gathering and integration of chromatograms.

An LC system consisting of a Waters 1525 binary HLPC pump, a Waters 717plus auto sampler and a Waters 2487 Dual λ Absorbance detector (Waters, Prague, Czech Republic) was utilized for HPLC analyses. The analytical column Zorbax SB C18 (50 mm × 4.6 mm, 1.8 μm, Agilent Technologies, Prague, Czech Republic) used was kept at 25 °C. The injection volume was 5 μL. The mobile phase contained 0.1% formic acid in water and methanol in a ratio dependant on the gradient profile, at a flow rate of 1.0 mL min^{-1}. The UV detector wavelength was set at 280 nm. Data acquisition was carried out by Breeze software.

2.3. Preparation of standard solutions

The stock solutions of standards were prepared with analytical accuracy dissolving 1 mg of standard substance in 1 mL of methanol. The working solutions for the standards used for the sensitivity comparisons, were prepared by diluting the stock solutions by a factor of 10, using methanol. These run solutions were also used for the HPLC system suitability test (SST) data measurement. UPLC SST run solutions were prepared by further dilution of the same stock solutions. The SST data run solution for phenolic acids was 50 times diluted, for catechins 80 times diluted, for coumarins and flavonoids 30 times diluted.

2.4. SST data measurement

Ten replicate standard solutions were injected into either the UPLC or HPLC systems. More diluted run solutions for UPLC than for HPLC were used in order to work in the linear detector range, according to the Lambert–Beer law. All HPLC analyses were performed at 25 °C. The UPLC analyses were performed at 50 or 25 °C. The R.S.D. data of retention times, peak areas and mean values of asymmetry and resolution were calculated for all components. The

rules for the measurements and the limits for the acceptance are given by appropriate guidelines [23] and pharmacopoeias [24,25]. The peak capacity values and the height equivalents of the theoretical plate were also calculated for each peak. Although the peak capacity is a more suitable tool to determine the quality of a gradient separation [26], height equivalents of the theoretical plate were presented as well in order to illustrate the correlation between both parameters.

2.5. Pretreatment of the samples

The wine samples were prepared without any extraction. They were filtrated through a 0.22 μm membrane and directly used for the injections. The tea infusions were prepared by pouring 100 mL of hot water (90 °C) over 1 g of dry tea mixture and 5 min of maceration with mild stirring. The then cooled down tea infusion was filtrated through a 0.22 μm membrane and was diluted, using methanol: water 50:50 (v/v) solution, by a factor of 2.

3. Results and discussion

Four groups of important phenolic compounds were chosen for the purpose of the study. The results presented in this work were obtained by comparing the methods of UPLC with HPLC using similar conditions and analytical columns with analogous particle size. The length of the analytical column for HPLC was 50 mm and for UPLC it was 100 mm. The reason behind this bipolar approach was the very high back-pressure in the HPLC system, generated by the column with the sub-2-microne particles, when keeping 100 mm column length. The UPLC system copes with this feature of sub-2-micron particle sorbents, but such a back-pressure is unacceptable for conventional HPLC system. Typical values of the back-pressures reached during the HPLC analyses, using 50 mm Zorbax analytical column, were around 30 MPa. Another reason for the usage of a short column was also the achievement of faster analysis.

The comparative data for the sensitivities of both systems were derived from the relationship between injection volumes and peak areas of the same concentrated solution.

In spite of the predominantly aqueous mobile phase, methanol was used as the solvent for the stock solutions. This was due to the poor solubility of some phenolic substances in water. The different solvent strength of methanol caused broadening and distortion of early eluting peaks. This phenomenon was observed despite using very small injection volumes.

3.1. Column temperature

The higher temperature (50 °C) was used during UPLC separations with the view of shortening the retention time and lowering backpressure and organic solvent consumption [27]. This high temperature was selected for the analyses because, in the case of phenolic acids, catechins and coumarins, it resulted in a high resolution of the chromatographic peaks. The negative effects on resolution were observed when the higher temperature was applied in the analysis of flavonoids. Due to these results, it was decided to keep lower temperature (25 °C). Higher column temperatures were applied to the HPLC analyses as well, but the impaired resolution for ionic compounds was evident. Therefore, the 25 °C column temperature was used.

3.2. Equilibration times

Both chromatographic methods were suitable for the analysis of phenolic compounds, but the UPLC method brought some advantages. It was not only faster, more sensitive and more efficient, but also a more reliable and ecologic method. The speed of the analysis was improved in two steps. Obviously the run time of analysis yielded first reduction, but secondly the equilibration time for bringing the column to the initial conditions after gradient analysis was much shorter. Between 10 and 15 min is recommended for returning a HPLC column to its equilibrated state, but according to our experiences, only 1.5–3 min were required for UPLC to regain its equilibrium. An equilibration time of 15 min was used for HPLC analyses. However, satisfactory results in the UPLC analyses were obtained using an equilibration time of 90 s for catechins and phenolic acids, and 3 min for flavonoids and coumarins. This disproportion was demanded by several factors. Firstly, methanol was used to flush out the system after these two gradient analyses, in order to prevent the retention of some residues on the column. Secondly, an increase of 50–60% of methanol was used in these gradient runs. Finally, the analysis of flavonoids was performed at a lower temperature.

3.3. Phenolic acids

UPLC analyses were performed 4.6 times faster than those by HPLC. Solvent consumption was decreased by a factor of 10. Injecting just 1.5 μL to the UPLC system decreased the peak area by half when compared to 5 μL injections into the HPLC, using the same concentrated solution. That means that about 1.7 times higher sensitivity was achieved with UPLC.

SST data comparing both methods are presented in Table 2. Retention time, peak area repeatability and symmetry factor values were significantly better for UPLC. The HPLC method indicated problems with the resolution of chlorogenic, vanillic and caffeic acid. Chromatographic peaks of these acids were partially overlapped. This same poor resolution of chlorogenic, vanillic and caffeic acid was not seen using the UPLC method. The separation

Table 2
SST data for phenolic acids

	SST parameters											
	Retention time (%R.S.D.)		Peak area (%R.S.D.)		Asymmetry factor		Resolution		Peak capacity		HETP (μm)	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Gallic acid	0.40	0.08	0.53	0.18	1.37	1.14	–	–	34.13	82.47	72.78	7.31
Protocatechuic acid	0.45	0.06	0.61	0.19	1.23	1.05	4.61	7.97	34.06	83.26	29.62	4.69
Chlorogenic acid	0.69	0.07	0.88	0.20	0.67	1.00	13.01	5.42	34.13	67.84	9.08	4.81
Vanillic acid	0.47	0.08	0.44	0.15	–	1.02	0.89	8.08	44.60	62.17	14.40	4.18
Caffeic acid	0.56	0.07	0.44	0.13	–	1.03	1.37	2.47	27.67	61.05	10.71	3.91
Syringic acid	0.44	0.08	0.22	0.16	0.88	0.91	4.39	3.02	31.02	51.84	5.01	3.98
p-Coumaric acid	0.39	0.07	0.49	0.26	0.87	1.02	6.74	9.78	30.96	44.78	2.61	3.98
Ferulic acid	0.27	0.07	1.02	0.26	0.86	0.99	5.17	7.58	37.95	39.36	1.34	3.74
Sinapic acid	0.23	0.09	0.46	0.28	0.83	1.01	2.97	1.56	28.44	41.15	0.92	3.33
o-Coumaric acid	0.20	0.05	0.60	0.31	0.85	1.03	6.06	12.30	39.37	113.33	1.08	0.27
Cinnamic acid	0.11	0.05	0.21	0.75	0.88	1.02	11.62	7.66	42.78	97.55	0.69	0.34

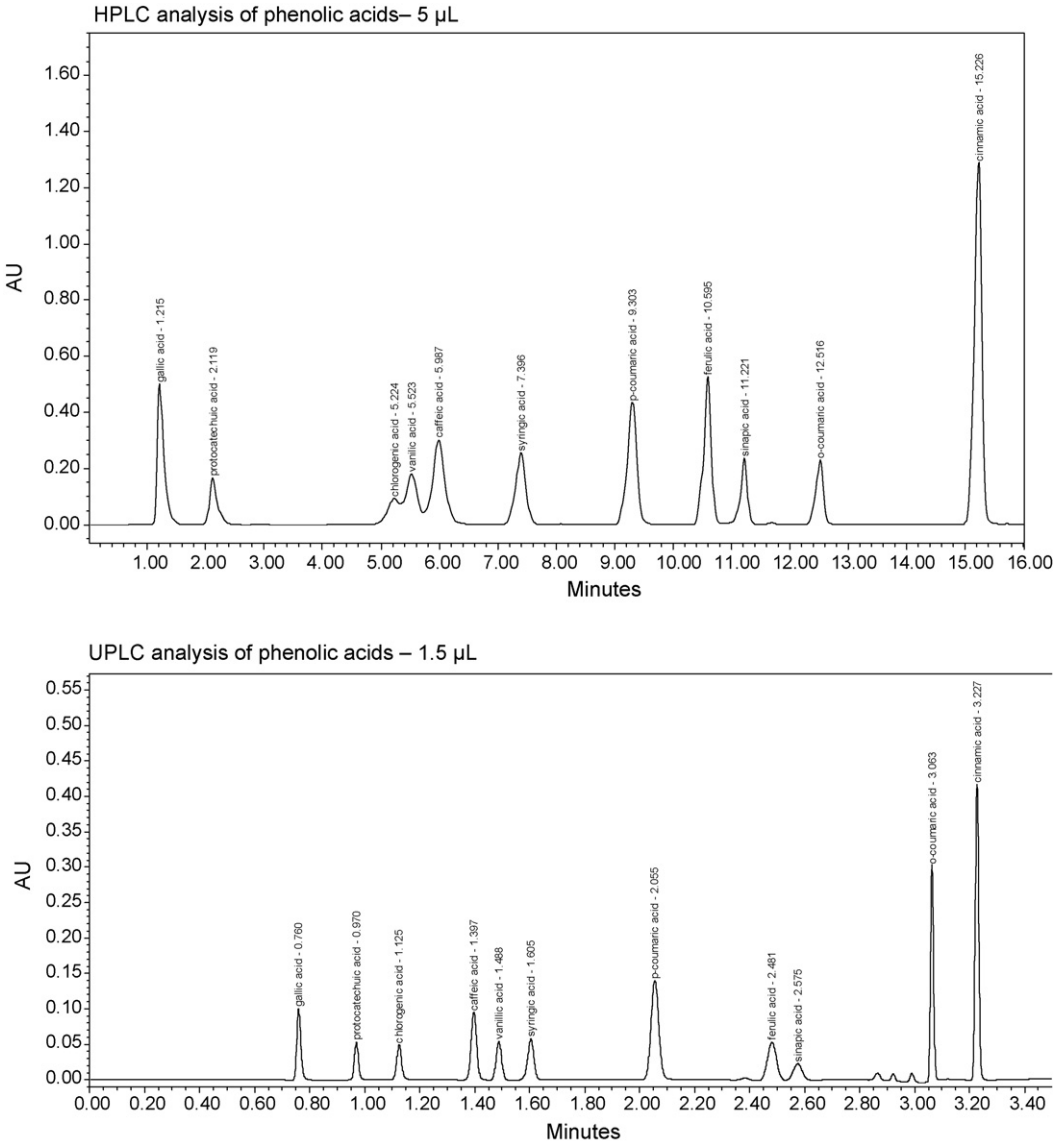


Fig. 2. Analysis of phenolic acids: HPLC, 0.1% formic acid–methanol, from 85:15 to 50:50 (v/v), 1.0 mL min^{-1}; UPLC, 0.1% formic acid–methanol, from 88.5:11.5 to 30:70 (v/v), 0.45 mL min^{-1}.

efficiency determined by peak capacity was higher for UPLC method.

There was found a difference in selectivity, since chromatographic peaks in HPLC conditions were currently eluting in the sequence of vanillic acid followed by caffeic acid. These peaks of vanillic and caffeic were switched over in the UPLC chromatogram (Fig. 2).

3.4. Catechins

The UPLC analysis time of the catechins was seven times shorter in comparison to the HPLC method. Solvent consumption for each individual analysis was reduced almost 16 times in this way, as can be seen in Fig. 3. Sensitivity of detection was 1.5 higher for UPLC. Peak retention time and the area repeatability parameters for UPLC

Table 3
SST data for catechins

	SST parameters											
	Retention time (%R.S.D.)		Peak area (%R.S.D.)		Asymmetry factor		Resolution		Peak capacity		HETP (µm)	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
GC	0.57	0.02	0.71	0.51	1.18	1.21	–	–	21.00	38.04	18.68	4.16
EGC	0.05	0.06	0.69	0.58	1.12	1.08	15.31	4.45	41.00	30.70	0.56	4.12
C		0.07		0.54		1.06		2.12		33.97		3.94
EGCg	0.03	0.09	0.76	0.42	1.19	1.09	9.05	2.45	31.00	26.66	0.40	4.19
EC	0.04	0.08	0.74	0.55	1.15	1.05	2.95	2.62	45.94	27.75	0.35	4.26
GCg	0.03	0.07	0.68	0.41	1.19	1.05	2.37	2.00	45.94	30.83	0.33	3.78
ECg	0.05	0.07	0.86	0.64	1.25	1.03	4.75	7.35	31.00	20.06	0.31	4.40
Cg	0.05	0.08	0.70	0.63	1.17	1.04	2.96	2.45	36.93	18.50	0.27	4.59

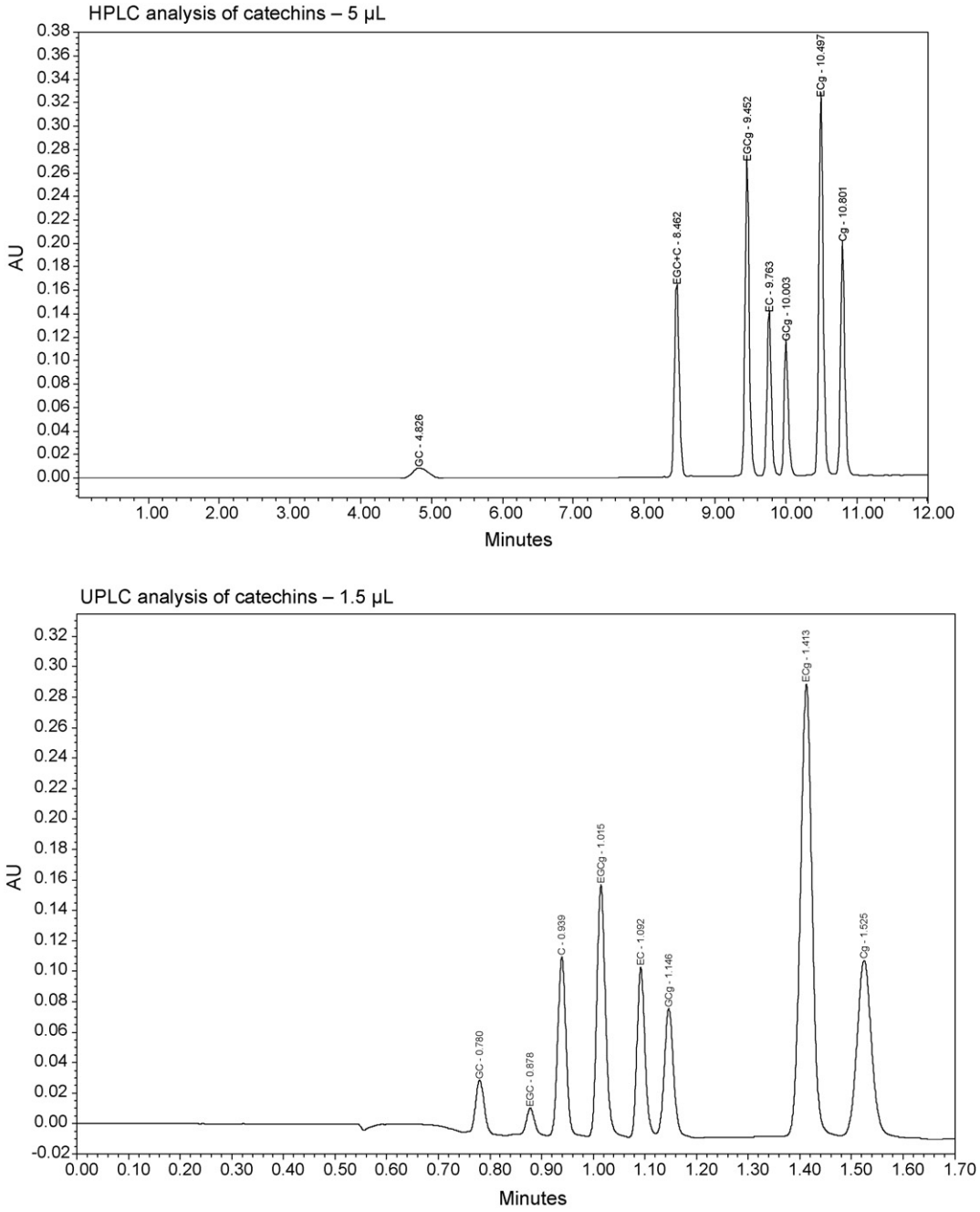


Fig. 3. Analysis of catechins: HPLC, 0.1% formic acid–methanol, from 95:05 to 40:60 (v/v), 1.0 mL min^{-1}; UPLC, 0.1% formic acid–methanol, from 88.5:11.5 to 60:40 (v/v), 0.45 mL min^{-1}.

analysis (Table 3) resembled the data gained for the HPLC method. Peak asymmetry values revealed less peak "tailing" in favour of the UPLC technique. The UPLC method has shown slightly lower peak capacity, probably due to the extremely fast analysis. On the other hand all peaks were separated by this method with satisfactory resolution, even the peaks of (−) -epigallocatechin and (+) -catechin. No separation was achieved using HPLC, even though several mobile phase modifications and different gradient methods were tested.

3.5. Coumarins

Using UPLC approach, the analysis time was three times shorter and the solvent consumption was decreased by a factor of 6.7 in comparison with HPLC. The sensitivity of the UPLC system was about twice as high. The use of 1.5 µL injection volumes caused a 1.7 multiple decrease in the peak area.

The comparison of the SST values is in Table 4. The UPLC analyses indicated excellent repeatability for retention time, but slightly worse repeatability for peak area. Other SST parameters such as asymmetry factor, resolution and peak capacity, were slightly superior for the UPLC analyses.

Despite of using the similar type of stationary phase, another change in selectivity was observed. The peak of daphnoretin, eluting at the end of chromatogram in HPLC analysis, moved ahead of 6-methyl-coumarin peak performing the UPLC method (Fig. 4).

Table 4
SST data for coumarins

	SST parameters											
	Retention time (%R.S.D.)		Peak area (%R.S.D.)		Asymmetry factor		Resolution		Peak capacity		HETP (μm)	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Aesculin	0.36	0.06	0.46	0.76	1.54	0.92	–	–	21.00	43.90	71.12	22.91
Umbelliferone	0.19	0.03	0.59	0.80	0.88	1.01	12.03	23.52	21.00	30.24	14.03	4.48
Scopoletin	0.18	0.04	0.59	0.70	–	0.96	1.31	2.30	18.14	34.33	15.67	2.89
4-Hydroxycoumarin	0.15	0.08	0.50	0.81	0.88	1.06	13.22	13.58	20.48	81.81	1.40	0.36
Daphnoretin	0.06	0.15	0.49	0.97	0.90	1.07	6.11	3.17	21.55	78.36	0.93	0.36
6-Methylcoumarin	0.11	0.10	0.41	0.71	0.99	1.06	4.99	2.59	31.00	69.42	0.45	0.42

3.6. Flavonoids

Analysis duration was 2.5 times shorter due to UPLC and solvent consumption was decreased by 5.5 times. The comparison of chromatograms can be seen in Fig. 5. The UPLC system also showed very good sensitivity (1.7 times higher), allowing injection of only 1.5 μL volume for reliable analysis results.

