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Entwicklung von LC-MS Methoden im Rahmen des BOKU Netzwerkes für Schad- und Naturstoffanalytik

Department für Chemie

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Danksagung

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Preface

The performed dissertation was accomplished ad the University of Applied Life Science and Natural Resources, BOKU, in Vienna between July 2006 and June 2009. The thesis was part of the BOKU network “SUNA” (Netzwerk für Schad- und Naturstoffanalytik) under the direction of Assoc. Prof. Stephan Hann and ao. Prof. Gunda Koellensperger. SUNA is a platform with the objective to extend inter-department collaboration.

The aims are the implementation and application of organic mass spectrometry for projects carried out at the following departments:

- Department of Chemistry
- Department of Forest and Soil Sciences
- Department of Water, Atmosphere and Environment
- Department of Material Sciences and Process Engineering
- Department of Sustainable Agricultural Systems
- Department of Biotechnology

As the first PhD student working within SUNA I had the challenging task to get familiar with the already available and the new instrumentation and software packages. The first application was the method development for the analysis of phenols and polyphenols in authentic wine. This project was performed in collaboration with the Faculty of Food Science of the Corvinus University in Budapest with the financial support of the Austrian and Hungarian Research Fund resulting in a poster presentation at an international scientific conference, the 27th International Symposium on Chromatography, 21.-25.09.2008 in Münster, Germany, an oral presentation at a national scientific convention, the ASAC meeting 2008 in Vienna, and a scientific paper accepted at the journal *Food Chemistry* 2010.
The second project within the SUNA framework was collaboration with the Department of Forest- and Soil Sciences, BOKU, Vienna and the objective for the project was the development of an analytical method for the determination of organic acids in soil and root related samples resulting in a poster presentation at an international scientific conference, ASMS in Philadelphia, 2009, and a scientific paper. The description of the experimental work and the applied analytical methods are detailed in the following sections.

Abstract

This thesis outlines the method development and the application of analytical methods on high performance liquid chromatography instruments coupled to organic mass spectrometers as LC-TOF-MS and LC-triplequad MS.

A part of this work is dedicated to the development of rapid and sensitive LC-MS/MS method for quantification of phenols and polyphenols in authentic wine samples performed on a triplequad mass spectrometer. Authentic wine samples were obtained from the Federal College and Research Institute for Viticulture and Pomology in Klosterneuburg (HBLAuBA). The samples used for the study were 97 authentically vinificated red wines from six different grape varieties. The grapes were obtained from eleven different wine regions in Austria including five vintages from 2003 to 2007 Phenols and polyphenols were separated via reversed phase HPLC employing sub-2 µm particles as stationary phase. Quantification was performed via external calibration, with internal standardization using multiple reaction monitoring by LC-MS/MS. Via canonical discriminant analysis the wines were successfully related according to their origin, grape variety and vintage. Finally this work resulted in a scientific paper in the peer reviewed journal *Food Chemistry.*

Within the second part of the thesis a novel method for sensitive and accurate quantification of organic acids as oxalic, malic and citric acid in soil and root related samples was developed. The method comprises a fast and simple derivatization procedure and subsequent analysis via LC-MSTOF. The carboxylic acid residues were esterified with benzyl alcohol, which increased the capacity factor due to reversed phase material and ionization efficiency. Quantification of the analytes was performed via internal standardization utilizing 13C- labeled oxalic acid. The method was applied on several soil related samples. The samples were purchased from the department of forest and soil science of the University of Applied life science and Natural Resources. The successful application of the novel method resulted in a scientific paper submitted to the journal *Analytical and Bioanalytical Chemistry.*

Kurzfassung

Die vorgelegte Dissertation befasst sich mit der Methodenentwicklung für Hochleistungsflüssigkeischromatographie gekoppelt mit organischen Massenspektrometern wie LC-TOF-MS and LC-triplequad MS sowie deren Anwendung.

Der erste Teil der Arbeit beinhaltet die Entwicklung einer schnellen und empfindlichen LC-MS/MS Methode zur Quantifizierung von Phenolen und Polyphenolen in authentischen Weinproben. Die Proben wurden von der HBLA und Bundesamt für Wein- und Obstbau Klosterneuburg zur Verfügung gestellt.

97 authentische Weine aus 11 Weinregionen Österreichs sowie 6 unterschiedliche Rebsorten von 5 aufeinander folgenden Jahrgängen wurden bezüglich ihres Phenolspektrums untersucht. Die Analyten wurden mittels reversed phase HPLC von einander getrennt. Die Quantifizierung erfolgte über externe Kalibrierung und der Verwendung eines internen Standards mittels eines triplequad Massenspektrometers. Anschließend erfolgte unter zu Hilfenahme der kanonischen Diskriminantenanalyse die Zuordnung der Weine nach Herkunft, Rebsorte und Jahrgang. Die erfolgreiche Applikation der Methode sowie die Datenanalyse wurden im wissenschaftlichen Journal *Food Chemistry* publiziert.