There are comparable values of retention time repeatability, peak area repeatability, asymmetry factor and resolution as well. The separation of rutin and naringin bears difficulties for both chromatographic methods. It was not possible to obtain satisfac-

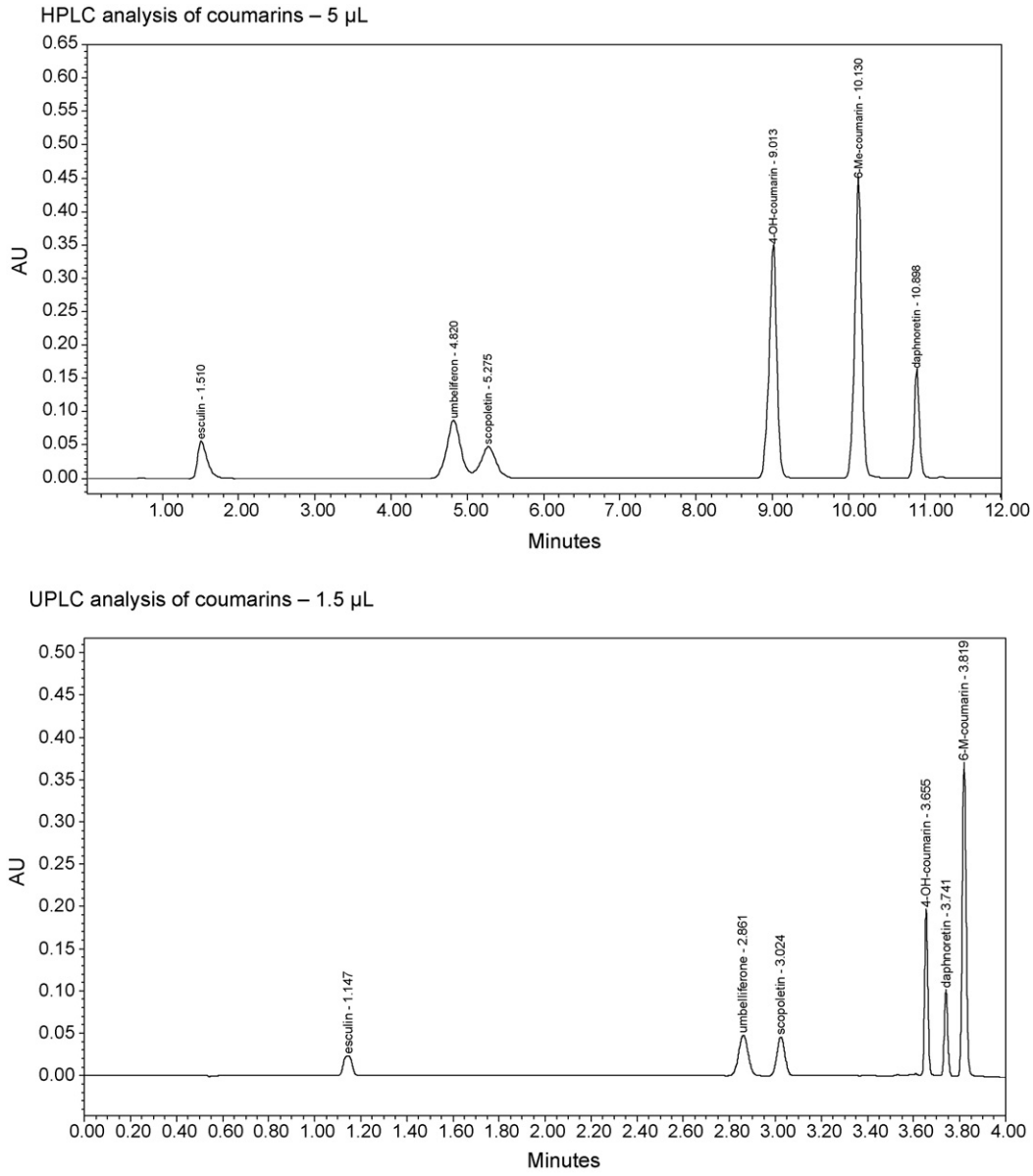


Fig. 4. Analysis of coumarins: HPLC, 0.1% formic acid–methanol, from 75:25 to 50:50 (v/v), 1.0 mL min^{-1}; UPLC, 0.1% formic acid–methanol, from 88.5:11.5 to 40:60 (v/v), 0.45 mL min^{-1}.

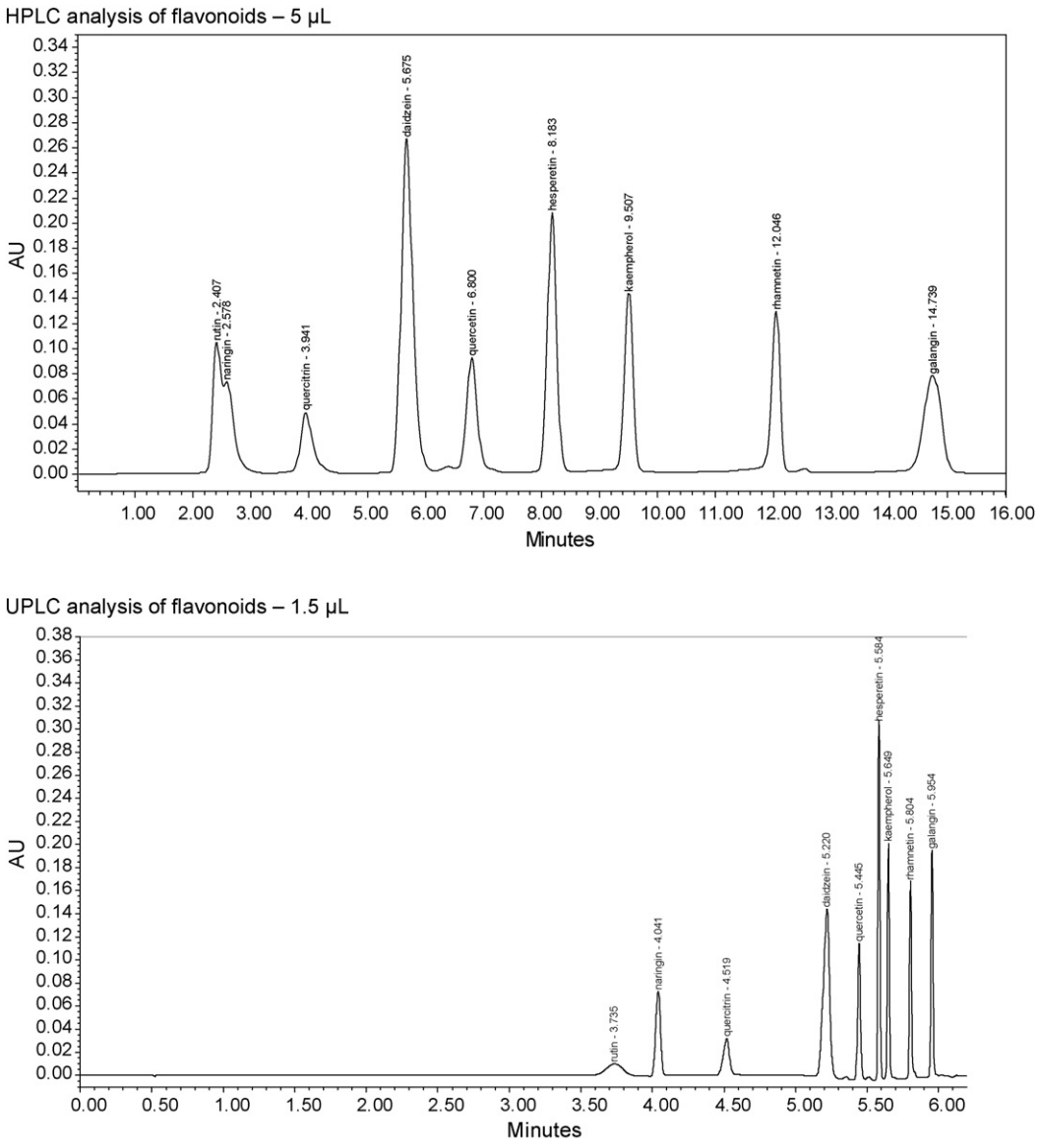


Fig. 5. Analysis of flavonoids: HPLC, 0.1% formic acid–methanol, from 60:40 to 40:60 (v/v), 1.0 mL min^{-1}; UPLC, 0.1% formic acid–methanol, from 69:31 to 10:90 (v/v), 0.45 mL min^{-1}.

tory resolution for these peaks using the HPLC method at all. In the UPLC method it was needed to use lower column temperature (25 °C) in order to reach acceptable chromatographic peak resolution. The UPLC system had significantly higher peak capacity number than the HPLC system. The first two peaks were excluded from this comparison, because of partial overlap in the HPLC analysis. The results of SST measurements could be seen in Table 5.

Table 5
SST data for flavonoids

	SST parameters											
	Retention time (%R.S.D.)		Peak area (%R.S.D.)		Asymmetry factor		Resolution		Peak capacity		HETP (µm)	
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC
Rutin	0.53	0.60	6.83	0.76	0.83	0.97	–	–	46.71	19.91	29.76	21.66
Naringin	0.66	0.21	8.36	0.84	–	0.97	–	2.06	29.22	58.71	–	2.80
Quercitrin	0.66	0.19	0.90	0.90	1.20	0.93	–	5.63	23.32	49.92	26.90	1.93
Daidzein	0.40	0.15	1.01	0.82	1.06	0.89	4.73	8.72	20.21	48.79	13.44	1.50
Quercetin	0.38	0.07	1.03	1.09	0.97	0.94	3.21	3.99	24.39	108.51	7.36	0.23
Hesperetin	0.26	0.02	1.02	0.83	0.93	0.95	4.29	4.73	30.09	155.30	4.33	0.13
Kaempferol	0.24	0.04	0.88	0.85	0.90	0.98	4.49	2.64	31.95	152.16	2.82	0.13
Rhamnetin	0.18	0.02	0.84	0.82	0.85	0.99	8.71	5.90	30.09	156.69	1.87	0.13
Galangin	0.22	0.02	1.04	0.90	0.99	0.99	5.74	5.82	20.21	152.31	5.46	0.12

Table 6
The analysis of grape wine and tea

	Blue Frankish (red wine) HPLC/UPLC	Kerner (white wine) HPLC/UPLC		Gunpowder (green tea) HPLC/UPLC	Nepal (black tea) HPLC/UPLC
Gallic acid	+/+	−/?	GC	?/+	−/?
Protocatechuic acid	+/+	+/+	EGC	?/+	?/+
Chlorogenic acid	−/−	−/−	C	?/?	?/?
Vanillic acid	+/+	?/?	EGCg	+/+	?/+
Caffeic acid	?/+	+/+	EC	+/+	?/?
Syringic acid	+/+	−/?	GCg	+/+	+/?
p-Coumaric acid	+/+	+/+	ECg	+/+	+/+
Ferulic acid	?/+	?/?	Cg	?/?	?/−
Sinapic acid	−/?	−/?			
o-Coumaric acid	?/?	−/−			
Cinnamic acid	−/−	−/−			

(+) detected; (−) not detected; (?) confirmation needed.

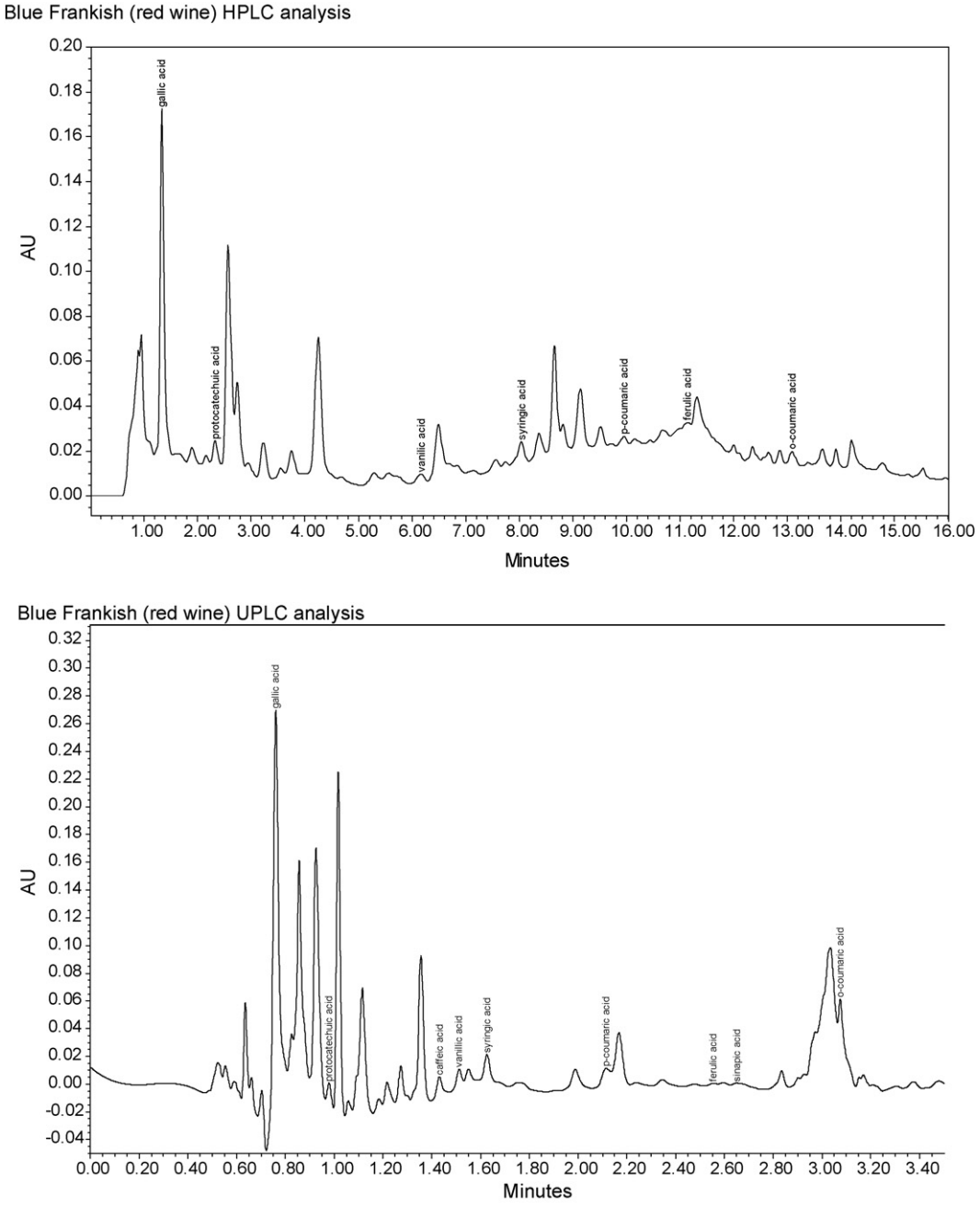


Fig. 6. Comparison of HPLC and UPLC method in the analysis of real samples.

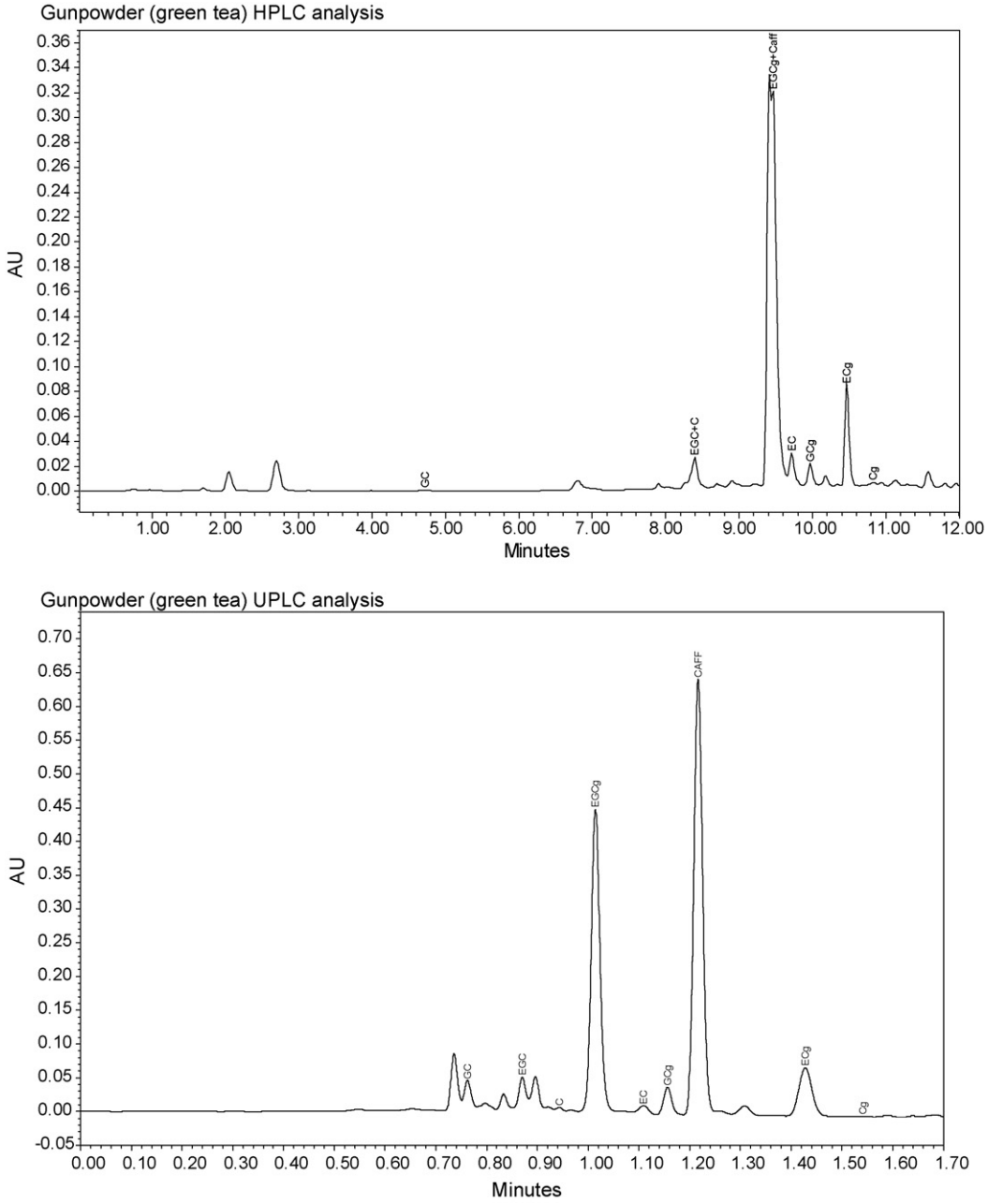


Fig. 6. (*Continued*).

Table 7
Comparison of analysis duration and solvent consumption

		Flow rate (mL min^{-1})	Analysis duration (min)	Solvent consumption (mL)	Time per analyte (min)
Phenolic acids	HPLC	1.00	16.0	16.0	1.5
	UPLC	0.45	3.5	1.6	0.3
Catechins	HPLC	1.00	12.0	12.0	1.5
	UPLC	0.45	1.7	0.8	0.2
Coumarins	HPLC	1.00	12.0	12.0	2.0
	UPLC	0.45	4.0	1.8	0.7
Flavonoids	HPLC	1.00	16.0	16.0	1.8
	UPLC	0.45	6.5	2.9	0.7

3.7. Analysis of phenolic acids in grape wines and catechins in teas

The real samples of grape wines and teas were selected in order to demonstrate the applicability of the methods for the analysis of real matrix samples. The results of the analyses are summarized in Table 6. Seven phenolic acids in red wine and three in white wine were reliably identified using the UPLC method. It is possible that other phenolic acids were observed. Nevertheless the confirmation using more selective detection is needed to be sure. The HPLC method enables to detect five phenolic acids, the presence of other two needs to be confirmed (Fig. 6).

In the matrix of the green tea infusion six catechins and caffeine were reliably identified. The remaining two catechins probably occur in minor concentrations. The black tea was a less abundant source of catechins, but in spite of this four catechins were identified. The presence of another three of them is suspected. On the other hand, the results obtained by the HPLC method are less informative due to the low sensitivity and in particular low separation efficiency. Only the peaks of GCg and ECg have good response and are sufficiently separated. The co elution of EGC and C was observed already on standard solution. Newly ascertained was the partial co elution of EGCg/caffeine and insufficient resolution of EC. The responses of GC and Cg were negligible (Fig. 6).

4. Conclusions

It is of course not possible to provide the strict comparison of HPLC and UPLC system, because there is more than one different parameter. The basic limitations are represented by the impossibility of the connection of both the columns to the same chromatographic system. The sorbent particle size and chemistry is different as well. On the other hand, the mobile phase constituents were the same and the gradient programs were constructed with respect to analysis speed and good resolution in both cases. The system suitability parameters gained for each method were compared. Generally, both analytical methods showed good results, but the UPLC system appeared to be superior.