Der zweite Teil der Arbeit beinhaltet die Entwicklung einer neuen Methode zur exakten Quantifizierung von organischen Säuren wie Oxalsäure, Äpfelsäure und Zitronensäure in Bodenproben beziehungsweise Wurzelexsudaten. Die Neu Methode besteht aus einer schnellen und einfach zu handhabenden Derivatisierung der Säurereste mit Benzylalkohol und der anschließenden Analyse mittels eines LC-TOFMS Systems. Aufgrund der Derivatisierung wurde die Polarität der zu untersuchenden Analyten drastisch herabgesetzt, somit konnte für die Chromatographische Trennung eine reversed phase Säule verwendet werden und weiters konnte dadurch die Ionisierungseffizienz erhöht werden. Die Quantifizierung erfolgte mittels externer Kalibration unter zu Hilfenahme einer mit ^{13}C angereicherten Oxalsäure als interner Standard. Die Boden,- sowie die Wurzelexsudatproben wurden von Department für Wald und Bodenwissenschaften der Universität für Bodenkultur bezogen. Die erfolgreiche Anwendung der neuen Methode zur Bestimmung von organischen Säuren in Böden sowie in Wurzelexsudaten wird im Journal *Analytical and Bioanalytical Chemistry* publiziert.

1 Introduction

1.1 Chromatography

1.1.1 Liquid chromatography

Chromatography is the widest spread analytical separation technique in science for the separation of analytes with similar characteristics. The applications for chromatography can be the identification, the quantification and the purification of analytes. Column chromatography was first used of the Russian biologist Michail Tsvet in 1903. The application concerned the separation of plant pigments via a glass column filled with calcium carbonate[1]. During the last century the number of application and techniques regarding chromatography was consequently increasing due to the rising requirements to characterize more and more complex compound mixtures. The principle of chromatography is the interaction of analytes between a stationary and a mobile phase.

1.1.2 Classification of chromatography

In gas chromatography the stationary phase is a liquid and the mobile phase is gaseous, in liquid chromatography the stationary phase is solid and the mobile phase is liquid. The major aim of both techniques is the separation of a mixture into its single components and their subsequent detection. The principle is the different interaction of the analytes with the stationary phase and the mobile phase, respectively. The movement of the analyte is limited to the mobile phase and consequently the retention time of an analyte depends on its distribution in the mobile phase. The efficiency of a chromatographic column depends on the relative velocities of the analytes in the system. Those velocities strongly depend on the Nernst distribution concentration [2,3,4]**.**

$$K = \frac{c_S}{c_M}$$

Equation 1: c_S and c_M are the molar concentrations in the stationary phase and the mobile phase respectively

The retention time t_R is the time an analyte needs after injection until detection. The dead time t_M is the time a not retarded substance (no interaction between analyte and stationary phase) needs from injection until its detection and it is equal the velocity of the mobile phase molecules

For analytes which are retained via e.g. lipophilic interactions on the stationary phase the retention time is longer and due to the retention time differences at the best the analytes are separated. However, during migration of the analytes through the separation system a separation counteracting effect is broadening the sample zones. This broadening is greater the longer the migration distance is. The relation between the retention time and the distribution coefficient is [5]:

$$v = \frac{L}{t_M} * \frac{1}{1 + \frac{KV_S}{V_M}} \qquad k' = \frac{KV_S}{V_M}$$

Equation 2: The relation between the retention time and the capacity coefficient; L is the length of the packed column, t_M is defined as the dead time, K is the Nernst distribution concentration, V_M and V_S are the volumes of the mobile and the stationary phase respectively

The capacity coefficient k' equ.3 is an important parameter for the description of retention times of analytes on chromatographic columns.

$$k' = \frac{t_R - t_M}{t_M}$$

Equation 3: The determination of the capacity coefficient via the retention time of an analyte and the dead time

The determination of the capacity coefficient is determined via the retention time of an analyte and the dead time. During the migration of an analyte through a column a broadening of the sample zone occurs resulting in a Gaussian bell shaped curve or normal distribution. Due to different residence of an analyte on the stationary phase and consequently different retention times of the individual analyte molecules a symmetric distribution around the zone center is observable. The Gaussian bell curve is described via 3 parameters i) the zone center witch is equal to the retention time t_R, ii) the height of the curve and iii) the width of the curve expressed as the standard deviation σ. As a rule the broadening of the zone increases with increasing retention time and distance [5].

1.1.3 Efficiency in chromatography

The efficiency in chromatography is described in terms of theoretical plate heights H and theoretical plate number N, respectively.

$$N = \frac{L}{H}$$

Equation 4: description of the efficiency of a chromatographic column via the length of the column L, the theoretical plate height H and the theoretical plate number N

The plate height H describes the dispersion of a peak caused by the mixing of the analyte with the mobile phase and the distribution of the individual analyte molecules in the stationary phase. Therefore the plate height can be described in terms of the standard deviation squared σ^2 (variance of a Gaussian distribution) and length of the column. As a consequence, the smaller the variance σ, the smaller is the plate height *H,* and the higher is the number of plates *N* [2,3,5].

$$H = \frac{\sigma^2}{L} \qquad N = 5.54\left(\frac{t_R}{b_{0.5}}\right)^2$$

Equation 5:

$$H = H_{conv} + H_{diff} + H_{C_S u}, H_{C_M u}$$

Equation 6: the plate height H is the sum of three contributors which have an impact on the number of H.