The UPLC gradient method development brought a substantial saving in time (a factor of 2–8), which was needed for method optimization (Table 7) The average analysis time of one analyte using UPLC was only 30 s, whereas in the developed HPLC method it was approximately 90 s. Accompanying the minimal solvent consumption (5–18 times lower) UPLC was more ecological and had lower analyses costs. The peak capacity measured as the gradient run time, divided by the peak width showed higher values for three UPLC gradient analyses. Only the peak capacity in the case of catechins was somewhat higher for HPLC, but it was outweighed by better utility of the UPLC method in the analysis of real samples. It is necessary to underline the excellent retention time and peak area repeatability of the UPLC method, most importantly in terms of use in routine pharmaceutical analyses. The UPLC method met all criteria required for pharmaceutical analyses. The great precision indicated for UPLC was achieved despite the use of negligible injection volumes in partial loop injection mode. Considering the shorter column washout time, UPLC methods can be rated as "green" methods. The UPLC method was more sensitive, the area of the detected peak increased by a factor 1.5–2, when using the same concentrated solution.

UPLC is becoming a more widely spread analytical method, nowadays found in many laboratories around the world. The practical value of both UPLC and HPLC, was confirmed by analysing real samples. The narrow peaks gained using the UPLC approach enable the detection of analytes at very low concentrations and with high resolution, making it appear preferable to HPLC.

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5.6. Supplement II

Lucie Nováková, Zdeněk Spáčil, Marcela Seifrtová, Lubomír Opletal, Petr Solich:

Rapid qualitative and quantitative ultra high performance liquid chromatography method for simultaneous analysis of twenty nine common phenolic compounds of various structures, Talanta 80 (2010) 1970-1979

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Rapid qualitative and quantitative ultra high performance liquid chromatography method for simultaneous analysis of twenty nine common phenolic compounds of various structures

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ABSTRACT

Twenty nine phenolic compounds comprising nine phenolic acids, sixteen flavonoids (including eight tea catechins, glycosides and aglycones), four coumarins plus caffeine were analysed within 20 min using ultra high performance liquid chromatography (UHPLC) with PDA detection. UHPLC system was equipped with C18 analytical column (100 mm × 2.1 mm, 1.7 μm), utilising 0.1% formic acid and methanol mobile phase in the gradient elution mode. The developed method was tested for the system suitability: resolution, asymmetry factor, peak capacity, retention time repeatability and peak area repeatability. The method was fully validated in the terms of linearity ($r^2 > 0.9990$ for all 30 compounds), range (typically 1–100 mg L^{-1}), LOD, LOQ, inter/intra-day precision (<3% and <9% respectively) and inter/intra-day accuracy (typically 100 ± 10%). Subsequently the method was applied to the identification (spectral information and peak purity calculations were profited) and quantification of phenolic compounds and caffeine present in tea infusions and extracts.

1. Introduction

Phenolic compounds attract the attention of research laboratories and literature especially due to their biological and physiological importance. They are able to scavenge free radicals [1,2], specifically reactive oxygen species (ROS) and reactive nitrogen species (RNS, e.g. nitric oxide). The oxidative stress, caused by the excess ROS/RNS, can damage cellular lipids, proteins or DNA inhibiting their normal function [3]. In this way it is implicated in various pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia, aging and other diseases [4]. Flavonoids and phenolic acids are two main groups of phenolic compounds with antioxidant activity.

Flavonoids form a large group of structurally related compounds with a chroman-type skeleton and with a phenyl substituent in the C_2 or C_3 position. They are often hydroxylated in positions 3, 5, 7, 3′, 4′ or 5′ and their hydroxylic groups are frequently modified (methylated, acetylated, prenylated or sulphated). In plants are flavonoids often present as *O*- or *C*-glycosides [5]. They are further divided into classes depending on the oxidation degree of the central pyran ring [6]. More than 6500 different flavonoid products of secondary plant metabolism, mainly responsible for the colour of flowers, fruits and sometime leaves, have been characterized in various plant species, but their real number may exceed 8000 [7].

The *phenolic acids* are secondary plant metabolites derived from either benzoic or cinnamic acid (Fig. 1). For instance gallic acid, caffeic acid, vanilic acid, ferulic acid and chlorogenic acid with esters can be frequently found in tea, wine and herbal infusions [8].

Coumarins are often found in phenolic fraction of plant extracts. Presently over 1000 coumarins have been described in the plant families *Fabaceae* (sweet clover, tonka bean), *Apiaceae* (khella, angelica) and *Rubiaceae* (sweet woodruff). Antioxidant activity of these 2H-1-benzopyran-2-ones (Fig. 1) is in general lower [9,10].

Tea is a rich source of antioxidant phenolic compounds, especially polyphenols, and it is after water the most often consumed beverage worldwide. Therefore it was chosen as matrix for validation in our study. Tea is made by steeping processed leaves, buds, or twigs of *Camellia sinensis* (L.) *Kuntze* (*Theaceae*) in hot water for a few minutes. Tea infusion is a rich natural source of alkaloids (caffeine, theobromine, theophylline, theacrine, adenin, xanthine), phenolic compounds (phenolic acids, flavanols, flavonols), amino acids (L-theanine), metals and vitamins (ascorbic acid) [11,12].

The polyphenolic fraction of tea contains flavanols (catechins), flavonols (myricetin, quercetin, kaempferol) and products

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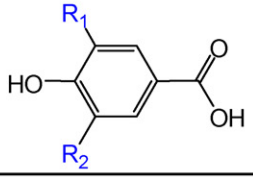
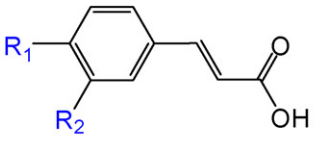
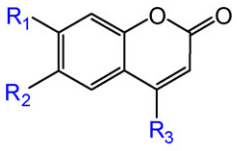
derivatives of benzoic acid	R1	R2
gallic acid	OH	OH
protocatechuic acid	H	OH
vanillic acid	H	OH_3
syringic acid	OCH_3	OCH_3



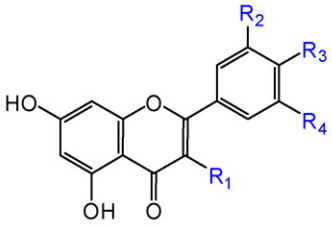
derivatives of cinnamic acid	R1	R2	Rx
caffeic acid	OH	OH	
chlorogenic acid	OH	OH	quinate
ferulic acid	OH	OCH_3	
o-coumaric acid	H	H	2´OH
cinnamic acid	H	H	



coumarins	R1	R2	R3
aesculin	OH	glucose	H
scopoletin	OH	OCH_3	H
4-hydroxycoumarin	H	H	OH
6-methylcoumarin	H	CH_3	H



Flavonoids	R1	R2	R3	R4
rutin	rhamno-glucose	OH	OH	H
myricetin	OH	OH	OH	OH
quercitrin	rhamnose	OH	OH	H
quercetin	OH	OH	OH	H
naringenin*	H	H	OH	H
luteolin	H	OH	OH	H
hesperetin*	H	H	OCH_3	OH
kaempferol	OH	H	OH	H



Catechins	R1	R2
gallocatechin (GC)	OH	OH
epigallocatechin (EGC)	OH	OH
gallocatechin gallate (GCG)	gallate	OH
epigallocatechin gallate (EGCG)	gallate	OH
catechin (C)	OH	H
epicatechin (EC)	OH	H
catechin gallate (CG)	gallate	H
epicatechin gallate (ECG)	gallate	H

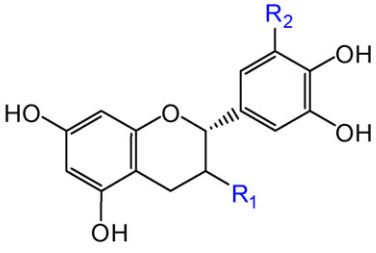


Fig. 1. Structural types of analysed phenolic acids, flavonoids, coumarins and caffeine. *Naringenin and hesperetin have no double bond on benzopyranone ring, they belong among flavanones.

of condensation (proanthocyanidins). Namely (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg) are the most abundant phenols in tea leaves, constituting up to 30% of the dry mass [13]. The representation of particular catechins in the tea infusion is dependent on the processing of tea leaves. EGCg is the major component of unfermented green tea [14], making up to about 10–50% of the total green tea catechins. Its antioxidant activity is 100 times more effective than that of vitamin C and 25 times more effective than that of vitamin E [15]. After the fermentation process the major catechin is GC, while the content of EGCg is decreased approximately 10× [16]. Besides catechins also small amounts of flavonols 3-*O*-glycosides occurs in tea leaves.

Analytical methods employed in analysis of flavonoids and phenolic acids were summarized in recently published review articles [5,17]. Basically all conventional separation methods are used such as capillary electrophoresis and chromatographic methods including HPLC, GC as well as TLC. Although HPLC was a method of choice, none of published works concerning with analysis of phenolic compounds in tea gave satisfactory results for separation of catechins [18–25]. The weak point of all published methods was limited separation efficiency, which press on to compromise separation time or resolution. The methods, which provided sufficient resolution for all peaks were extremely time-consuming, which means analysis time about 25–40 min for typically 7–12 analytes. For instance 15 analytes within 92 min [25] or 24 analytes within

Table 1
Recent LC methods dealing with the analysis of tea flavonoids and phenolic acids.

Ref	Matrix	Analysis		Detection	LOD	Duration [min]
		Qualitative	Quantitative			
(a) UHPLC methods						
[33]	Wine; grapefruit juice; green tea	17 phenolic acids		ESI-MS/MS	0.15–10 pmol/inj	10
[34]	Plant materials; Trifolium pratense; Glycine max; Pisum sativum; Ononis spinosa	(1) Gallic acid, (2) protocatechuic acid, (3) *p*-hydroxybenzoic acid, (4) vanillic acid, (5) caffeic acid, (6) syrinngic acid, (7) *p*-coumaric acid, (8) daidzin, (9) ferulic acid, (10) glycitin, (11) sinapic acid, (12) genistin, (13) ononin, (14) daidzein, (15) glycetin, (16) sissotrin, (17) genistein, (18) formonetin, (19) biochanin A		PDA 270 nm	0.2–0.4 μg L^{-1}	1.9
[35]	*Epimedium*	15 flavonoids – incl. epimedins, icariin, baohuoside, caohuosides and sagittatosides		UV 270 nm	0.05–0.13 ng	13
[36]	Leaves of *Isatis indigotica*	(1) Isoorientin, (2) isoorientin-3-glucoside, (3) isovitexin, (4) isovitexin-3-glucoside, (5) isocoparin, (6) isocoparin-3-glucoside		PDA, ESI-MS	0.8–4.5 μg.L^{-1}	6.5
[37]	Rat plasma	(1) Puerarin, (2) daidzein, (3) baicalin, (4) wogonoside, (5) liquiritin		ESI-MS	2.54–10.2 μg.mL^{-1}	6
[38]	*Rosa damascene*, *Rosa bourboniana*, *Rosa brunonii*	(1) Gallic acid, (2) tetra-O-galloyl hexoside, (3) di-O-galloyl hexoside, (4) galloyl tannin, (5) rutin, (6) quercetin-3-O-hexoside, (7) quercetin-3-O-dipentoside, (8) kempferol-3-O-rutinoside, (9) kaempferol-3-O-rhamnoside, (10) kaempferol-3-O-hexoside, (11) quercitrin, (12) kaempferol-3-0-pentoside, (13) quercetin-3-O-pentoside, (14) flavonol glycoside, (15) myricetin, (16) kempferol-3,7-hexose-rhamnoside, (17) quercetin, (18) kaempferol		ESI-MS/MS, PDA	NA	3
[39]	Tea samples, wine samples	11 phenolic acids, 8 catechins, 6 coumarins, 9 flavonoids – as separated groups		UV 280 nm	NA	3.5, 1.7, 4.0, 6.5
(b) HPLC methods						
[18]	Tea leaves, non-fermented tea dry extracts	(1) Gallic acid, (2) caffeine, (3) theobromine	(4) C, (5) Cg, (6) EC, (7) ECg, (8) EGC, (9) EGCg, (10) GC, (11) GCg	DAD (210 and 280 nm)	0.19–1.16 mg L^{-1}	25
[19]	Non-fermented, decaffeinated non-fermented, partially fermented and fermented tea	(1) Theaflavin, (2) caffeine, (3)C, (4) EC, (5) ECg, (6) EGC, (7) EGCg		DAD (280 nm)	0.02–1.80 mg L^{-1}	10
[20]	Fresh buds and leaves	(1) Theobromine, (2) caffeine, (3) theacrine, (4) C, (5) EC, (6) ECg, (7) EGC, (8) EGCg, (9) GC, (10) GCg		DAD (231 nm)	0.84–2.98 mg L^{-1}	20
[21]	Fresh young shoots of tea	(1) Theogallin, (2) gallic acid, (3) theobromine, (4) isochlorogenic acid, (5) GC, (6) EGC, (7) C, (8) *p*-coumarylquinic acid, (9) chlorogenic acid, (10) caffeine, (11) EGCg, (12) EC, (13) *p*-coumaric acid, (14) 3-(*p*-hydroxyphenyl)propionic acid, (15) GCg, (16) quercetin 3-rhamnosylglucoside, (17) epigallocatechin 3,5-digallate, (18) ECg (19) Cg, (20) quercetin 3-glucoside, (21) quercetin glycoside (22) kaempferol 3-rhamnosylglucoside, (23) kaempferol glycoside, (24) epicatechin 3,5-digallate		DAD (280, 310, 340, 380, 450 and 510 nm)	NA	40
[22]	Non-fermented tea extracts and fermented tea	(1) Theamine, (2) theophylline, (3) theobromine, (4) caffeine, (5) chlorogenic acid (6) C, (7) Cg, (8) EC, (9) ECg, (10) EGC, (11) EGCg, (12) GCG		PDA (200 nm)	0.05–0.20 mg L^{-1}	40
				MS (ESI+/−)	0.01–0.10 mg L^{-1}	
[23]	Non-fermented, partially fermented, fully fermented and post-fermented tea	(1) 3,5-Dihydroxybenzoic acid, (2) *p*-anisic acid, (3) myricetin, (4) 3,4,5-trimethoxycinnamic acid	(5) EC, (6) ECG, (7) EGC, (8) EGCG, (9) CG, (10) gallic acid, (11) caffeine	UV (280 nm)	NA	25
[24]	Non-fermented tea extracts	(1) C, (2) EC, (3) ECg, (4) EGC, (5) EGCg, (6) GC, (7) GCg		DAD (270 nm)	0.40–3.85 mg L^{-1}	18
				MS (ESI+)	0.33–0.71 mg L^{-1}	
[25]	Peanut skin extract	(1) Gallic acid, (2) protocatechuic acid, (3) EGC, (4) C, (5) caffeic acid, (6) procyanidin B2, (7) EC, (8) EGCg, (9) *p*-coumaric acid, (10) ferulic acid, (11) piceid, (12) ECg, (13) Cg, (14) resveratrol, (15) quercetin	PDA 250 nm, 280 nm, 306 nm, 320 nm, 370 nm		0.1–0.9 ppm	92

Table 2
System suitability test, calibration and sensitivity results.

No.	Component	Resolution	Asymmetry factor	Peak Capacity	Retention Time [%RDS]	Peak Area [%RSD]	Range [μg/ml]	Correlation Coefficient	LOD [mg L^{-1}]	LOQ [mg L^{-1}]
1	Gallic acid	–	1.17	122.35	0.22	0.21	0.20–105.88	0.9996	0.06	0.20
2	GC	5.38	1.13	118.26	0.36	0.52	2.70–135.00	0.9998	0.82	2.70
3	Protocatechuic acid	6.20	1.07	71.58	0.26	0.26	0.18–54.31	0.9997	0.05	0.18
4	Esculin	10.92	1.05	75.35	0.35	0.28	0.43–129.20	0.9997	0.13	0.43
5	EGC	6.14	–	73.10	0.52	0.76	1.50–109.95	0.9999	0.45	1.50
6	C	1.34	1.06	55.35	0.51	0.24	0.34–102.24	0.9997	0.10	0.34
7	Chlorogenic acid	5.27	–	37.50	0.33	0.68	0.41–122.44	0.9993	0.12	0.41
8	Vanillic acid	1.37	–	71.85	0.25	0.22	0.19–58.38	0.9998	0.06	0.19
9	Caffeic acid	2.23	0.97	75.77	0.28	0.55	0.04–64.60	0.9995	0.01	0.04
10	Caffeine	1.59	1.04	80.88	0.19	0.20	0.42–125.91	0.9997	0.13	0.42
11	Syringic acid	7.78	1.00	76.39	0.16	0.17	0.23–70.22	0.9997	0.07	0.23
12	EGCg	2.50	1.04	70.98	0.18	0.46	3.20–161.70	0.9997	0.97	3.20
13	EC	5.00	0.97	91.38	0.15	0.20	0.38–112.84	0.9997	0.12	0.38
14	GCg	6.32	1.00	62.83	0.14	0.49	3.00–148.30	0.9995	0.91	3.00
15	Scopoletin	3.97	0.95	85.29	0.10	0.22	0.22–66.59	0.9996	0.07	0.22
16	Ferulic acid	5.26	0.94	80.63	0.11	0.21	0.23–67.87	0.9996	0.07	0.23
17	ECg	2.37	0.99	58.41	0.11	0.83	3.10–156.70	0.9996	0.94	3.10
18	Cg	5.75	0.98	62.80	0.11	0.41	2.90–146.70	0.9994	0.88	2.90
19	o-Coumaric acid	10.85	0.91	69.35	0.10	0.18	0.20–58.58	0.9996	0.06	0.20
20	Rutin	3.40	0.94	57.83	0.12	0.20	0.70–210.75	0.9996	0.21	0.70
21	4-OH-Coumarin	2.14	0.91	65.98	0.09	0.18	0.19–55.54	0.9995	0.06	0.19
22	Myricetin	3.58	0.84	41.09	0.13	0.84	22.98–229.75	0.9992	6.96	22.98
23	Quercitrin	2.98	–	74.08	0.12	0.40	0.24–72.72	0.9994	0.07	0.24
24	6-Me-coumarin	1.30	1.01	56.21	0.07	0.75	0.19–55.56	0.9991	0.06	0.19
25	Cinnamic acid	5.58	0.90	57.36	0.09	0.21	0.17–52.26	0.9993	0.05	0.17
26	Quercetin	5.76	0.88	44.93	0.13	0.91	45.78–228.88	0.9991	13.87	45.78
27	Naringenin	2.01	0.91	54.65	0.11	0.26	1.25–93.81	0.9990	0.38	1.25
28	Luteolin	3.17	0.92	52.94	0.13	0.85	3.66–188.00	0.9994	1.11	3.66
29	Hesperetin	2.63	0.91	49.30	0.10	0.39	1.39–104.46	0.9992	0.42	1.39
30	Kaempferol	5.19	0.89	36.00	0.15	0.73	4.05–202.75	0.9993	1.23	4.05

LOD: limit of detection, LOQ: limit of quantification.

40 min [21]. An overview of HPLC methods used in analysis of phenolics with the emphasis to catechin analysis is displayed in Table 1b.

Recently, Ultra high performance liquid chromatography (UHPLC) has become widely spread technique and new trend in separation sciences. Higher separation efficiency of sub-2-μm particle sorbents allows faster chromatographic separation keeping the same resolution compared to HPLC sorbents with conventional particle size [26]. This is expressed by Knox curves, where lower height equivalent of theoretical plate indicates improved separation efficiency and higher optimum linear velocity, which means decrease in analysis time [27]. Generated back pressure of particle sorbents is inversely related to the cubed particle size, therefore UHPLC system is necessary (back pressures up to 100 MPa). A higher sensitivity is achieved using column with small internal diameter (typically 2.1 mm) and a low volume optical cell for UV detection. UHPLC systems have been demonstrated to be beneficial in the pharmaceutical development [28], drug monitoring [29], metabolite profiling [30], proteomics [31], environmental chemistry [32] or food chemistry [33].

Several new UHPLC methods for the determination of flavonoids and phenolic acids in various matrices have been recently developed [33–39]. An overview is displayed in Table 1a. These methods have typically analytical runs shorter than 10 min, in extremes they are able to separate 19 analytes in 2 min [34]. High applicability is of value especially in work of Gruz et al. [33], who determined 17 common phenolic acids or the work of Klejdus et al. [34], who determined common phenolic acids together with some flavonoids. The main drawback of latter method lays in determination only of vanillic and caffeic acid without chlorogenic acid, as these three phenolic acids typically cause coelutions in real matrices and they are often present together. Kumar et al. developed UHPLC-DAD and UHPLC–ESI-MS/MS method only for determination of phenolic acids and flavonoids [38].

In our previous work four individual methods were developed for four groups of phenolics (catechins, less polar flavonoids, coumarins and phenolic acids) using UHPLC-UV [39]. The aim was to stress the benefit of UHPLC above HPLC analysis in analysis of phenolic compounds. The four newly developed methods used various four completely different gradient elution profiles and temperatures. The convenience of UHPLC was clearly proven in terms of speed of analysis and separation efficiency however previously reported UHPLC methods might be excessively rapid not providing enough peak capacity for separation of all structurally similar compounds. Also selectivity issue was not addressed as single UV detection does not provide sufficient confirmatory results.

The aim of this work was to develop and fully validate fast, efficient routine UHPLC method with PDA detection for the simultaneous qualitative and quantitative analysis of a wide range of the most commonly occurring phenolic compounds of various structures plus caffeine (to exclude the interference) in plant matrices or dietary supplements. Structures have been chosen based upon the occurrence of phenolic compounds in plant material – see Fig. 1, to represent important examples from each group of phenolic compounds of various polarities. Tea infusions and tea extracts were selected in order to demonstrate sufficient separation efficiency, selectivity and sensitivity. The proposed method can be used in other applications, e.g. for the quantification of phenolic compounds in other plant extracts. Notably dietary supplements with contents of phenolic compounds are potential field for application, since their popularity is growing. Manufactures have not been required to demonstrate declared amount of active ingredients of their products so far [40] however, this may change soon. Therefore, commercial dry tea extracts were also included in our study.

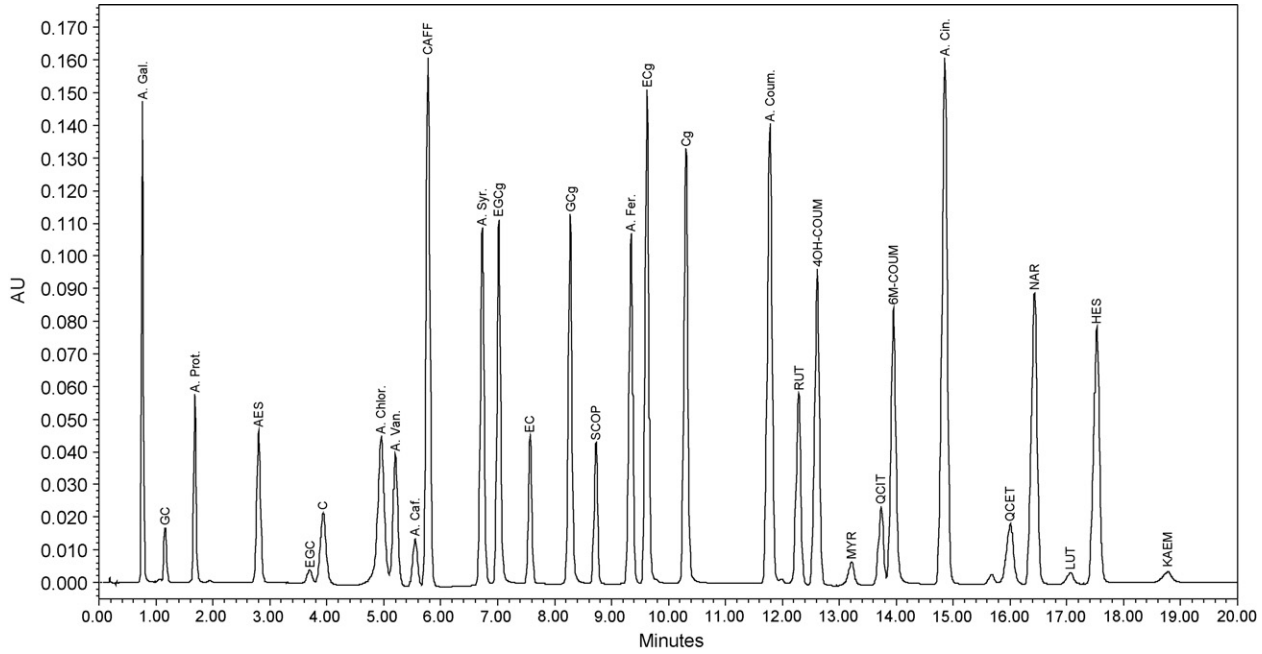


Fig. 2. Chromatogram of a standard mixture of 29 phenolic compounds and caffeine. (UV 280 nm, 0.1% formic acid–methanol from 88.5:11.5 to 50:50 (v/v), 0.45 mL min^{-1}).

2. Experimental

2.1. Chemicals and reagents

Reference standards of phenolic compounds were used in this study. They were purchased from Sigma–Aldrich (Prague, Czech Republic) as follows: phenolic acids: gallic (A. Gal.), protocatechuic (A. Prot.), chlorogenic (A. Chlor.), vanillic (A. Van.), caffeic (A. Caf.), syringic (A. Syr.), ferulic (A. Fer.), o-coumaric (A. Coum.), cinnamic (A. Cin.); flavonoids: catechin (C), catechin gallate (Cg), epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC), epigallocatechin gallate (EGCg), gallocatechin (GC), gallocatechin gallate (GCg), rutin (RUT), myricetin (MYR), quercitrin (QCI), quercetin (QCE), naringenin (NAR), luteolin

Table 3
Spectral data and peak purity calculations. Peak purity criteria are fulfilled when *peak purity angle* is lower than *peak purity threshold*.

Component	t_R	Standard mixture				Formosa gunpowder tea sample			
		λ_{max} 1	λ_{max} 2	Peak purity angle	Peak purity threshold	λ_{max} 1	λ_{max} 2	Peak purity angle	Peak purity threshold
Gallic acid	0.96	216	271	0.436	1.583	216	271	0.860	3.943
GC	1.27	270	335	1.654	12.569	270	–	7.887	32.516
Protocatechuic acid	1.74	260	294	0.344	1.569	260	294	0.632	4.339
Esculin	2.67	334	–	0.176	1.493	335	–	0.280	2.248
EGC	3.24	269	332	2.169	15.980	270	–	3.342	10.448
C	3.46	279	389	2.270	10.522	280	–	5.307	42.763
Chlorogenic acid	4.44	246	324	0.423	2.003	246	323	0.459	3.470
Vanillic acid	4.71	260	292	0.558	2.038	261	292	1.535	7.208
Caffeic acid	4.98	242	323	0.333	0.932	243	323	0.220	1.546
Caffeine	5.45	273	–	0.614	2.506	273	–	0.451	2.989
Syringic acid	6.43	217	274	0.442	1.647	217	274	0.912	5.919
EGCg	6.73	274	–	1.055	3.532	274	–	0.734	2.982
EC	7.31	279	–	2.196	6.782	279	–	3.103	15.093
GCg	8.08	274	–	0.999	2.772	274	–	1.889	8.828
Scopoletin	8.54	228	344	0.137	0.714	228	345	0.168	1.153
Ferulic acid	9.09	235	323	0.151	0.684	236	323	0.633	1.128
ECg	9.46	278	–	0.890	2.297	278	–	1.191	5.098
Cg	10.17	279	–	1.004	2.427	278	–	18.880	7.391
o-Coumaric acid	11.39	277	325	0.216	0.776	277	325	1.154	1.501
Rutin	12.05	256	355	0.318	1.479	256	354	0.930	2.181
4-OH-Coumarin	12.24	281	303	0.588	1.072	281	303	6.485	2.688
Myricetin	12.89	252	373	0.730	1.167	252	371	1.378	1.972
Quercitrin	13.46	256	350	0.274	1.137	256	351	0.327	2.027
6-Me-coumarin	13.70	279	323	0.475	1.286	279	323	0.650	2.859
Cinnamic acid	14.42	217	278	0.346	0.859	217	278	0.622	2.661
Quercetin	15.58	255	370	0.451	1.142	255	370	0.388	1.977
Naringenin	15.96	213	290	0.747	1.603	213	290	0.934	4.070
Luteolin	16.56	254	250	0.223	0.924	254	351	0.231	1.525
Hesperetin	17.09	288	–	0.775	1.572	288	–	0.874	3.808
Kaempferol	18.16	265	365	0.269	1.273	265	366	0.315	2.118

Table 4
Validation results for intra- and inter-day precision.

Method precision [% RSD]							
Component	Concentration level [mg L^{-1}]	Level 1		Level 2		Level 3	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Gallic acid	25, 50, 100	0.05	2.30	0.11	1.31	0.07	0.63
GC	25, 50, 100	0.08	0.93	0.18	0.59	0.15	0.73
Protocatechuic acid	5, 25, 50	0.42	1.53	0.14	1.31	0.23	1.52
Esculin	5, 25, 50	0.38	0.80	0.31	2.80	0.07	0.26
EGC	25, 50, 100	0.26	1.29	0.28	0.59	1.93	2.14
C	5, 25,50	0.36	0.69	0.86	1.37	0.20	1.94
Chlorogenic acid	1, 5, 25	1.09	8.32	0.45	2.83	0.30	1.37
Vanillic acid	5, 25, 50	0.45	2.68	0.15	2.43	0.04	0.28
Caffeic acid	5, 25, 50	0.20	1.71	0.14	1.15	0.16	1.44
Caffeine	25, 50, 100	0.05	0.39	0.06	0.34	0.02	0.39
Syringic acid	1, 25, 50	0.24	3.51	0.21	2.14	0.10	2.39
EGCg	25, 50, 100	0.10	0.63	0.13	0.70	0.09	0.52
EC	25, 50, 100	0.08	0.67	0.06	0.16	0.04	0.21
GCg	5, 25, 50	0.22	4.88	0.35	3.26	0.28	0.57
Scopoletin	5, 25, 50	0.09	3.33	0.09	2.34	0.10	0.51
Ferulic acid	5, 25, 50	0.19	1.29	0.24	0.93	0.10	1.28
ECg	25, 50, 100	0.08	0.42	0.03	0.28	0.04	0.09
Cg	5, 25, 50	0.17	0.84	0.16	2.15	0.24	1.90
o-Coumaric acid	5, 25, 50	1.31	2.28	0.19	1.49	0.04	1.43
Rutin	25, 50, 100	0.10	0.64	0.07	0.51	0.04	0.47
4-OH-Coumarin	5, 25, 50	0.06	1.51	0.20	1.84	0.25	1.91
Myricetin	1, 25, 50	1.10	5.61	0.25	3.27	0.10	2.37
Quercitrin	1, 25, 50	0.70	3.79	0.37	2.78	0.13	2.04
6-Me-coumarin	5, 25, 50	0.18	2.38	0.16	2.13	0.14	0.48
Cinnamic acid	5, 25, 50	0.16	2.66	0.14	2.38	0.04	0.59
Quercetin	5, 25, 50	0.20	5.87	0.27	4.15	0.10	1.60
Naringenin	1, 25, 50	2.23	3.86	0.11	1.75	0.20	1.97
Luteolin	5, 25, 50	0.27	4.28	0.22	3.79	0.25	1.24
Hesperetin	5, 25, 50	0.12	2.92	0.15	2.63	0.17	0.53
Kaempferol	5, 25, 50	0.21	4.44	0.21	3.82	0.13	1.46

Table 5
Validation results for intra- and inter-day accuracy.

Method accuracy [% of recovery]							
Component	Concentration level [mg L^{-1}]	Level 1		Level 2		Level 3	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Gallic acid	25, 50, 100	106.11	107.40	112.50	113.10	110.28	111.39
GC	25, 50, 100	100.03	96.84	99.74	98.19	94.38	93.39
Protocatechuic acid	5, 25, 50	93.50	89.17	96.66	93.10	100.03	96.94
Esculin	5, 25, 50	91.11	90.49	101.45	97.42	99.90	100.84
EGC	25, 50, 100	76.89	76.15	92.98	91.82	97.47	96.77
C	5, 25,50	98.72	88.61	97.50	92.70	99.66	96.06
Chlorogenic acid	1, 5, 25	100.67	105.16	95.10	92.15	102.31	104.45
Vanillic acid	5, 25, 50	95.92	93.65	101.36	97.77	100.84	101.29
Caffeic acid	5, 25, 50	92.27	89.01	96.86	92.65	101.62	97.29
Caffeine	25, 50, 100	82.96	82.63	94.35	94.14	95.38	96.77
Syringic acid	1, 25, 50	94.81	93.46	101.85	94.30	100.77	97.84
EGCg	25, 50, 100	87.45	86.54	99.73	99.54	101.14	101.54
EC	25, 50, 100	96.24	95.51	101.06	101.38	95.31	95.00
GCg	5, 25, 50	97.96	92.63	99.12	99.42	105.52	106.87
Scopoletin	5, 25, 50	100.73	97.58	98.85	101.36	101.01	102.17
Ferulic acid	5, 25, 50	96.76	92.00	102.34	96.17	100.28	103.47
ECg	25, 50, 100	99.71	99.56	105.10	105.15	100.68	100.89
Cg	5, 25, 50	100.25	102.13	102.01	96.70	98.53	99.96
o-Coumaric acid	5, 25, 50	93.76	88.05	102.79	96.29	100.50	103.70
Rutin	25, 50, 100	101.11	101.90	104.10	104.75	99.92	100.48
4-OH-Coumarin	5, 25, 50	94.97	89.81	97.36	92.50	100.82	96.17
Myricetin	1, 25, 50	99.82	103.46	103.26	95.38	103.94	107.99
Quercitrin	1, 25, 50	100.20	100.22	103.08	96.06	99.59	99.86
6-Me-coumarin	5, 25, 50	97.39	95.45	102.10	98.89	102.45	102.85
Cinnamic acid	5, 25, 50	97.36	95.26	99.51	102.55	102.55	103.08
Quercetin	5, 25, 50	96.37	92.97	96.94	98.52	106.15	106.88
Naringenin	1, 25, 50	95.12	93.78	95.82	90.22	99.55	94.42
Luteolin	5, 25, 50	96.35	93.64	97.32	101.62	103.13	103.96
Hesperetin	5, 25, 50	97.66	94.97	102.79	98.51	102.42	103.08
Kaempferol	5, 25, 50	97.06	93.99	97.89	102.36	103.79	105.04

(LUT), hesperetin (HES), kaempferol (KAEM); coumarins: aesculin (AES), scopoletin (SCOP), 4-hydroxycoumarin (4OH-COUM), 6-methylcoumarin (6M-COUM) and caffeine (CAFF).

The non-fermented teas Kokaicha and Formosa Gunpowder, the fermented teas Pu-Erh and Nepal SFTGFOP-Maloom, the mixture of non-fermented and fermented tea leaves "Festival", the fruit teas "Messina" and "Cinnamon-Apple" were all obtained from OXALIS (Slušovice, Czech Republic). The non-fermented Vietnam tea with jasmine was purchased from a specialized tea shop. The green tea extracts "GT ex. p." (min. 80% catechins, max 8% caffeine) and "Ethyl Acetate Free Green Tea extract" (80% catechins/50% EGCg) were obtained from PORTA s.r.o (Prague, Czech Republic).

The mobile phase additive formic acid 98% p.a. as well as HPLC grade methanol and acetonitrile were obtained from Sigma–Aldrich. HPLC grade water, prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA), was additionally filtrated through a 0.22 μm membrane filter immediately before use.

2.2. Instrumentation

Acquity UPLC system (Waters, Prague, Czech Republic) consisted of Acquity binary solvent manager (BSM), Acquity sample manager (SM), Acquity column manager and Acquity PDA detector. All UPLC analyses were performed on a BEH C18 analytical column (100 mm × 2.1 mm, 1.7 μm, Waters, Czech Republic).

The mobile phase consisted of 0.1% formic acid (solvent A) and methanol (solvent B). The following gradient program was employed: 0–4 min isocratic elution (88.5% A:11.5% B) and 4–20 min linear gradient elution from 88.5% A:11.5% B to 50% A:50% B. The initial flow rate 0.45 mL min^{-1} was reduced to 0.42 mL min^{-1} in the time interval from 4.1 to 20 min. Column oven temperature

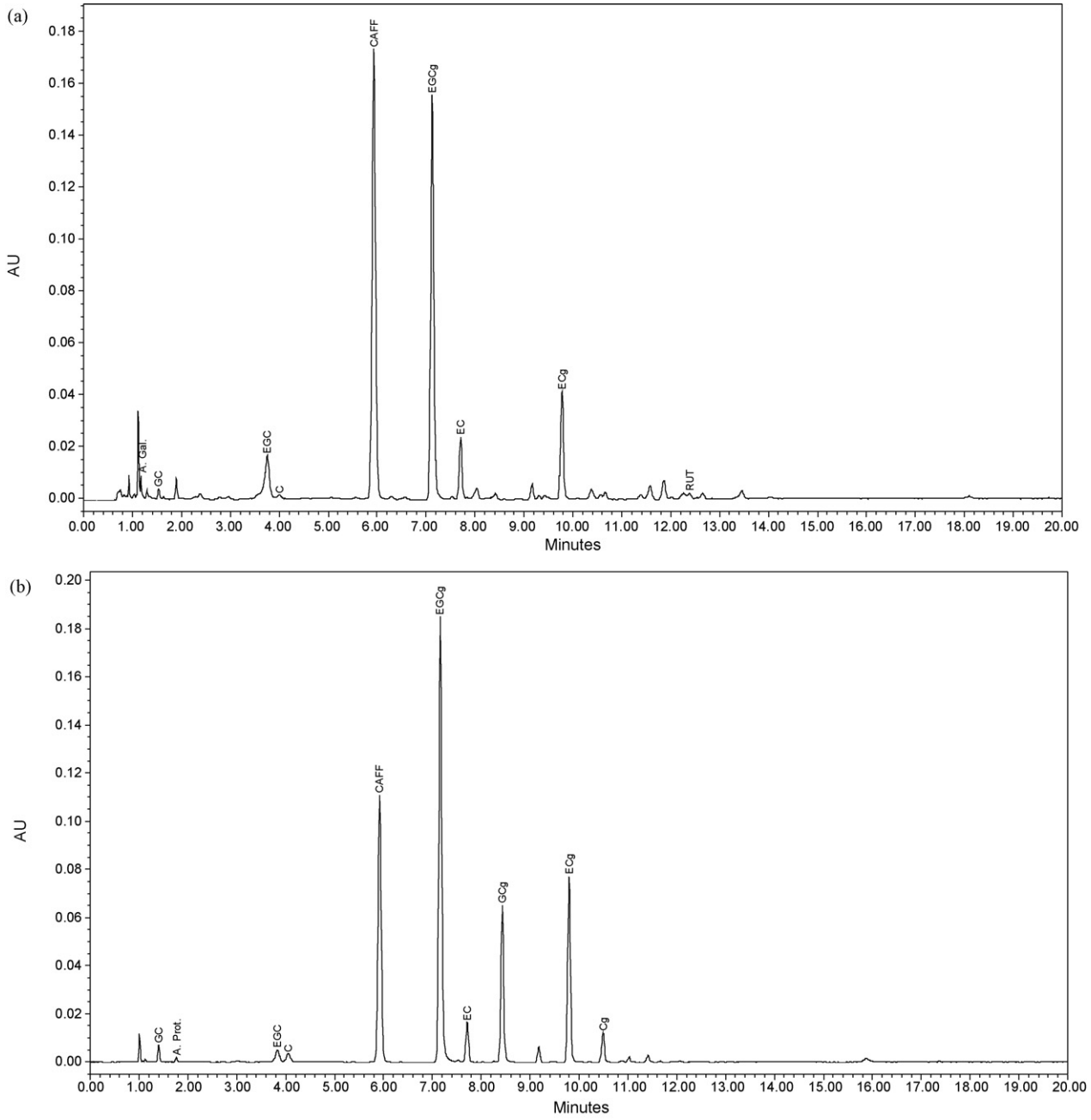


Fig. 3. Chromatograms of Kokaicha (water infusion) (a) and GT ex. p. extract (b).

was set up to 25 °C and the samples were kept at 4 °C in the sample manager. The partial loop injection mode with needle overfill injected a volume of 2.0 μL using 5 μL injection loop. Methanol was used as a strong wash solvent and 20% methanol as a weak wash solvent. The PDA detection was conducted at 280 nm for quantitative purposes with data acquisition rate of 20 Hz. Spectral data and peak purity calculation were used for identity confirmation. Chromatographic software Empower 2 was used for gathering data and processing of the chromatograms. This software enables a calculation of peak purity and operation with spectral data. Two UV absorbance maxima (if available) were used for identity confirmation of all analytes. Peak purity calculations uses two variables – peak purity angle and peak purity threshold. The criteria of peak purity are fulfilled when peak purity angle value is lower than the value of peak purity threshold.

2.3. Stock solutions and tea samples pre-treatment

The *stock solutions* of phenolic acids, flavonoids, catechins, coumarins and caffeine were prepared in methanol at the concentrations ranging from 1 to 5 g L^{-1}, keeping approximately equimolar ratios. All 30 stock solutions were combined into one mixed working solution and further diluted by mobile phase.