- The 1st term in equ.6 is the Eddy- or turbulent diffusion and it describes distribution of an analyte due to turbulent mixing in a chromatographic column with a packed bed. The paths of the individual analyte molecules can vary within the retention times and due to of inhomogeneities regarding the flow velocity of the mobile phase and the particle diameters. Consequently the turbulent diffusion is a function of the diffusion coefficient in the mobile phase D_M, the particle diameter d_P and the velocity of the mobile phase *u* [2,5].

$$H_{conv} = f(u, d_P, D_M)$$

Equation 7:

- The 2nd contributor H_{diff} is the longitudinal diffusion according to 1st Fick's law and the term describes the concentration gradient along and against the flow direction of the mobile phase.

$$\frac{dc}{dt} = -D\frac{dc}{dz}$$

Equation 8: 1st Fick's law

$$\frac{B}{u} = \frac{2k_D D_M}{u}$$

Equation 9: Description of the longitudinal diffusion with B as the longitudinal diffusion coefficient, u as the velocity of the mobile Phase, k_D as a constant related longitudinal diffusion and D_M as the diffusion coefficient of the mobile phase.

The dependency of the concentration on time demonstrates the relation of the duration of the chromatographic process and the velocity of the mobile phase (the higher the flow rate the smaller is the contribution of the longitudinal diffusion to H [2].

- The 3rd term of equ.6 describes the mass transfer of the stationary phase $C_S u$ and $C_M u$ and its contribution to H. For the characterization of $C_S u$ it is important to distinguish between a liquid stationary phase equ. 9 and a solid stationary phase equ. 10.

$$C_S u = \frac{qk' d_f^2 u}{(1+k')^2 D_S}$$

Equation 10:

$$C_S u = \frac{2t_d k' u}{(1+k')^2}$$

Equation 11:

The mass transfer coefficient, $C_S u$, for a liquid stationary phase (equation 9) is a function of the thickness of the liquid stationary phase squared d_f^2, the constant q, the capacity factor k' and the diffusion coefficient of the analyte related to the stationary phase D_S. A general rule regarding the thickness of the stationary phase is the thicker the phase the longer is the time of mass transfer in the stationary phase.
For solid stationary phases is the mass transfer coefficient $C_S u$ dependent on the time t_d an analyte needs for adsorption or desorption respectively and the velocity constant k'.
The mass transfer term of the mobile phase $C_M u$ is determined via a function including the column diameter d_c, the particle diameter d_p the velocity of the mobile phase u and the diffusion coefficient of the mobile phase D_M [2,3,5].

The summed up three terms is resulting in the van Deemter equation, equ.12 and it is the simplified description of the theoretical plate height of a chromatographic column dependent on the flow velocity of the mobile phase[6].

$$H = A + \frac{B}{u} + Cu$$

Equation 12: van Deemter equation with the incremental contributions of the Eddy diffusion A, the longitudinal diffusion B/u and the mass transfer coefficients Cu

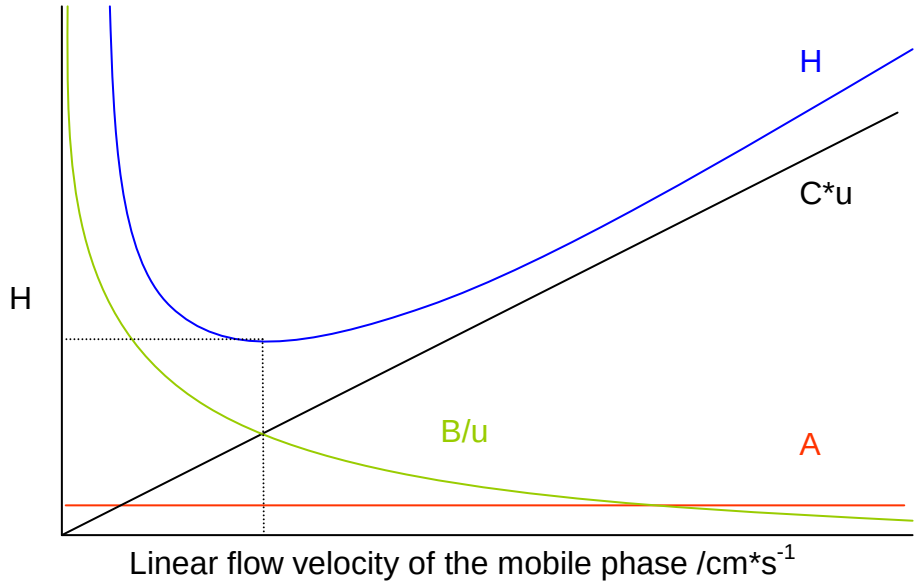

Figure 1: van Deemter plot with the contributions (A, C*u, B/u) on the plate height according to the van Deemter equation. The dotted line shows the optimal flow rate due to the minimum plate height

1.1.4 Chromatographic resolution

The chromatographic resolution *R* is per definition the ability to separate two analytes A and B and it is a measure for the distance between the peaks. The distance is not determined in absolute numbers but based on the scale of peak widths. The accepted definition for resolution is based on the expression of the peak width by the use of plate heights *N* and the retention time *t* is substituted by the capacity factor *k'* (average of k'_A and k'_B of the individual analytes) [2,3,7].

$$\alpha_{B,A} = \frac{k'_B}{k'_A}$$

Equation 13:

$$R_{A,B} = \frac{1}{4}(\alpha_{B,A} - 1)(\frac{k'}{1+k'})\sqrt{N}$$

Equation 14:

$$N = 16R^2{}_{A,B}(\frac{1}{\alpha - 1)})^2(\frac{1+k'}{k'})^2$$

Equation 15:

The equation describing the resolution $R_{A,B}$ includes 3 terms: i) the selectivity term *(α-1),* ii) the retardation term *k'/(1+k')* and iii) the efficiency term $N^{½}$.

It is possible to adjust these terms with diverse impact on the resulting resolution.

i) The selectivity term is probably the term with the highest potential regarding the optimization of resolution. Changes which have a high impact are possible via the composition of the solvent, the adjustment of pH, column temperature and the choice of stationary phases.

ii) Via manipulation of the retardation term the quality of a separation can be drastically increased. However an increase regarding the capacity factor *k'* results in an increase of analysis time. Optimal values for the capacity factors due to analysis time and resolution are within 1-5. Changes relating to the retardation term can be made via the eluent composition; even marginal changes can have a high impact.

iii) Changes regarding the number of theoretical plates via changing the length of the chromatographic column to increase the resolution is not very effective because the analysis time increases as well and N is found under the square root in the equ.13. A way to increase the resolution via the efficiency term could be to decrease the plate height utilizing smaller particle diameters or via a reduction of the solvent viscosity in the mobile phase hence the diffusion coefficient in the mobile phase increases [2,3,7].

1.1.5 Types of stationary phases

In the last century the number applications for chromatography was increasing enormously due to the increasing demand of methods for the identification, quantification or purification of analytes in complex matrices. An extraordinary application is the high performance liquid chromatography HPLC because of its wide range of applications and its available stationary phases [2,8].

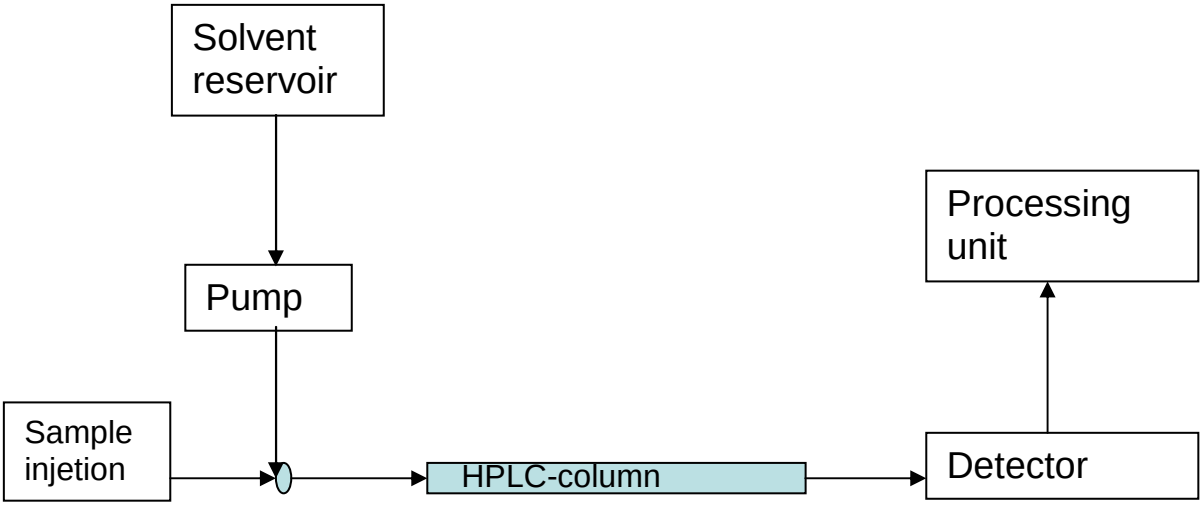

Figure 2: Schematic assembly of a HPLC-system

Regardless of the age and additional technical features the principal assembly of a HPLC instrument stays the same (Figure 2). The solvent, stored in a solvent reservoir is delivered through the system via the pump assembly followed by a device for injection, to the separation column and to the detector. Data storage and processing is done computer aided including the particular software packages.
Column materials are highly significant components of HPLC systems. The widest spread representatives are reversed phase RP, normal phase/HILIC (hydrophilic interaction liquid chromatography, ion chromatography IC and size exclusion chromatography SEC [2,3,8].

1.1.6 Reversed phase liquid chromatography RPLC

The RP material is a stationary phase with determining properties applicable for a wide range of analytes with various characteristics. The crucial point is the synthesis and production of reproducible stationary phases regarding particle diameter and functionalization degree (coverage) in order to obtain predictable, reproducible and robust retention characteristics.
The functional groups of a RP material are nonpolar hydrophobic organic species attached to either silica via silyl ether bonds (-Si-O-Si-), or polymers. The principle of retention and separation of the analytes is the hydrophobic interaction between the analyte and the stationary phase. Eluents are water or buffers and an organic solvent mixable in any arbitrary percentage with water e.g. acetonitrile or methanol. The classification of the solvents follows the eluotropic series starting at water with the lowest elution power and ends with n-hexane with the highest elution power. For the RP

stationary phase of choice with the best properties regarding the adsorbens, diameter, chain length, column length and particle size exist plenty of applications from various suppliers [9,10].