The *tea infusions* were prepared by pouring 100 mL of hot water (90 °C) over 1 g of dry tea mixture and 5 min of maceration with mild stirring. Then the cooled down tea infusion was filtrated through 0.20 μm membrane filter and further 2.5 times diluted by the mobile phase. The diluted tea infusions were used for injections immediately.

The *methanolic tea extracts* were prepared by pouring 50 mL of methanol over 1 g of tea mixture and sonication for 20 min. The following steps were the same as in the case of water infusion; the solutions were filtrated and stabilized by low pH of the mobile phase.

The *tea extract samples* prepared from commercially available powder extracts were diluted by methanol, filtrated and further diluted by the mobile phase prior to analysis. Fresh samples prepared daily were used for the analysis.

2.4. System suitability test and validation

A mixed standard solution of all phenolic compounds at the concentration corresponding to the middle of the calibration range was injected in ten replicates for the system suitability test (SST) measurements. Evaluated parameters were the mean values of resolution, asymmetry factor and peak capacity. The repeatability of retention time and peak area expressed as %RSD were checked and they should meet the requirement of RSD < 1%. The rules for measurement and the limits for the acceptance are given by official pharmacopoeias [41,42].

Determined validation parameters were following: range, linearity, LOD, LOQ, precision, accuracy and selectivity. Linearity was established using mixed standard solutions at seven calibration levels for each compound. Method precision was tested at three concentration levels in three replicates using tea samples to calculate RSD of the determination, which describes the closeness of agreement between series of measurements. Tea samples spiked with standard solution at three concentration levels were used for determination of method accuracy and recovery. Spiked samples were run in three replicates and agreement between theoretical and measured value was confronted with appropriate guidelines [43]. Both accuracy and precision were determined as intra-day and inter-day measurements (measurements were performed on three different days). Method selectivity was confirmed by calculation of peak purity and spectral data. Peak purity was calculated using two variables (calculated by software) – peak purity angle and peak purity threshold. The value of peak purity angle must be lower than the value of peak purity threshold. Formosa gunpowder tea was used as tea matrix for precision and accuracy experiments.

3. Results and discussion

3.1. Method development

The different portions of organic modifier present in mobile phase were tested in order to achieve optimal separation. The key issue was to achieve separation between peaks of chlorogenic, vanillic and caffeic acid, due to very similar retention properties. A sufficient separation was obtained eluting 0.1% formic acid and methanol isocratically in the ratio 88.5:11.5 (v/v) for 4 min. Consequently gradient curves of convex and concave profile and several target ratios were tested in order to achieve satisfactory separation of later eluted analytes. The final gradient profile with the target ratio 50:50 (v/v) enabled quick elution of relatively low polar flavonoid aglycons. A less steep gradient profile prolonged the analysis and undesirably affected symmetry of late eluting peaks. Finally the slightly convex gradient curve was performed to optimize the resolution.

The choice of wash liquids and standard/sample dissolution media is of special importance in UHPLC. Concerning wash liquids – weak wash solvent participate on injection procedure, therefore

Table 6
Quantification of tea components in water infusions (mg g^{-1} of dry tea mixture).

	Kokaicha	Vietnam tea with jasmine	Formosa gunpowder	Nepal-Maloom	Pu-Erh	Festival	Messina	Cinnamon-apple
Gallic acid	0.39	0.49	1.05	2.21	3.18	0.94	0.13	0.12
GC	2.53	3.08	3.66	LOD	0.80	LOD	–	–
Protocatechuic acid	–	–	0.18	0.11	–	LOD	0.10	–
EGC	33.82	35.04	37.05	24.71	–	4.88	–	–
C	0.75	1.12	1.69	0.40	–	1.25	–	–
Chlorogenic acid	–	–	0.93	–	–	–	2.20	0.84
Vanillic acid	–	0.47	–	–	–	–	–	–
Caffeic acid	–	–	–	0.05	–	–	–	0.05
Caffeine	14.18	20.72	19.73	24.39	16.04	6.90	–	–
EGCg	22.82	25.90	33.42	12.87	–	4.63	–	–
EC	6.30	5.81	5.94	1.04	0.39	1.25	–	–
GCg	–	0.99	1.02	0.83	–	LOD	–	–
Ferulic acid	–	–	–	0.17	–	0.08	–	–
ECg	4.84	6.21	9.65	5.02	–	1.84	–	–
Cg	–	0.92	0.92	–	–	LOD	–	–
Rutin	1.31	1.15	2.22	0.67	–	–	1.21	0.68
Quercitrin	–	0.49	1.13	0.54	–	–	–	–
Total catechins	71.07	79.06	93.33	45.82	1.19	13.85	0.70	–

Table 7
Quantification of tea components in methanolic tea extracts and in commercial dried tea extracts (mg g^{-1} of dry tea mixture/powder).

	Kokaicha	Vietnam tea with jasmine	Formosa gunpowder	Nepal-Maloom	Pu-Erh	Festival	Messina	Cinnamon-Apple	GT ex. p.	Ethyl acetate free GT extr.
Gallic acid	0.09	0.07	1.85	0.81	0.58	0.97	0.06	0.07	5.57	2.97
GC	0.59	0.92	2.42	LOD	–	0.79	–	–	56.64	13.61
Protocatechuic acid	0.05	–	0.14	–	–	–	0.06	–	–	–
EGC	5.67	7.22	15.13	4.37	–	8.02	–	–	107.67	84.71
C	0.22	0.41	1.27	0.14	–	0.39	–	–	19.63	6.63
Chlorogenic acid	–	–	0.48	–	–	–	0.97	0.30	–	–
Vanillic acid	–	–	–	–	–	–	–	–	–	–
Caffeic acid	–	–	0.04	–	–	0.02	–	–	–	–
Caffeine	3.64	8.01	18.33	13.32	–	13.35	–	–	107.87	49.40
EGCg	7.08	11.90	37.88	13.00	–	14.41	–	–	337.02	524.57
EC	1.53	2.05	5.49	–	–	2.52	–	–	51.47	32.85
GCg	–	0.48	0.69	–	–	0.56	–	–	106.65	26.7
Ferulic acid	–	–	–	–	–	0.09	–	–	–	–
ECg	2.02	3.53	13.27	4.76	–	4.94	–	–	101.57	116.38
Cg	–	0.47	0.52	–	–	0.50	–		23.32	9.86
Rutin	0.40	0.40	1.19	–	–	0.66	1.60	0.42	–	–
Quercitrin	–	–	0.94	0.24	–	–	–	–	–	–
Total catechins	17.11	26.99	76.66	22.27	0	32.12	–	0	803.98	815.31

it was necessary to use one corresponding to initial gradient conditions (20% methanol). The same applied to dissolution media as using narrow diameter columns the choice of dissolution solvent can influence chromatographic separation significantly more than in conventional HPLC analysis.

3.2. System suitability test and validation

SST parameters including resolution – R_s, peak asymmetry, repeatability of retention time and peak area were measured for all 30 compounds. The values of the resolution were in the range specified by pharmacopoeias (>1.5) corresponding to baseline separation, except of the values for the three pairs of substances (EGC and C, chlorogenic and vanillic acid, quercitrin and 6-methylcoumarin, where R_s was around 1.3). The asymmetry factor values varied between 0.84–1.17 fully meeting the criteria for symmetric peaks (0.8–1.5) according to EP [41]. The average peak capacity was 67.7, which is fully acceptable by general recommendations.

RSD for the retention time ranged from 0.09 to 0.52% and for the peak area from 0.17 to 0.91%, respectively. This is again in agreement with the requirements of EP [41]. Details of SST results are given in Table 2 and a typical chromatogram of standard mixture separation is shown in Fig. 2. The developed gradient method met all claims obligatory for SST.

To demonstrate method selectivity, spectral data were acquired and peak purity was calculated – data are displayed in Table 3. Two UV maximum absorbance wavelengths (if available) were compared in measurement of standard mixture and real sample. UV spectra matched for all maximum absorbance wavelengths ± 1 nm. These criteria for the peak purity were met for all 30 compounds in standard mixture, however only for 28 compounds in spiked real tea sample in spite of seeming agreement in UV spectra. Thus it could be concluded, that peak of Cg and 4-hydroxymethylcoumarin probably co-eluted with some interfering compound from matrix and quantitative results will not be reliable.

The calibration curves were measured using mixed standard solutions in a wide concentration range, typically from 1 to 100 mg L^{-1}, details are given in Table 2. The response was linear in tested range with correlation coefficients >0.9990 for all analytes. The last point of calibration curve was considered to be method LOQ (S/N = 10). LOD were calculated by extrapolation (S/N = 3). Typical LOD and LOQ were 0.06 mg L^{-1} or 0.2 mg L^{-1} respectively. Intra-day method precision for all thirty compounds expressed as RSD was less than 3%, while inter-day precision was less than 9%, the results are displayed in Table 4. Intra-day method accuracy expressed as recovery was within the range 77–110%, while inter-day method accuracy within 76–111% with typical values 100 ± 10%. The results are shown in Table 5.

3.3. Quantification of tea samples and extracts

In fact a cup of tea is a simple water infusion. Hence we used the extraction by hot water to study concentrations of polyphenolic tea components available for common tea consumers. Results achieved using the hot water extraction were compared to those achieved by sonication in cold methanol. Although compared to water methanol is theoretically more preferable solvent for polyphenols, the extraction by methanol appeared to be less efficient in case of non-fermented teas than the hot water infusion. Typical chromatogram is given in Fig. 3a. Results of the tea samples quantification can be seen in Tables 6 and 7. The indicated numbers represent the milligram content of tea components in 1 g of dry tea mixture. Excellent sensitivity of proposed UHPLC method allowed quantification of phenolic compounds present even in trace concentrations such as C, Cg, GCg, protocatechuic acid or rutin however, the care must be taken with each sample to verify its selectivity using peak purity calculation and spectral data.

According to the expectations, high content of catechins was found in non-fermented teas, typically ranging from 7 to 9% (w/w). The highest total quantity of catechins at all was found in Formosa gunpowder (93 mg g^{-1}), while very similar quantities were reported by Wang et al. in gunpowder tea [18]. Content of caffeine in the non-fermented teas was around 2% (w/w). Apart from catechins and caffeine also other phenolic compounds were quantified in tea samples. Phenolic acids (gallic, chlorogenic, protocatechuic, vanillic, caffeic and ferulic acid) were present at concentrations around 1 mg g^{-1}. Myricetin, quercetin and kaempferol-3-glycosides were also previously reported in tea infusions [44], although only quercitrin and rutin were found in our samples in detectable concentration. The fermented teas and fruit mixtures contained much lower quantities of monitored phenolic compounds.

The commercially available green tea extracts were tested in order to verify correct quantification of phenolic compounds by UHPLC – see Fig. 3b. Determined quantities were compared to the quantity guaranteed by the producer (>80%) and good agreement between both values was found (Table 7).

4. Conclusions

A newly developed UHPLC method with PDA detection enables simultaneous determination of 29 commonly occurring phenolic compounds and caffeine in tea matrices within only 20 min.

Apart from catechins (the most common antioxidant phenolic compounds abundant in tea and many other plant materials) also other natural phenolic compounds from groups of flavonoids, phenolic acids and coumarins were chosen in order to extend the utilization of this method to a number of various applications. High separation efficiency predestines this analytical method to be, e.g. useful screening tool in pharmaceutical botany or fingerprinting for the quality control of tea and tea extracts. The system suitability test and validation parameters were meeting requirements for all thirty compounds and thus the method was convenient for use in the quantitative analysis. Method selectivity was enhanced using spectral data from PDA detector and peak purity information. Furthermore it is important to highlight the minimal consumption of time and solvents when UHPLC is used contrary to conventional HPLC methods.

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5.7. Supplement III

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Analytical Methods

Comparison of positive and negative ion detection of tea catechins using tandem mass spectrometry and ultra high performance liquid chromatography

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ABSTRACT

Tea catechins are an important group of natural compounds associated with health promoting effects and desired commodities for the growing market of dietary supplements and functional foods. Consequently these compounds attract more interest of research groups worldwide. A reliable quantitative analysis of tea catechins is essential for human intervention studies, manufacturers of dietary supplements and quality control by authorities. UHPLC–ESI-MS/MS analytical method was chosen due to rapid runtime, high sensitivity and selectivity. The chromatographic separation of eight tea catechins was achieved within 2.5 min on C_{18} BEH analytical column (100 mm × 2.1 mm i.d.; 1.7 μm), whilst the gradient elution mode was employed using water:methanol mobile phase with addition of volatile organic acid. The concentration of organic acids in the mobile phase was optimised within the range of 0.01–0.1% (v/v). High sensitivities were achieved in positive (10.2-16.8 fmol/inj.) and negative ion detection mode (102.1-168.1 fmol/inj.), through accurate and complex tuning of MS parameters. The UHPLC-ESI-MS/MS method was validated in terms of linearity (>0.9997; >0.9990), range (0.02–2.40 mg L^{-1}; 0.15-24.00 mg L^{-1}), LOD (3.0–4.8 μg L^{-1}; 30.1–48.0 μg L^{-1}), LOQ (9.9–15.8 μg L^{-1}; 150.5-240.0 μg L^{-1}), intra-day precision (4.4-7.1% RSD; 3.3-5.1% RSD), accuracy (94.06-113.7%; 89.5-108.4%), retention time repeatability (0.0-0.5% RSD; 0.0-0.6% RSD), and peak area repeatability (1.2-4.0% RSD; 2.4-3.5% RSD) for positive and negative ion detection modes, respectively. The statistical comparison of the quantitative results obtained in positive and negative ion detection mode was performed.

1. Introduction

Tea infusion brewed from *Camellia sinensis* (L.) Kuntze (Theaceae) is the most popular beverage after water and it is a rich natural source of polyphenols (PPs). Namely (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg) (Fig. 1) constituting up to 30% of the dry mass (Wilson & Clifford, 1992). EGCg is the major component of unfermented tea with 100-fold higher antioxidant activity than vitamin C (Hu & Kitts, 2001). It makes up about 10–50% of the total green tea catechins (Liang, Ma, Lu, & Wu, 2001). Over the fermentation process the major catechin is (−)-gallocatechin (GC), whilst the content of EGCg is decreased approximately 10-fold (Liang, Lu, Zhang, Wu, & Wu, 2003). There is growing evidence that these PPs have beneficial effects on living organisms in context of their antioxidant activity, such as reduced risk of cardiovascular diseases or cancer (Barbosa, 2007; Khan & Mukhtar, 2007). Also anti-inflammatory and anti-obesity effects have been reported by Khan and Mukhtar (2007). Liquid chromatography (LC) represents 65% of all analytical methods used for the analysis of PPs, but separation efficiency or detection sensitivity is often insufficient to handle complex natural sample (Kartsova & Alekseeva, 2008). These shortcomings can be avoided using up-to-date instrumentation, such as ultra high performance liquid chromatography (UHPLC) systems, which has already shown to be practical in many applications (Gruz, Novak, & Strnad, 2008; Wren & Tchelitcheff, 2006; Yuan, Wang, Tu, Peng, & Li, 2008). Higher separation efficiency compared to conventional HPLC system is provided, due to exploitation of sub-2-μm particle sorbents operated at backpressures up to 15,000 psi (100 MPa). The sensitivity is improved using column with small internal diameter (typically 2.1 or 1 mm) more suitable for mass spectrometry (MS) detection. Only 20% of LC methods published on analysis of PPs derivate benefit from sensitivity and selectivity of employed MS detection (Kartsova & Alekseeva, 2008). The main drawback of existing LC methods appears to be not taking full advantage of LC sorbents, which leads to the compromise between separation time and resolution. Thus reported separation time is usually 20-45 min in the average. The lengthy analysis dictate that some samples may not be analysed for several hours, potentially leading to auto-oxidation and epimerization of instable analytes (Sang, Lee, Hou, Ho, & Yang,

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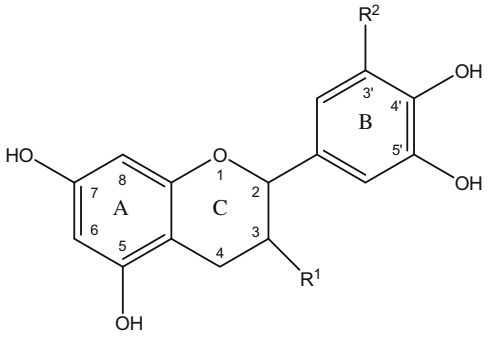

Name	Substitution
(+)-catechin	3S –OH
(+)-gallocatechin	3S –OH; 3′ –OH
(+)-catechin gallate	3S –gallate
(+)-gallocatechin gallate	3S –gallate; 3′ –OH
(-)-epicatechin	3R –OH
(-)-epigallocatechin	3R –OH; 3′ –OH
(-)-epicatechin gallate	3R –gallate
(-)-epigallocatechin gallate	3R –gallate; 3′ –OH

Fig. 1. Structures of tea catechins.

2005). Nevertheless accomplished separation of all eight tea catechins in single run is reported rarely (Li, Xiaohong, Xianggang, Jiaxian, & Chuangxing, 2008; Masukawa et al., 2006; Spacil, Novakova, & Solich, 2008; Wang, Provan, & Helliwell, 2003). The other methods tolerate poor resolution (Fu, Liang, Han, Lv, & Li, 2008; Neilson, Green, Wood, & Ferruzzi, 2006; Yang, Ye, Xu, & Jiang, 2007) or straight-out co-elutions of chromatographic peaks (Pelillo et al., 2004; Zhu et al., 2004; Zuo, Chen, & Deng, 2002). On the other hand additional sample pretreatment (e.g. extraction) can be omitted in some cases, due to increased sensitivity/selectivity (Zhu et al., 2004). Rapid LC run associated with significantly lower solvent consumption will reduce the costs and instrument time per analysis.

The aim of this work was to develop and validate rapid, sensitive and selective UHPLC-MS/MS method for simultaneous determination of all eight naturally occurring tea catechins and to demonstrate applicability of method to the analysis of real sample (tea infusion). Production of nutrition supplements became a multi-billion dollar business, but manufactures are required to demonstrate quantity of the active compounds only for pharmaceutical formulation registered in official pharmacopoeias (European Pharmacopoeia 6th edition, 2007; United States Pharmacopoeia 33, 2008). However the worldwide trend points towards more strict regulations. Therefore method will be invaluable tool for quality control authorities or producer himself.

2. Experimental

2.1. Chemicals and reagents

The following standards of flavonoids: (+)-catechin (C), (−)-catechin gallate (Cg), (−)-epicatechin (EC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCg), (−)-gallocatechin (GC), (−)-gallocatechin gallate (GCg) were purchased from Sigma–Aldrich (Steinheim, Germany). The non-fermented tea Formosa Gunpowder was obtained from OXALIS (Slušovice, Czech Republic).

The mobile phase additive ammonia 10%, acetic acid 99.0% (Fluka, Steinheim, Germany) and formic acid 98% p.a. as well as LC–MS grade methanol were obtained from Riedel-de Haen (Seelze, Germany). HPLC grade water, prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA), was additionally filtrated through a 0.22 μm membrane filter immediately before use.

2.2. Instrumentation and analytical conditions

UHPLC–MS/MS system was Acquity UPLC (binary solvent manager, sample manager) and Micromass Quattro micro API benchtop triple quadrupole mass spectrometer (both from Waters, Milford, MA). Electrospray ionization (ESI) source was operated in positive and negative ion detection mode. Following parameters of MS source were set up for positive and negative ion detection mode respectively (tuning range specified in the brackets): desolvation temperature 400 °C, 450 °C (200–450 °C), capillary voltage 3.2 V, 1.5 V (0.5-3.5 V), extractor voltage 2 V, 2 V (0.0–10.0 V), RF lens (hexapole) voltage 1.5 V, 1.5 V (0.0–2.0 V), nebulisation gas flow rate 400 $L.h^{-1}$, 500 $L.h^{-1}$ (200-800 $L.h^{-1}$), cone gas flow rate 150 $L.h^{-1}$, 100 $L.h^{-1}$ (0-150 $L.h^{-1}$) and cone voltage 20 V, 30 V (15–80 V). Selected reaction monitoring (SRM) was performed with dwell time of 150 ms in a) transitions for positive ion detection mode: 307→139 (CE 15 eV), 291→139 (CE 15 eV), 459→139 (CE 20 eV), 443→139 (CE 15 eV) b) transitions for negative ion detection mode: 305→125 (CE 20 eV), 289→123 (CE 30 eV), 457→169 (CE 15 eV), 441→169 (CE 15 eV). The data were acquired and processed using MassLynx software version 4.1 (Waters, Milford, MA, USA). The analytical column C_{18} BEH (100 mm x 2.1 mm i.d.; 1.7 μm, Waters, Milford, MA, USA) was kept at constant temperature (35 °C) during chromatographic separation and injection volume was 1.5 μL. The samples were stored at 4 °C in the sample manager. The eluent A was 0.05% acetic acid in positive ion detection mode and 0.01% formic acid in negative mode. The eluent B was always methanol 100%. The mobile phase flow-rate was 0.45 $mL.min^{-1}$, whilst gradient elution program was applied as follows: initial 25% B; after 1 min percentage of B ramped to 30% at once, followed with isocratic part till 2.5 min; in 2.51 min percentage of B ramped to original conditions (25% B). The equilibration time before next run was 3 minutes.