1.1.7 Ion exchange chromatography IC

The stationary phase in IC is separating the analytes (ions or polar compounds) based on their charge via coulomb interactions. The material for the stationary phase can be a resin or gel with active ionic groups e.g. SO_3^-, COO^-, NH_3^+ etc. on its surface. The principle of the IC is that the charged analytes interact with the contrary charged stationary phase. In dependence of the analyte and its charge the IC is classified into anion- and cation exchange chromatography. To optimize conditions for an IC are the right choice of IC-resin, the pH of the, the ionic strength and the choice of counter ions in the mobile phase [2,3].

1.1.8 Size exclusion chromatography SEC

The principle of SEC is the classification of the analytes due to their size. The material is porous (defined diameter) and the analytes, which are oversized for the pores elute without retention whereas smaller molecules enter the pores and elute later. Applications for SEC are rough determination of the molecular weight or a prefractionation and purification prior further analysis of typically biologically polymers such as proteins [2,3].

1.1.9 Normal phase/ HILIC

Normal phase is chromatography utilizing any highly polar stationary phases as spherical silica particles, aluminium oxide or chemically bond phases with cyano-, diol-, amino-, diethylamino- etc. functional groups. For normal phase the eluotropic series is in the reversed order compared to RPLC with water as the eluent with the strongest elution power. HILIC is a mode of normal phase chromatography and was first defined 1990 by Alpert [11]. The difference is that he grouped the phases due to their character e.g. simple unbonded silica silanol or diol bonded phases, amino or anionic bonded phases, amide bonded phases, cationic bonded phases and zwitterionic bonded phases [12,13].

1.2 Mass spectrometry

1.2.1 History of MS

The history of mass spectrometry goes back to J.J. Thomson in 1897 who discovered the electron and determined its mass to charge ratio m/z. in 1912 [14]. He constructed the first mass spectrometer and called it parabola spectrograph. He obtained mass spectra of O_2,N_2,CO, CO_2 $COCl_2$ and he was able to show that neon has at least exists in two isotopic forms. In 1922 Aston was able to determine the isotopic composition of several elements utilizing a so called mass spectrograph [15]. However the list of scientists who contributed the development of mass spectrometry is very long and some of them were even awarded with the Nobel price. A continuous improvement of the instruments regarding sensitivity, resolution, mass range and accuracy resulted in sensitivities in the attomole levels[16,17], spectral resolutions of 8000000 [18], mass accuracies around 1 ppm and the detection of macromolecules with an atomic weights of 40.5 MDa [19]. Especially the coupling of mass spectrometry to other chromatographic systems e.g. HPLC, GC and CE are enormous powerful analytical tools and allow in the best case the identification of an isolated analyte [20]. A brief overview of some ion sources, mass analyzers and detectors will be given in the following section.

1.2.2 Ion Sources

In the ion source the analyte is ionized via energy transfer prior to analysis. There is a large variety of different ion sources considering the characteristics and the chemical and physico chemical properties of the analyte (polarity, thermal stability, volatility, etc) or the ionization process e.g. electron-, field-, photo- and chemical ionization. In the following section the most important and most frequently used ionization sources for organic mass spectrometry will be briefly described.

1.2.3 Electron impact ionization EI

This ionization technique was first introduced by Dempster and improved by Bleakney and Nier [21,22] and it is widely used ion source in gas chromatography coupled to mass spectrometers (GC-MS). The principle of this method is that a heated filament is emitting electrons which are colliding with molecules in the gas phase. Gaseous molecules are introduced directly into the source and electrons with wavelength between 2.7 and 1.4 Å respectively 20 and 70 electron volts (eV) collide with the analytes (the fragmentation increases with increasing eV). The wavelength and the eV are associated via equ. 16 [23].

$$\lambda = \frac{h}{mv}$$

Equation 16: m is the mass, h Planck's constant, λ the wavelength and v is the velocity

$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

Equation 17: the gas phase reaction of EI

The gas phase reaction for the production of ions follows equ.17 but usually 10 eV are enough for the ionization of a molecule, hence the excess of energy leads to extensive but significant fragmentation of the analyte molecule. Due to the fragmentation pattern EI provides structural information and can be helpful by the identification of unknown analytes [20].

1.2.4 Chemical ionization CI

The principle of chemical ionization is the production of ions through a collision of the analyte with collision gas present in the ion source. Widely used reaction gases are methane, isobutene and ammonia [24]. In contrary to EI, the spectrum contains less fragmentation and the analyte molecule can be easily recognized. Although a variety of chemical ionization reactions exist the prevalent is the proton transfer reaction. The analyte molecule M is introduced introduced into the into the ionization plasma and the reagent gas $[G+H]^+$ transfers a proton to the analyte molecule resulting in a protonated molecular ion $[M+H]^+$. The formation of $[M+H]^+$ requires the affinity for protons as a character of the analyte molecule [20].