2.3. Stock solutions and tea samples pretreatment

The daily fresh stock solution of each catechin was prepared to the concentration of 5.10^{-3} M, when accurately weighted amount of analyte was dissolved in methanol. Subsequently adequate volumes of stock solutions were blended resulting in the mixed stock solution containing all 8 catechins in concentration of 5.10^{-4} M. The mixed solution was injected after appropriate dilution by initial mobile phase. The acidic pH of mobile phase enhances stability of catechins (Chen, Zhu, Tsang, & Huang, 2001; Labbé, Têtu, Trudel, & Bazinet, 2008).

Approximately 1.0 g of dry tea mixture (non-fermented tea Formosa Gunpowder), accurately weighed, was extracted with 100 mL of hot water (90 °C) for 5 min with mild stirring. The cooled down tea infusion was filtrated through 0.22 μm membrane filter and further diluted using initial mobile phase by the factor of 20 and 200 for the negative and positive ion detection mode, respectively. Above mentioned dilutions were prepared in six replicates and used to determine intra-day precision. Furthermore 50-fold diluted tea infusion was prepared and spiked with mixed standard solution (GC = 0.31 $mg.L^{-1}$; EGC = 0.31 $mg.L^{-1}$; C = 0.30 $mg.L^{-1}$; EGCg = 0.48 $mg.L^{-1}$; EC = 0.33 $mg.L^{-1}$; GCg = 0.46 $mg.L^{-1}$; ECg = 0.30 $mg.L^{-1}$; Cg = 0.44 $mg.L^{-1}$) to determine accuracy in positive ion detection mode and 400-fold diluted tea infusion was prepared and spiked with mixed standard solution (GC = 2.33 $mg.L^{-1}$; EGC = 2.34 $mg.L^{-1}$; C = 2.26 $mg.L^{-1}$; EGCg = 3.60 $mg.L^{-1}$; EC = 2.44 $mg.L^{-1}$; GCg = 3.42 $mg.L^{-1}$; ECg = 2.26 $mg.L^{-1}$; Cg = 3.32 $mg.L^{-1}$) to determine accuracy

in negative ion detection mode. These experiments were performed in three replicates.

2.4. Method validation

A mixed standard solution at the concentration 5.10^{-6} M was injected in ten repetitions in order to perform system suitability test (SST) measurements. The monitored parameters were the repeatability of retention time and peak area (%RSD). Basic method validation was done in the terms of range, linearity, LOD, LOQ, intra-day precision, and accuracy. The rules for measurement and the limits for the acceptance are given by official pharmacopoeias (European Pharmacopoeia 6th edition, 2007) and appropriate guidelines (International Conference on Harmonization (ICH). Q2(R1): Text on Validation of Analytical Procedures, 2005). Differences between quantitative results achieved in positive and negative ion detection mode were statistically evaluated using t-Test (Bland, 2000).

3. Results and discussion

3.1. UHPLC method development

The initial UHPLC conditions for the separation of catechins were based on the gradient method published by Spacil et al. (2008). However several changes were necessary e.g. extremely rapid gradient elution was slightly prolonged, due to technical parameters of MS instrument (acquisition rate). The different mobile phase additives were tested with consideration of ionisation efficiency, namely acetic and formic acid in the concentration ranging from 0.01-0.1% (v/v). Ammonium hydroxide was also considered as mobile phase additive possibly increasing ionisation efficiency in the negative ion detection mode, but chromatographic separation was precluded at these conditions (data not shown). Maximal response in positive and negative ion detection mode was achieved with 0.05% acetic acid (v/v) and 0.01% formic acid respectively. Despite of reciprocal proportion between concentration of organic acid additive and the response height observed in negative ion detection mode, the concentration of organic acid additive below 0.01% (v/v) resulted in distorted chromatographic peaks. In reference to sensitivity different composition of A eluent was chosen in dependence on ion detection mode, eluent B was always methanol 100%.

3.2. LC elution conditions

An appropriate gradient elution program as described in Section 2.2 enabled to separate all eight catechins mutually including the four non-epi-forms and the four epi-forms within the bare 2.5 min. Fig. 2 displays UHPLC–MS/MS chromatograms acquired using SRM in positive ion detection mode. Catechins were eluted in the order of GC, EGC, C, EGCg, EC, GCg, ECg and Cg, meaning that the epi-forms with gallate (EGCg and ECg) were eluted prior to the non-epi-forms with gallate (GCg and Cg), whilst the non-epi-forms without gallate (GC and C) were eluted prior to the epi-forms without gallate (EGC and EC). The elution order is influenced by present organic modifier, thus LC methods designed for separation of tea

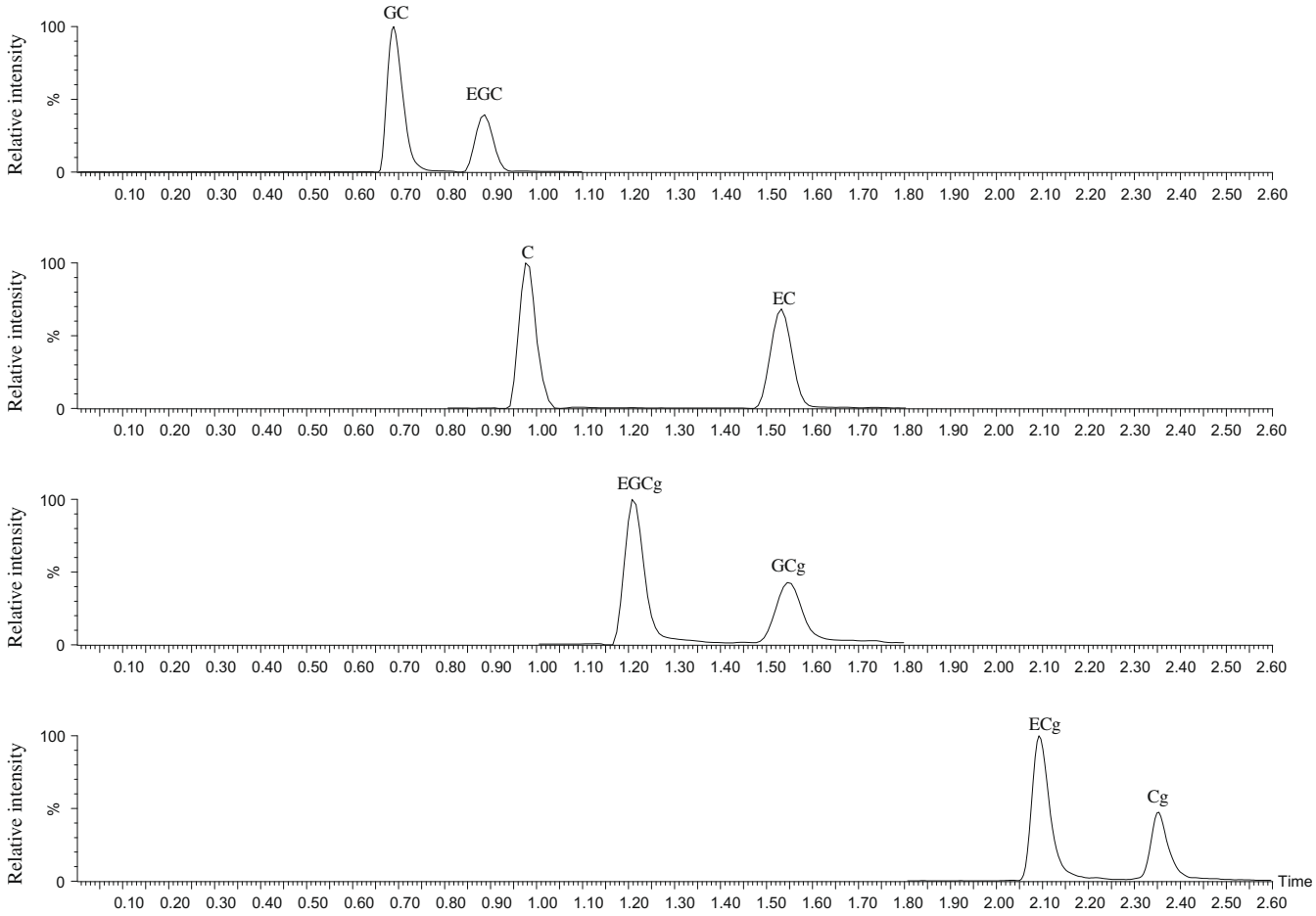

Fig. 2. Typical SRM chromatogram in positive ion detection mode: GC (307 → 139), EGC (307 → 139), C (291 → 139), EC (291 → 139), EGCg (459 → 139), GCg (459 → 139), ECg (443 → 139), Cg (443 → 139).

catechins on reversed phase were using either acetonitrile (Fu et al., 2008; Li et al., 2008; Masukawa et al., 2006; Neilson et al., 2006; Yang et al., 2007; Zhu et al., 2004) or methanol (Pelillo et al., 2004; Spacil et al., 2008; Wang et al., 2003; Zuo et al., 2002). The different selectivity was observed using conventional C_{18} columns, as the peak of EC was eluted ahead of the peak of EGCg, when acetonitrile was applied and the elution order was reversed, when methanol was applied. This observation was consistent in all reported works.

3.3. MS tuning

ESI source parameters were attentively tuned both in positive and negative ion detection mode firstly using single ion monitoring (SIM) to achieve the highest possible signal intensity of precursor ion. Whilst some factors affected the signal intensity minimally (e.g. capillary, extractor and RF lens voltage; cone gas), the others had fundamental effects; e.g. desolvation temperature and nebulising gas flow rate have to be elevated considering mostly aqueous mobile phase and relatively high flow rate. Product ions scans were acquired for both ion detection modes using optimal settings for precursor ion and collision energies ranging from 10 to 40 eV. The most intensive transitions were selected for each monitored precursor *m/z* to obtain optimal selectivity and sensitivity, collision energies were closely optimised and responses plotted in the bar graph (data not shown). Subsequently the optimal transition showing the highest peak area and S/N for detection of each analyte was picked.

3.4. SRM transitions

The same ascertained transitions were used for a non-epi-form and an epi-form of isomeric pairs, since the different configuration on C_2 does not play any role in fragmentation mechanism. The findings were consistent with previous reports (Cren-Olivé, Déprez, Lebrun, Coddeville, & Rolando, 2000; Gonzáles-Manzano, Gonzáles-Paramás, Santos-Buelga, & Duenas, 2009; Miketova et al., 2000). In the positive ion detection mode fragment 139 *m/z* is the most preferable for catechins with absence of gallate. This very important fragment is formed after cleavage of two bonds in the ring C and can originate from: (i) phenolic ring A of all eight catechins or (ii) phenolic ring B of galocatechins (Figs. 3 and 4). This is hypothesised on the basis of occurrence related fragment 123 *m/z* in the MS2 spectra of C, EC, Cg and ECg (Fig. 3a) and not in the MS2 spectra of GC, EGC, GCg and EGCg (Fig. 3b), which can be explained by absence/presence of hydroxyl substitution in position 3′. The optimal collision energies for 139 and 123 *m/z* fragments were also very similar. The lower intense ions 291 and 307 *m/z* were detected applying mild collision energies and corresponded to the loss of gallate. Both of them were associated with even more intense dehydrated ions 273 and 289 *m/z*, respectively.

The occurrence of 125 *m/z* was observed in the negative ion detection mode. Its origin is probably similar to the 139 *m/z* in the positive ion detection mode (Figs. 3 and 4). The reasons for this presumption were two: (i) 125 *m/z* was observed in the MS2 spectra of all catechins, meaning it was certainly derived from the aromatic core A; (ii) ion 109 *m/z* (125 m/z –OH) was present only in the spectra of C, EC, Cg and ECg (Fig. 4a) and not in spectra of galocatechins (GC, EGC, GCg and EGCg) Fig. 4b. High optimal collision energy (40 eV) was needed for generation of fragment 125 *m/z* in the case of catechins gallates. However the loss of gallate (441 → 169 and 457 → 169) was the most intense transition in this case (Fig. 4).

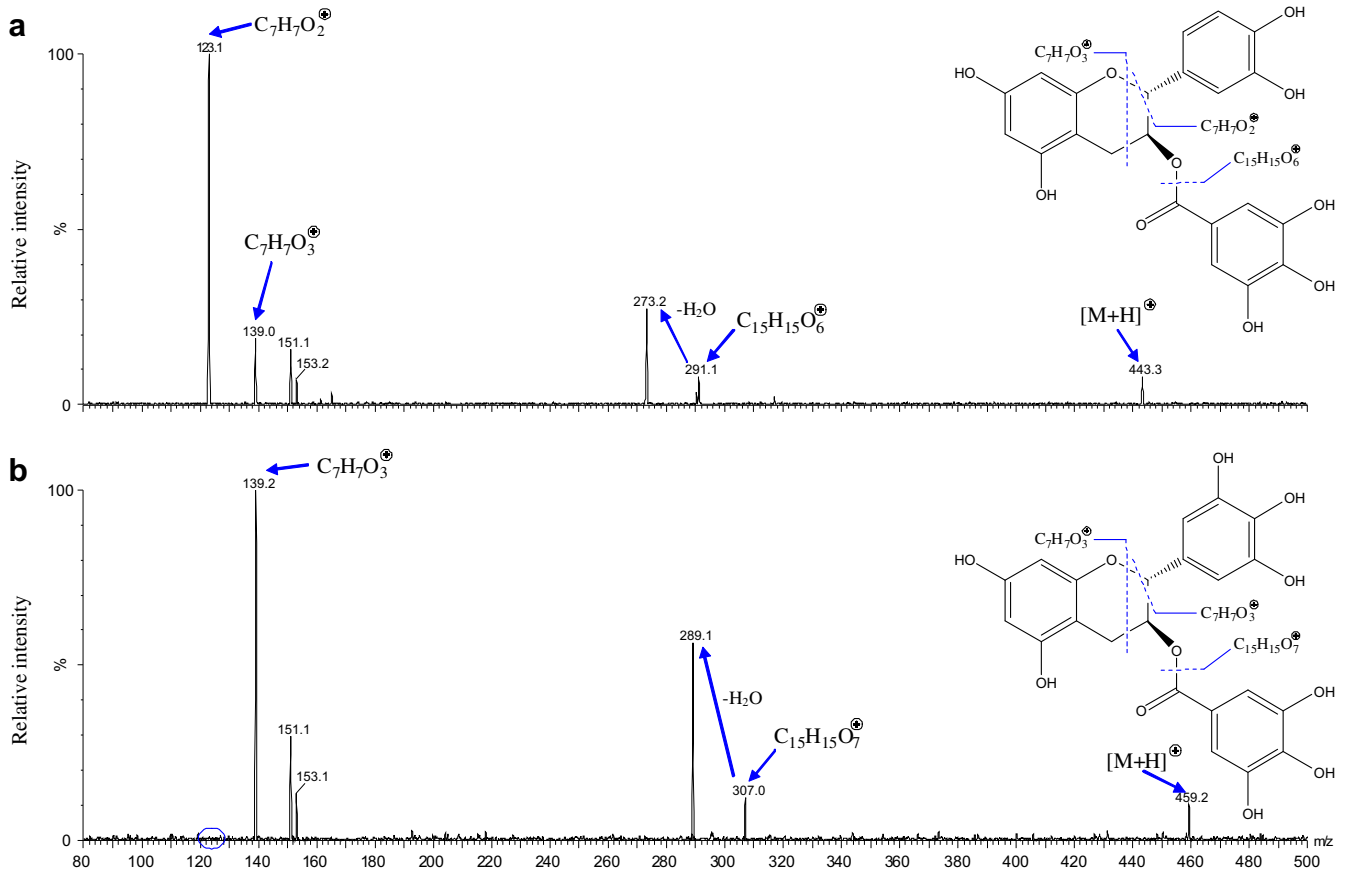

Fig. 3. MS2 spectra of: (a) ECg and (b) EGCg in positive ion detection mode (collision energy 10 eV).

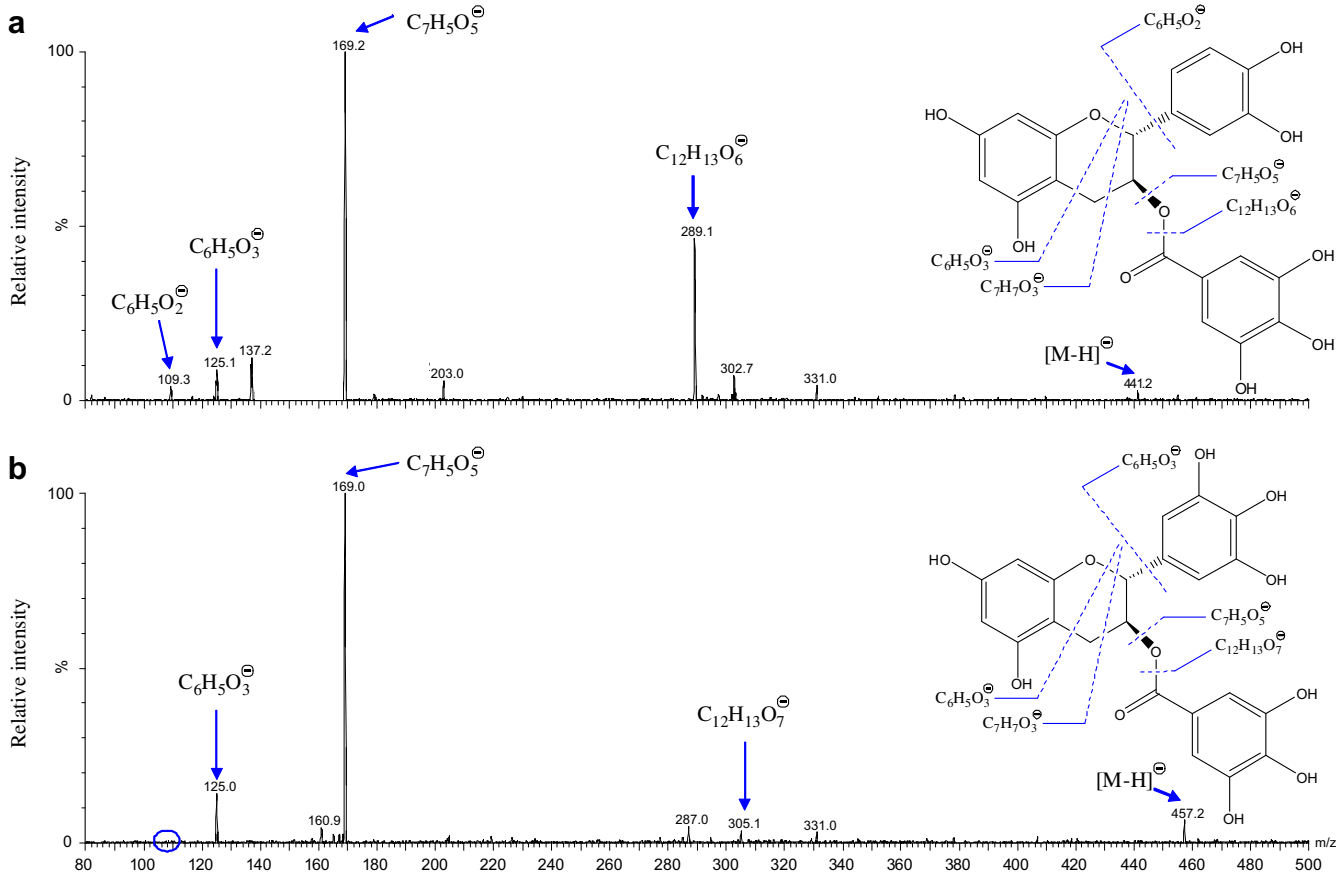

Fig. 4. MS2 spectra of: (a) ECg and (b) EGCg in negative ion detection mode (collision energy 15 eV).

Table 1
Parameters of method validation.

	R_t (% RSD)	Peak area (% RSD)	R^2	Range (mg L^{-1})	LOD (μg L^{-1})	LOQ (μg L^{-1})	Intra-day precision (% RSD)	Accuracy (%)
SRM positive ion detection								
Gallocatechin	0.00	1.16	0.9999	0.02–1.55	3.1	10.2	5.47	113.70
Epigallocatechin	0.54	1.81	0.9999	0.02–1.56	3.1	10.3	4.43	94.06
Catechin	0.00	1.56	0.9999	0.02–1.51	3.0	9.9	5.26	112.55
Epigallocatechin gallate	0.43	2.07	0.9999	0.02–2.40	4.8	15.8	5.03	103.37
Epicatechin	0.21	1.31	0.9997	0.02–1.63	3.3	10.7	5.39	113.38
Gallocatechin gallate	0.31	3.32	0.9999	0.02–2.28	4.6	15.0	–	104.56
Epicatechin gallate	0.42	2.88	0.9999	0.02–1.51	3.0	9.9	7.09	94.67
Catechin gallate	0.44	3.98	0.9999	0.02–2.21	4.4	14.6	–	96.58
SRM negative ion detection								
Gallocatechin	0.00	2.36	0.9998	0.16–15.53	31.1	155.3	4.44	90.39
Epigallocatechin	0.58	3.13	0.9998	0.16–15.59	31.2	155.9	4.20	89.45
Catechin	0.00	3.47	0.9997	0.15–15.05	30.1	150.5	5.14	102.24
Epigallocatechin gallate	0.35	3.35	0.9992	0.24–24.00	48.0	240.0	3.27	102.50
Epicatechin	0.34	2.66	0.9991	0.16–16.26	32.5	162.6	4.54	98.66
Gallocatechin gallate	0.00	3.39	0.9990	0.23–22.80	45.6	228.0	–	103.44
Epicatechin gallate	0.24	3.01	0.9996	0.15–15.05	30.1	150.5	4.41	107.50
Catechin gallate	0.22	3.53	0.9995	0.22–22.10	44.2	221.0	–	108.36

3.5. Method validation

Linearity, range, LOD, LOQ, intra-day precision, accuracy, R_t and peak area (A_p) repeatability expressed as %RSD were determined for SRM both in positive and negative mode. Typical chromatogram is depicted in Fig. 2 and summary of validation results is listed in Table 1. There is notably impaired A_p repeatability in the negative mode (3.1% RSD in average) compared to positive (2.3% RSD in average), due to lower concentration of organic acid in mobile phase, whilst the repeatability of R_t remains the same (0.2% RSD and 0.3% RSD in average for negative and positive ion detection mode, respectively). Five point calibration curve was plotted through the concentration range of two orders of magnitude obtaining an excellent linearity ($R^2 > 0.9990$) for all analytes in both ion detection modes. The typical sensitivity ranged from 10.2 to 16.8 fmol/inj. (LOD) and from 33.7 to 55.5 fmol/inj. (LOQ) in positive ion detection mode and from 102.1 to 168.1 fmol/inj. (LOD) and from 336.8 to 554.6 fmol/inj. (LOQ) in negative ion

Table 2
Quantification of Formosa Gunpowder tea sample.

	Positive ion detection SRM mg/g ± RSD (n_1 = 6)	Negative ion detection SRM mg/g ± RSD (n_2 = 6)	t-test[a] ($n_1 = n_2 = 6$)
Gallocatechin	2.46 ± 0.14	2.34 ± 0.13	1.539
Epigallocatechin	24.14 ± 1.15	23.05 ± 1.06	1.707
Catechin	0.61 ± 0.05	0.57 ± 0.05	1.386
Epigallocatechin gallate	39.69 ± 2.14	39.30 ± 1.38	0.375
Epicatechin	6.49 ± 0.40	6.90 ± 0.37	1.843
Gallocatechin gallate	1.83 ± 0.17	1.89 ± 0.10	0.745
Epicatechin gallate	5.85 ± 0.44	5.89 ± 0.27	0.190
Catechin gallate	Not detected	Not detected	–

[a] At 95% confidence level; $t_{critical}$ = 2.228 (Bland, 2000).

detection mode. These LODs were approximately 10-fold lower than those reported for determination of phenolic acids with the same instrumentation as was used for our study (Gruz et al., 2008). Our results in sensitivity were from 2 to 3 orders of magnitude lower than those reported using UV detection at 231 nm (Yang et al., 2007) or 200 nm (Zhu et al., 2004). Intra-day precision was ranging from 4.43 to 7.09% RSD and 3.27 to 5.14% RSD in the positive and negative ion detection mode, respectively (Table 1). The variances in the recovery of spiked samples within ±14% limit were acceptable considering that plant material was analysed.

3.6. Quantification of tea sample

UHPLC–ESI-MS/MS method was applied to the quantification of catechins in the non-fermented tea Formosa Gunpowder sample. Total content of all catechins 81.1 and 79.9 mg/g was determined using positive and negative ion detection mode, respectively. Detailed representation of each catechin is listed in Table 2. The difference between quantitative data determined using positive and negative ion detection mode are considered to be not statistically significant, by conventional criteria. Taking example from previous reports very similar content of catechins in the same kind of green tea was determined by Wang et al. (2003). Also other recent works (Li et al., 2008; Neilson et al., 2006; Yang et al., 2007) reported cognate constitution of green tea.

4. Conclusions

Newly described UHPLC–MS/MS method represents approximately a 12-fold reduction in total analysis time (2.5 min, as opposed to 30 min on average) with proportional approximately 12-fold reduction in solvent usage associated with typical HPLC methods applied to analyze catechins. The validation data indicate that extremely rapid UHPLC method is repeatable, reproducible and sensitive. Although the method was about 10-fold more sensitive, when performing in positive ion detection mode, negative ion detection mode provides higher selectivity and hence can be useful in case that matrix compound(s) interfere with analyte. The use of this rapid method will reduce risk of sample degradation before analysis and also increase analytical throughput required for complex studies investigating biological activities of catechins. It is important to note that UHPLC is turning to be standard in the family of LC methods, especially when coupled with MS. Therefore this method guarantee broad spectrum of research applications in the analysis of catechins in dietary supplements, tea extracts, tea infusions and also in very complex matrices such as biological fluids and tissues studies involving the administration of tea flavonoids.

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5.8. Supplement IV

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Matrix-less laser desorption/ionisation mass spectrometry of polyphenols in red wine

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Matrix-assisted laser desorption/ionisation (MALDI) of small molecules is challenging and in most cases impossible due to interferences from matrix ions precluding analysis of molecules <300–500 Da. A common matrix such as ferulic acid belongs to an important class of compounds associated with antioxidant activity. If the shared phenolic structure is related to the propensity as an active MALDI matrix then it follows that direct laser desorption/ionisation should be possible for polyphenols. Indeed matrix-less laser desorption/ionisation mass spectrometry is achieved whereby the analyte functions as a matrix and was used to monitor low molecular weight compounds in wine samples. Sensitivity ranging from 0.12–87 pmol/spot was achieved for eight phenolic acids (4-coumaric, 4-hydroxybenzoic, caffeic, ferulic, gallic, protocatechuic, syringic, vanillic) and 0.02 pmol/spot for *trans*-resveratrol. Additionally, 4-coumaric, 4-hydroxybenzoic, caffeic, ferulic, gallic, syringic, vanillic acids and *trans*-resveratrol were identified in wine samples using accurate mass measurements consistent with reported profiles based on liquid chromatography (LC)/MS. Minimal sample pre-treatment make the technique potentially appropriate for fingerprinting, screening and quality control of wine samples.

Phenolic compounds are abundant secondary plant metabolites associated with antioxidant activity. So far more than 200 polyphenols from the group of phenolic acids, flavonoids, stilbenes or anthocyanins have been identified in red wine.[1,2] *trans*-Resveratrol and phenolic acids (PHAs) comprising derivatives of benzoic acid (4-hydroxybenzoic, protocatechuic, vanillic, gallic and syringic acids) and cinnamic acid (4-coumaric, caffeic and ferulic acids) naturally present in grape berries (*Vitis vinifera* sp.) are shown in Fig. 1. Qualitative and quantitative information about PHAs in wine can predict the wine's origin, the wine-making process or can be used for tracking changes during aging.[3] PHAs can also be used as markers of the progression of wine spoilage.[4] In recent years considerable attention has been focused on their health-promoting effects, due to increased incidence of serious pathologies such as cancer, cardiovascular diseases and diabetes.[5,6] Furthermore there are reports on antibacterial effects of these PHAs on pathogenic organisms, e.g. *Listeria monocytogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* or *Staphylococcus aureus*.[7]

Matrix-assisted laser desorption/ionisation (MALDI) is conventionally used for the analysis of biomolecules or large organic molecules. Despite development of several techniques and surfaces the analysis of molecules with mass <300–500 Da is hampered due to interferences from the matrix added to facilitate desorption and ionisation. Different matrices and surfaces have been used to eliminate/reduce matrix effects, such as 9-aminoacridine,[8] high molecular weight matrices,[9] porous silicon,[10–12] carbon allotropes,[13–21] and inorganic materials.[22–25] Desorption/ionisation on porous silicon (DIOS) and surface-assisted laser desorption/ionisation (SALDI) on carbon materials are the most common examples. Silicon surfaces utilised for DIOS have to be converted into porous silicon by electrochemical etching to obtain the desired physical properties. Processed porous silica, though efficient for analysis of small molecules, is unstable in air due to easy oxidation of surface groups causing dramatic changes in wetting properties.[10]

Carbon allotropes such as carbon nanotubes, activated carbon and graphitised carbon black have been evaluated as a surface for desorption/ionisation of small molecules. In spite of the benefits they deliver to the small molecule mass spectrometry community they suffer from several drawbacks. Low sensitivity, probable contamination of the source and a need to use an adhesion agent to keep the particles attached to the stainless steel plate (in case of using larger particle size) are the main disadvantages.

Conventional MALDI matrices are often organic acids able to provide protons to ionise analytes. They also have conjugated aromatic systems which makes them able to absorb more laser energy which also facilitates desorption. Phenolic acids similar to those present in wine have been

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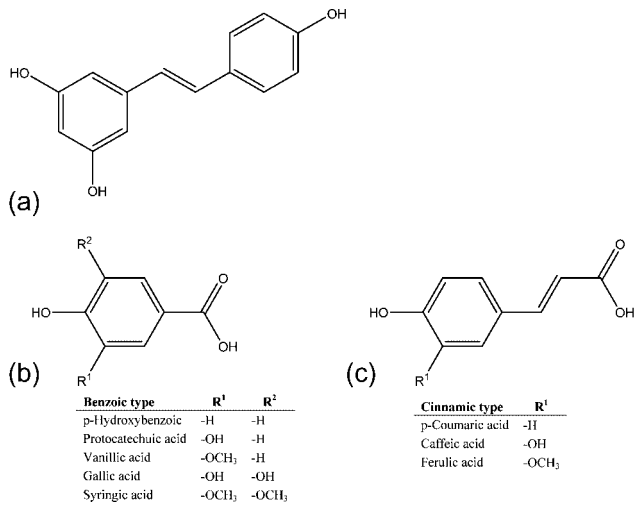

Benzoic type	R^1	R^2
p-Hydroxybenzoic	-H	-H
Protocatechuic acid	-OH	-H
Vanillic acid	$-OCH_3$	-H
Gallic acid	-OH	-OH
Syringic acid	$-OCH_3$	$-OCH_3$

Cinnamic type	R^1
p-Coumaric acid	-H
Caffeic acid	-OH
Ferulic acid	$-OCH_3$

Figure 1. Structural types of analysed polyphenols: (a) *trans*-resveratrol, (b) phenolic acids derived from benzoic acid, and (c) phenolic acids derived from cinnamic acid.

used as matrices in MALDI (e.g. ferulic acid)[26,27] and these shared structural characteristics prompted us to hypothesise that this group of compounds can be analysed by direct laser desorption without use of any matrix or special surface modifications and also assist other compounds present in the sample to be ionised/desorbed. The molecular weights of the target analytes range from 138 to 198 Da; therefore, the matrix-less direct laser desorption/ionisation (LDI) approach offers the advantage of avoiding the interferences from matrix ions in the low molecular weight region.

EXPERIMENTAL

Chemicals and materials

Methanol, trifluoroacetic acid (TFA, ≥99%), cesium acetate (≥99.99%), standard substances: caffeic acid, 4-hydroxybenzoic acid, ferulic acid, gallic acid, 4-coumaric acid, protocatechuic acid, syringic acid, vanillic acid and *trans*-resveratrol, were all obtained from Sigma-Aldrich (Prague, Czech Republic). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) and had a resistance >18 MΩ.cm^{-1}. The red grape wines Cabernet Sauvignon and Merlot were purchased in Systembolaget AB (Stockholm, Sweden). One pair of wine samples came from Concha y Toro winery (Santiago, Chile) and the other pair used for cross-comparison was from Lindemans Winery (Karadoc, Australia). All four wines were of the same vintage 2007. The solid-phase extraction (SPE) cartridge used for pre-treatment of wine samples was a Discovery DPA-6S (polyamide resin base material, 50–160 μm particle size, 3 mL volume, 250 mg packing) from Supelco (Bellefonte, PA, USA).

Pre-treatment of wine samples

Stock solutions of concentration 1.0 g/L were prepared by accurately weighing 1 mg of each standard substance and dissolving it in 1 mL of methanol. These stock solutions were further diluted by factors of 10, 20, 50, 100, 1000 and 10000 and used as working solutions for the LDI experiments.

The SPE cartridges were conditioned by passing 3 mL of methanol followed by 3 mL of water. Each wine sample (2 mL) was passed through a cartridge and the cartridges were washed with 3 mL of 0.1 M TFA. The elution was performed using 2 mL of methanol/0.1 M TFA (80:20 w/w).

Mass spectrometry

LDI spectra were obtained using a Voyager DE-STR time-of-flight mass spectrometer (Applied Biosystems, Framingham, MA, USA) in positive reflectron mode equipped with a 2.0 m flight tube (3.0 m in reflector mode) and pulsed (3 ns) nitrogen laser (337 nm). Voyager Control Panel 5.10 and Data Explorer 4.0.0.0 were used to acquire and process data. Two-point external calibration was made using 4-hydroxybenzoic acid ($[M+H]^+$ monoisotopic mass 139.04 Da) and syringic acid ($[M+H]^+$ monoisotopic mass 199.06 Da) as calibrants and resulted in 80 ppm mass accuracy. The resolving power of the acquired mass spectra was typically 5000 FWHM. The limit of detection (LOD) and quantification (LOQ) were defined as signal-to-noise (S/N) ratio = 3 and 10, respectively, and were determined by spotting standard solutions in the concentration range from 100 ppb to 100 ppm in duplicates. To acquire mass spectra, 200 laser shots were collected randomly within a radius of 700 microns from the centre of the spot.

RESULTS AND DISCUSSION

Preliminary LDI analyses

The LDI spectra of standard solutions (10 ppm) of PHAs and *trans*-resveratrol were collected in the positive ion detection mode without the addition of any matrix (see Fig. 2(a)). Matrix-less LDI is suited for these compounds, due to their structure which has not been reported previously. The spectrum of a blank sample is presented in Fig. 2(b). Spectra were recorded also in negative ion detection mode given the acidic nature of the analytes, but no signal was observed even if ammonium hydroxide was used to enhance ionisation.

In the positive ion spectra it was not possible to distinguish the sodiated and potassiated PHAs with *m/z* values 177.0, 193.0 and 203.0 (see Table 1) due to the resolution of the instrument. However, they are most likely sodiated protocatechuic, gallic and caffeic acids since the occurrence of these ions is more common. The attempt to overcome this shortcoming was made using cesium acetate, in order to distinguish overlapping adducts of phenolic acids. Mostly $[M+Cs]^+$ adducts were formed when the concentration ratio between Cs^+ and each analyte was approx. 1:1 (Fig. 3(a)) and predominantly $[M+2Cs–H]^+$ adducts were observed when the ratio was 10:1 (Fig. 3(b)). Moreover, suppression of signals belonging to PHAs was observed in the presence of Cs^+, when working with lower concentrations of PHAs (<10 ppm) or greater abundance of Cs^+ compared to PHA (>10:1), while the signal of *trans*-resveratrol (228.1 *m/z*) appeared to be unaffected. The suppression observed is apparently due to the PHA-Cs^+ adduct being less efficiently desorbed/ionised compared to the PHA-Na^+/K^+ adduct. Significant contamination of the MALDI plate was caused by Cs^+, which was difficult to remove by applying conventional cleaning protocols. Due to the above mentioned reasons, Cs^+ was not used further.

Apart from the ions at 227.1 *m/z* for oxidised and 229.1 *m/z* for non-oxidised forms of *trans*-resveratrol which were

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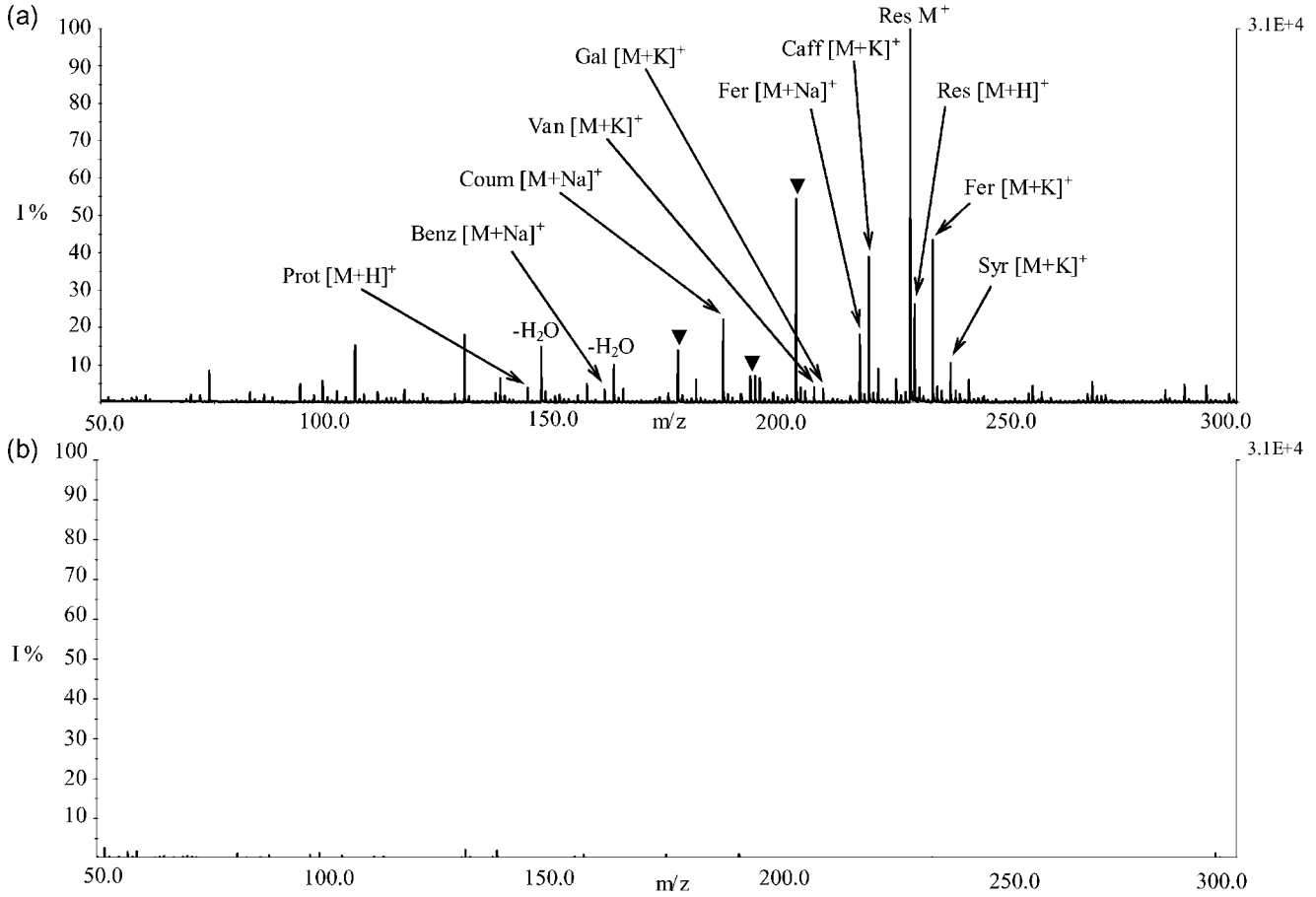

Figure 2. Positive-ion matrix-less LDI mass spectra of (a) phenolic acids and *trans*-resveratrol standards with a concentration of 10 ppm; (b) 0.1% TFA in water. Peaks corresponding to Na^+/K^+ adducts of phenolic acids which cannot be assigned due to low resolution of the instrument are labelled with triangles. Peaks marked '-H_2O' correspond to the H_2O-loss fragments.

expected, the major signal was at 228.1 *m/z*. The same ion (228.1 *m/z*) has been reported by Domínguez *et al*. when using liquid chromatography/mass spectrometry (LC/MS) for the analysis of *trans*-resveratrol.[28] This ion is probably the radical molecular ion of *trans*-resveratrol.