1.2.5 Matrix assisted laser desorption ionization MALDI

MALDI was first introduced by Karas and Hillenkamp 1988 [25,26,27] and since that time this ionization technique became a widely spread method for the analysis of e.g. biopolymers, synthetic polymers or large inorganic compounds. The principle is that laser pulses with power output between 10^6 and 10^{10} Wcm^{-2} vaporize and ionize the analytes and the matrix from a usually solid surface. The choice of matrix is dependent on the analytes e.g. trihydroxyacetophenone (THAP) for oligonucleotides or α-cyano-4-hydoxycinnamic (CHCA) acid for peptides and proteins etc. [20] and must have a high absorption regarding the laser wavelength. Before the analysis the analyte is mixed with the matrix and subsequently the mixture is dried resulting in a solid solution where the analyte is

embedded into matrix crystals The ionization mechanisms are still not completely understood [28,29] but the mechanisms include gas phase photoionization, proton transfer from excited matrix and ion-molecule reaction. Subsequently the analyte ions are accelerated via electrostatic fields and transferred into the analyzer [20].

1.2.6 Atmospheric pressure ionization API

The atmospheric pressure ionization interfaces ionize the analytes at atmospheric pressure and a subsequently transfer into the mass spectrometer. The problem is the difference in pressure between the ion source and the mass analyzer and it is usually solved utilizing differential vacuum pumping systems. The vacuum compartments are separated by the so called skimmers or cones (lenses with small orifices). The ions can pass through the lenses and reach the mass analyzer. The advantages of API sources are the coupling of LC-systems to mass spectrometers and the easy introduction of samples hence there exist different API sources and the most important are electrospray ESI, atmospheric pressure chemical ionization APCI, and atmospheric pressure MALDI [20,30].

1.2.7 Atmospheric pressure MALDI AP-MALDI

The method of operation for an AP-MALDI source is the same as a MALDI source under vacuum [31,32,33]. The ions produced by the laser beam are transported via high voltage (usually 2-3 kV) into the mass analyzer and a flow of dry nitrogen is surrounding the MALDI probe. The transfer rate of ions into the analyzer is lower but the AP ionization is softer compared to the conventional MALDI source and produces mainly single charged ions and the ions do not fragment but the ions tend to form adducts with the matrix. These unwanted adducts can be eliminated increasing the laser energy, or the transfer capillary temperature [20].

1.2.8 Electrospray ESI

ESI was first introduced by Fenn et al. [34,35] and he showed multiple charged ions obtained from a protein with an ESI source and he was rewarded with a Nobel price in chemistry. An ESI source works with a capillary (the liquid is passing through), a sheath flow surrounding the capillary and high voltage in the kV range. The liquid is passing the capillary and the high voltage disperses the liquid resulting in highly charged droplets. A heated gas (mostly nitrogen) inside the ESI source supports the desolvation of the

droplets and due to the electric field a Taylor cone formation is observable. From this Taylor cone smaller highly charged droplets are released. Due to the ongoing desolvation the smaller droplets accumulate charges on their surface. Regarding the ion formation two theories are discussed i) the charge residue model CRM and ii) the ion evaporation model IEM [23].

i) The CRM explains the ion formation due to coulomb explosions until a droplet contains a single analyte ion

ii) The IEM explains the ion formation due to the reduction of radius of the droplet has reached a diameter of 10-20 nm and a direct ion formation of the ion into the gas phase occurs.

However an explicit explanation of the ion formation regarding ESI is currently not possible. The advantages of an ESI interface are that the ionization occurs directly from solution consequently thermally labile-, ionic as well as polar analytes can be studied. The flow rates from the HPLC can range between a few nL min^{-1} until approx. 1 mL min^{-1} hence a wide range of liquid chromatographic systems can be applied with limitation in the use of buffers with non volatile salts e.g. sulfate, borate, phosphate etc. Depending on the molecular weight ESI can produce multiply charged ions therefore small molecules with single charges as well as analytes with high molecular weight e.g. proteins carrying multiple charges can be explored almost without limitations regarding the mass analyzer and its mass range limit [20,23].

1.2.9 Atmospheric pressure chemical ionization APCI

APCI is an ionization technique at atmospheric pressure that enables mass spectra from highly polar and ionic compounds. The HPLC effluent flows into a pneumatic nebulizer and the liquid stream is converted into an aerosol. In the desolvation chamber the solvent is removed and the hot gas is ionized utilizing a corona discharge electrode. The process of ionization is comparable to CI, the electrons interact with the surrounding gas resulting in ionization of the gas and that gas interacts with the analyte molecule via ion-molecule reaction. The advantages of APCI are that it is a relatively soft ionization technique that means analytes can be studied without decomposition, it is more tolerant to high buffer concentrations compared to ESI and flow rates from the HPLC up to 2 mL min^{-1} are possible but it works less effective at very low flow rates [20,23,30].

1.2.10 Atmospheric pressure photo ionization APPI

The principle of an APPI ion source is the utilization of photons emitted by a discharge lamp for the ionization gas phase molecules [36,37]. The analyte molecules are vaporized

via a heated nebulizer followed by a reaction with the emitted photons. The APPI source is often used in case the analyte molecules are barely ionizable via APCI or ESI. In most cases those particular compounds have non polar characteristics. Due to the low efficiency of the direct ionization of the analyte molecules doping molecules are added in order to increase the ionization efficiency of the analyte. The dopants must have the characteristics to be ionizable via photons and to transfer the charge in order to ionize the analyte. Widely used dopants are toluene and acetone and the ionization efficiency can be increased by the factors 10-100 [20]. The first step of the ionization process using a dopant is the formation of a radical molecule of the dopant followed by a transfer of the charge to the solvent molecules and the final step is the transfer of the charge to the analyte equ. 18.

$$D + h\nu \rightarrow D^{\bullet +} + e^{-}$$

$$D^{\bullet +} + S \rightarrow [S + H]^{+} + (D - H)^{-}$$

$$M + [S + H]^{+} \rightarrow [M + H]^{+} + S$$

Equation 18: Reaction cascade of an APPI-ion source with D as a dopant, S as solvent molecule and M as the analyte molecule

In case the ionization of the analyte molecule is lower than the dopant the direct charge transfer is possible equ. 19.

$$D^{\bullet +} + M \rightarrow M^{\bullet +} + D$$

Equation 19: Direct ionization of the analyte molecule M via charge transfer of the dopant D

APPI is also applicable for the negative ionization so long a dopant is used equ. 20.

$$M + e^{-} \rightarrow M^{\bullet -}$$

Equation 20: Formation of the negatively charged analyte M via APPI.

The formation of the negatively charged analyte is produced via the thermal electrons originating from the photo ionization of the dopant. APPI is a complementary ionization technique to APCI and ESI however the influence of solvents, buffers or additives can have strong impact on the ionization efficiency. For substance classes as flavonoids, steroids, pesticides, polyaromatic hydrocarbons, etc. APPI is suitable ionization technique [38].

1.2.11 Mass analyzers

The ions produced via the ion sources are separated in the mass analyzer. There exist several mass analyzers based on various principles but all of them determine the mass to charge ratio *m/z* of the analytes. For the determination of m/z all mass analyzers use either static or dynamic electric or magnetic fields to achieve a separation of the ions. There are five main characteristics to determine the performance of a mass analyzer i) the mass range, ii) the analysis speed, iii) the transmission, iv) the mass accuracy and v) the resolution.

i) the mass range corresponds to the highest and lowest m/z the instrument can measure, it is expressed in Thomson Th or in mass units (u can be expressed in Dalton Da and it is defined as the twelfth of the mass of a ^{12}C atome; Th is the m/z unit, 1 Th = 1 u/e with e as charge unit)

ii)the analysis speed is the time a mass analyzer needs for scanning a certain mass range, it is either expressed in mass units per second $u*s^{-1}$ or mass units per milli second $u*ms^{-1}$

iii) the transmission is the ratio between the ions detected and the ions which have entered the mass analyzer

iv) the mass accuracy is the difference between the measured m/z and the theoretical m/z, it is expressed by milli mass units mmu or in parts per million ppm

v) resolution is the ability to distinguish signals of ions with small differences according their m/z, depending on the type of instrument peaks can be considered as resolved when the valley between them is smaller or equal to 10% (magnetic sector or ion resonance instruments) of the weaker peak intensity respectively 50% (e.g. quadrupole, ion trap, time of flight instruments), the definition of the resolution shown in equ.18 [20,23,39].

$$R = \frac{m}{\Delta m}$$

Equation 21: Δm ist the smallest mass difference for which two peaks with the masses m and m+ Δm are resolved

For various applications various mass analyzers have been developed, some are presented in the following section.

1.2.12 Quadrupole analyzer

A quadrupole mass analyzer consists of four circular or hyperbolic rods arranged perfectly parallel. The opposite rods are electrically connected to radio frequency RF and

direct current. For distinct voltages ions of certain m/z values are accelerated along the x-axis and follow stable trajectories through the rods. The applied voltages stabilize or destabilize the ions and therefore a quadrupole is also named mass filter. Quadrupole mass analyzers are cost effective devices with fast scanning times but they only can provide nominal resolution and have limited mass ranges (max. 4000 Th).

In order to increase selectivity in mass spectrometric analysis instruments utilizing three quadrupoles (triple quadrupole mass spectrometer) in series were introduced [40] (Figure 3).