The LODs are presented in Table 2. Despite the vast acceptance of LDI-MS, the mechanism of the desorption/ionisation process is still not well understood. For instance, desorption/ionisation efficiencies of MALDI for various biomolecules can be diametrically diverse depending on the dihydroxybenzoic acid isomer.[29] This example demonstrates the different properties of very similar molecules analogous to the large variance observed in LODs of PHAs and *trans*-resveratrol. Looking at the structures of polyphenols we can speculate that the efficiency of desorption/ionisation in matrix-less LDI is related to the number of delocalised electron pairs. Indeed *trans*-resveratrol (LOD = 0.02 pmol/spot) has 13 delocalised electron pairs (7 conjugated double bonds and 6 electron pairs belonging to -OH groups); on the other hand, 4-hydroxybenzoic acid (LOD = 14.06 pmol/spot) includes only 8 delocalised electron pairs (4 conjugated double bonds and 4 electron pairs belonging to -OH groups). This trend towards easier desorption/ionisation of the analyte with the higher number of delocalised electron pairs as a primary attribute and the higher number of conjugated double bonds as secondary attribute is consistent for our analytes.

Apart from analysis of anthocyanins there is limited literature on MALDI or LDI dealing with polyphenols and even less data for LOD comparison. However, Grant and Helleur[30] analysed 4-coumaric acid by MALDI with α-cyano-4-hydroxycinnamic acid as matrix and used several surfactants to suppress generation of matrix-related ions. Approximately 10 ng (65 pmol) of standard was used for spotting on the MALDI plate in this work, which is more than 10-fold higher compared to our LOD for the same ion. Moreover, about 60% reduction in the peak intensity was observed after adding surfactant. Recently published ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS) analysis of phenolic acids[31] presented LODs of all eight phenolic acids in the range of 1–15 pmol on-column, which corresponds to our LODs in the range of 0.12–87 pmol/spot. In comparison with the above-mentioned studies our LODs are fully comparable with LDI or LC/MS methods.

Pre-treatment and spotting of wine samples on the MALDI plate

It is not possible to spot and analyse wine samples directly by LDI, without any sample pre-treatment, due to the presence of high concentrations (>2 g.L^{-1}) of glucose and fructose which form a glassy film concealing analytes.[32] Therefore, a SPE cartridge with polyamide resin base material was used for sample cleanup. This type of sorbent adsorbs PHAs from

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Table 1. Determined LOD and LOQ values for phenolic acids and *trans*-resveratrol using the matrix-less LDI method

			LOD		
		Exact mass	mg/L	ng spotted	pmol spotted
4-Hydroxybenzoic acid	$[M+H]^+$	139.0	14.06	2.81	20.37
	$[M+Na]^+$	161.0	60.00	12.00	86.88
	$[M+K]^+$	177.0	—	—	—
Protocatechuic acid	$[M+H]^+$	155.0	1.22	0.24	1.58
	$[M+Na]^+$	177.0	—	—	—
	$[M+K]^+$	193.0	—	—	—
4-Coumaic acid	$[M+H]^+$	165.1	3.88	0.78	4.73
	$[M+Na]^+$	187.0	0.36	0.07	0.44
	$[M+K]^+$	203.0	—	—	—
Vanillic acid	$[M+H]^+$	169.1	ND	ND	ND
	$[M+Na]^+$	191.0	1.02	0.20	1.21
	$[M+K]^+$	207.0	35.83	7.17	42.61
Gallic acid	$[M+H]^+$	171.0	ND	ND	ND
	$[M+Na]^+$	193.0	—	—	—
	$[M+K]^+$	209.0	0.10	0.02	0.12
Caffeic acid	$[M+H]^+$	181.1	2.77	0.55	3.07
	$[M+Na]^+$	203.0	—	—	—
	$[M+K]^+$	219.0	0.24	0.05	0.27
Ferulic acid	$[M+H]^+$	195.1	1.46	0.29	1.50
	$[M+Na]^+$	217.0	0.40	0.08	0.41
	$[M+K]^+$	233.0	0.90	0.18	0.93
Syringic acid	$[M+H]^+$	199.1	0.93	0.19	0.93
	$[M+Na]^+$	221.0	0.15	0.03	0.15
	$[M+K]^+$	237.0	2.00	0.40	2.02
trans-Resveratrol	ox. $[M+H]^+$	227.1	0.10	0.02	0.09
	$M^{\cdot+}$	228.1	0.02	0.00	0.02
	$[M+H]^+$	229.1	0.35	0.07	0.31

ND: not detected

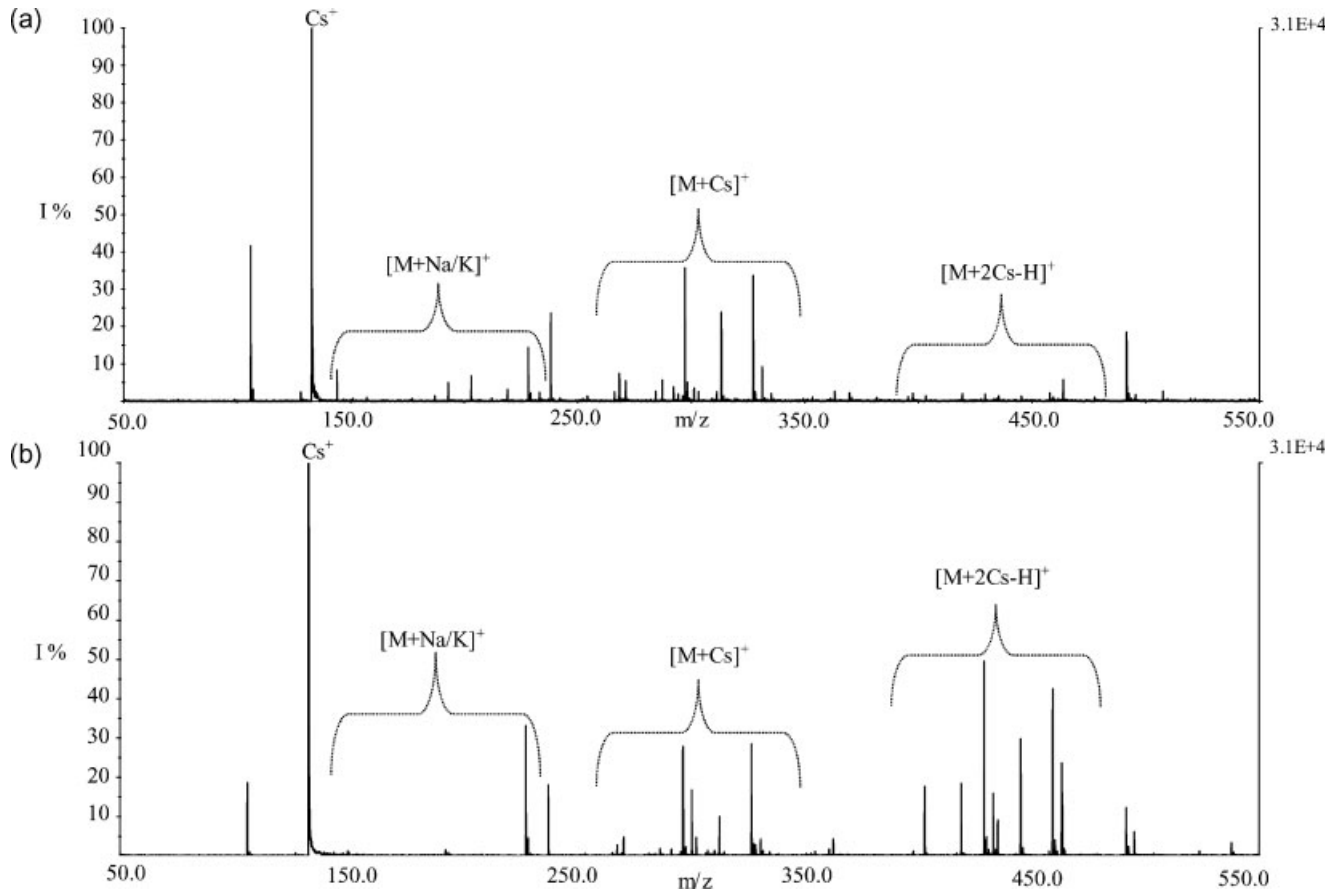

Figure 3. Positive-ion matrix-less LDI mass spectra of phenolic acids and *trans*-resveratrol standards (10 ppm) (a) in the presence of Cs^+ (10 ppm) and (b) in the presence of Cs^+ (100 ppm).

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Table 2. Analysis of red wine Cabernet Sauvignon (Concha y Toro, Chile). $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were identified with semi-quantitative determination for the most intense peaks (n = 6). Results compared to literature[35–37]

Compound	$[M+H]^+$ (S/N)	$[M+Na]^+$ (S/N)	$[M+K]^+$ (S/N)	Cabernet Sauvignon (mg/L)	Hamoudova *et al.*[35] (mg/L)	del Alamo *et al.*[36] (mg/L)	Lee and Rennaker[37] (mg/L)
4-Hydroxybenzoic acid	9.2	6.2	—	<LOQ	—	—	—
Protocatechuic acid	ND	—	—	ND	2.40	<LOD-0.87	—
4-Coumaic acid	ND	7.8	—	3.22	2.12	<LOD-2.21	—
Vanillic acid	ND	6.0	6.1	<LOQ	0.61	0.09–1.04	—
Gallic acid	ND	—	11.2	3.80	22.04[a]	1.0–16.4	—
Caffeic acid	22.6	—	29.8	2.28		<LOD-2.26	—
Ferulic acid	ND	ND	ND	ND	1.38	<LOD	—
Syringic acid	ND	14.7	9.8	2.60	3.96	<LOD-1.79	—
	ox. $[M+H]^+$	$M^{\cdot+}$	$[M+H]^+$				
trans-Resveratrol	ND	12.8	ND	0.09	—	—	<LOD-3.07

ND: not detected.
[a] Sum total gallic acid and caffeic acid.

aqueous or methanolic solutions under the reversed-phase mechanism through strong hydrogen bonding between hydroxyl groups of the compound and amide groups of the resin. It is useful for extracting PHAs from the wine samples and removing the neutral sugars, as reported in detail by Matejicek *et al.*[33] This study carried out on Cabernet Sauvignon and Merlot wines provides excellent recoveries (85.2–98.4%) with relative standard deviation (RSD) = 1.9–3.5%.

The signals corresponding to six PHAs and *trans*-resveratrol can be found in the LDI mass spectrum of red grape wine (Fig. 4). The phenolic acid profile is consistent with that obtained by the UPLC[34] method coupled to MS detection (data not shown). To determine concentrations of four major PHAs and *trans*-resveratrol in the Cabernet Sauvignon wine from Chile, S/N ratio comparisons were made between standard solutions and samples of interest (Table 2). Although this is a semi-quantitative determination very similar concentrations of PHAs were reported by Hamoudova *et al.*[35] in red wine of the same variety and by del Alamo *et al.*[36] in young Spanish wines. The estimated concentration of *trans*-resveratrol is in agreement with the results reported by Lee and Rennaker[37] on 12 samples of Cabernet Sauvignon wine grown in Idaho. Furthermore, all four wine samples were compared using S/N ratios of detected analytes (Table 3). Generally, there is a higher content of all detected PHAs in wines from Chile, comparing

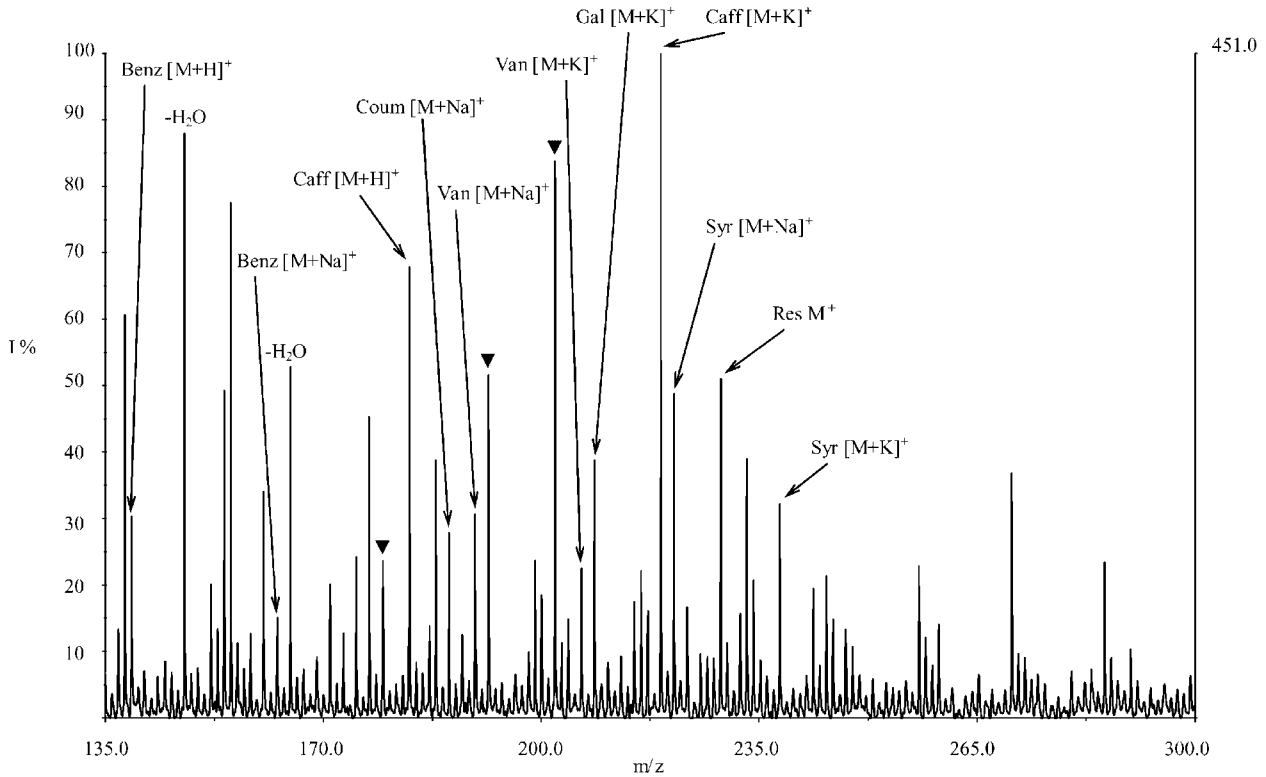

Figure 4. Positive-ion matrix-less LDI mass spectrum of Cabernet Sauvignon indicating identified phenolic acids. Peaks corresponding to Na^+/K^+ adducts of phenolic acids which cannot be assigned due to low resolution of the instrument are labelled with triangles. Peaks marked with '$-H_2O$' correspond to the H_2O-loss fragments.

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Table 3. Signal-to-noise (S/N) ratio comparison of wine samples (n = 3)

			Valle Central, Chile		South-east Australia	
		Exact Mass	Cabernet Sauvignon	Merlot	Cabernet Sauvignon	Merlot
4-Hydroxybenzoic acid	$[M+H]^+$	139.0	9.5	9.9	7.5	10.1
	$[M+Na]^+$	161.0	6.2	8.3	3.4	4.1
	$[M+K]^+$	177.0	12.6[a]	13.4[a]	3.5[a]	8.3[a]
Protocatechuic acid	$[M+H]^+$	155.0	ND	ND	ND	ND
	$[M+Na]^+$	177.0	12.6[a]	13.4[a]	3.5[a]	8.3[a]
	$[M+K]^+$	193.0	18.9[b]	21.0[b]	4.0[b]	6.0[b]
4-Coumaic acid	$[M+H]^+$	165.1	ND	ND	ND	ND
	$[M+Na]^+$	187.0	7.2	14.9	ND	ND
	$[M+K]^+$	203.0	18.2[c]	31.9[c]	5.3[c]	17.1[c]
Vanillic acid	$[M+H]^+$	169.1	ND	ND	ND	ND
	$[M+Na]^+$	191.0	6.0	7.6	ND	3.6
	$[M+K]^+$	207.0	6.9	8.3	ND	ND
Gallic acid	$[M+H]^+$	171.0	ND	ND	ND	ND
	$[M+Na]^+$	193.0	18.9[b]	21.0[b]	4.0[b]	6.0[b]
	$[M+K]^+$	209.0	16.8	13.7	ND	5.8
Caffeic acid	$[M+H]^+$	181.1	32.1	31.0	18.3	18.4
	$[M+Na]^+$	203.0	18.2[c]	31.9[c]	5.3[c]	17.1[c]
	$[M+K]^+$	219.0	13.7	15.5	6.3	9.8
Ferulic acid	$[M+H]^+$	195.1	ND	ND	ND	ND
	$[M+Na]^+$	217.0	ND	ND	ND	ND
	$[M+K]^+$	233.0	ND	ND	ND	ND
Syringic acid	$[M+H]^+$	199.1	ND	ND	ND	ND
	$[M+Na]^+$	221.0	18.7	21.4	3.7	9.9
	$[M+K]^+$	237.0	6.7	12.1	3.2	8.3
trans-Resveratrol	ox. $[M+H]^+$	227.1	ND	ND	ND	ND
	$M.^+$	228.1	15.1	26.2	ND	31.5
	$[M+H]^+$	229.1	ND	5.1	ND	4.0

ND: not detected.
[a] Sum of $[4\text{-hydroxybenzoic acid} + K]^+$ and $[\text{protocatechuic acid} + Na]^+$.
[b] Sum of $[\text{protocatechuic acid} + K]^+$ and $[\text{gallic acid} + Na]^+$.
[c] Sum of $[4\text{-coumaic acid} + K]^+$ and $[\text{caffeic acid} + Na]^+$.

geographical origin and higher content of the most of PHAs in Merlot wines, comparing varieties. Signals of PHAs in Merlot and Cabernet Sauvignon samples from Chile are on average 2- or 3-fold higher compared to Australian Merlot and Cabernet Sauvignon, respectively. On the other hand the content of *trans*-resveratrol is significantly higher in Merlot compared to Cabernet Sauvignon wines regardless of the geographical origin of the wine. Taken together, results show that identified peaks associated with various PHAs can be used for fingerprinting. Furthermore, the intense peaks at 177.0, 193.0 and 203.0 *m/z*, although they cannot be assigned to the particular PHA due to the resolution of the instrument, can also aid in fingerprinting.

CONCLUSIONS

The described method details the analysis of phenolic acids and *trans*-resveratrol without addition of any external matrix. This allows a versatile and potentially high-throughput analysis of natural samples for the purpose of profiling and quality control. The minimal sample preparation involved ensures wide applicability for various natural and manufactured food products at very low cost. Furthermore, this can be used as a screening method for subsequent LC/MS quantitative analysis. In this way time-consuming LC/MS separation can be avoided in the case of negative results from LDI. The method can be envisaged as a component of analytical protocols, for example, to assay functional foods. To the best of our knowledge this is the first sufficiently detailed attempt to fingerprint low molecular weight phenolics in wine by LDI apart from analysis of anthocyanins.

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6. CONCLUSIONS

Pharmaceutically important polyphenols (PPs) were analyzed in this doctoral thesis utilizing novelty aspects of LC and MS. Specifically, four groups of phenolic compounds (phenolic acids, catechins, flavonoids and coumarins) were chromatographically separated by gradient elution using conventional HPLC and UHPLC to assess the advantages of UHPLC over HPLC. The feasibility and advantages of using rapid LC methods to separate PPs were further evaluated analyzing a wide range of different PPs in a single chromatographic run, thus achieving simultaneous separation and quantification of 29 PPs in 20 minutes. The benefits of UHPLC-MS/MS coupling were demonstrated analyzing tea catechins. Alternative way to analyse PPs is represented by LDI-TOF technique, while acquired results were confirmed by UHPLC-MS. The author's idea was based on shared structural characteristics of PPs and commonly used MALDI matrices, potentially leading to the efficient absorption of laser energy and thus facile desorption/ionisation. This is illustrated on the experiments with phenolic acids, but is applicable to the other groups of PPs with extended system of conjugated π electrons.

The fundamentals of physics and chemistry are essential for complete understanding of the analyte properties and its interaction with other entities present in the sample. The LC based analytical methodology was improved, clearly showing that the exploitation of insights gained from understanding the structure and chemical properties of analytes, and the operating principles of available equipment facilitates the development of innovative analytical techniques.

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