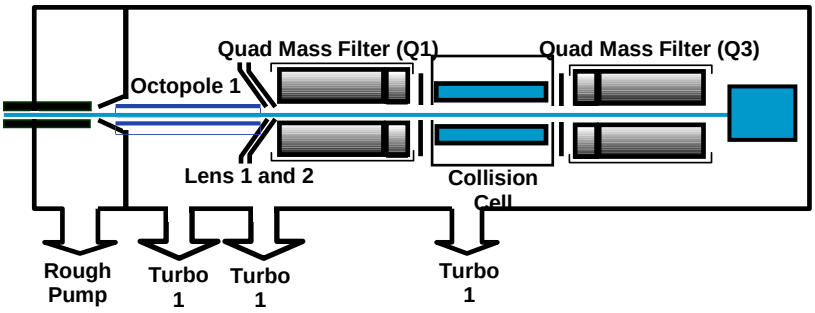

Figure 3: scheme of a triple quadrupole mass spectrometer from Agilent Technologies

Such instruments can provide not only the molecular weight of an ion but via a collision with an inert gas or reactant gas in the collision cell it can provide structural information. The collision cell is a quadrupole or a hexapole filled with gas (either inert or reaction gas) and the ions fragment via collision with the inert gas provided that kinetic energy is converted into internal energy and in case of the reaction gas an ion-molecule reaction can be induced. The resulting product ions are focused and separated in the third quadrupole.

1.2.13 Ion trap analyzer

The ion trap analyzer is a mass analyzer invented by Paul and Steinwendel 1960 [41,42] and it is made up of a ring electrode and two “end cap” electrodes at the top and the bottom and it is in the most cases pressurized with a collision gas e.g. helium. In contrary to the quadrupole mass analyzer not only ions with distinct m/z pass through the rods but ions with different m/z are trapped at the same time and via applying resonant frequencies along the z-axis and ions with distinct m/z are expelled. The ion trap works as a three dimensional quadrupole and the ions follow complex trajectories until RF is applied to the ring electrode then ions with certain m/z become unstable and are ejected from the analyzer while others with m/z according to the applied RF frequencies are stable. The frequency stays the same but changing the amplitude of the frequency a

collision induced fragmentation with the reaction gas fragment spectra of the analytes can be obtained. Alternatively fragment ions can be trapped again and thus multistage MS experiments (MS^n) can be performed. Like the quadrupole mass analyzer is the ion trap limited in resolution (4000) and mass range (6000 Th) [20,23]. Recent developments regarding ion trap mass analyzers have shown that resolutions up to 30000 are possible [43].

1.2.14 Time of flight analyzer TOF

The TOF was first described by Stephens 1946 [44] and the principle is the separation of ions after an initial acceleration in an electric field free region according to their m/z. The acceleration of the ions is performed applying a potential between an electrode and an extraction grid resulting in that all ions have acquired the same kinetic energy. In the flight tube the ions are separated according to their m/z before detection and the velocity of each ion is inversely proportional to the square root of its mass equ. 19.

$$t^2 = \frac{m}{z}\left(\frac{L^2}{2eV_s}\right)$$

Equation 22: principal of a flight time determination with a TOF mass analyzer. The ion m/z, accelerated by potential V_s needs the time t is to cover the distance L.

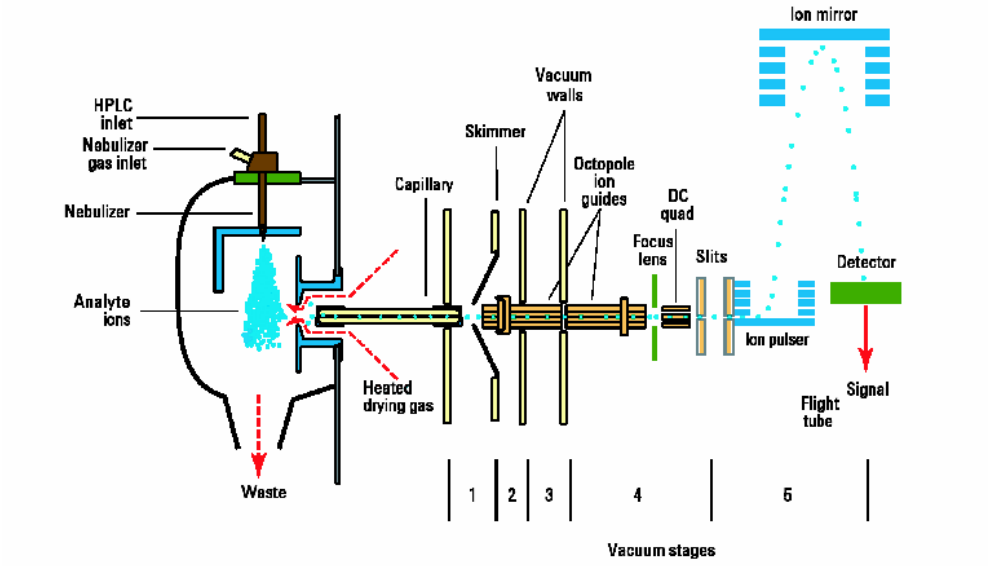

Figure 4: scheme of a TOF instrument including the ESI source and the ion optical devices for focussing the ion beam (figure from Agilent technologies)

To improve the mass resolution an electrostatic reflector called reflectron (figure 4) is used. The reflectron increases the flight path of the ions without increasing the dimensions of the instruments. A reflectron acts as an ion mirror decreasing the kinetic energy dispersion of the ions with the same m/z. An ion with more kinetic energy and consequently with more velocity will enter the reflectron deeper and vice versa for an ion with lower kinetic energy. TOF-instruments with reflectrons have mass limits up to 10000 Th and resolutions around 20000 [45,46,47].

1.2.15 Fourier transform mass spectrometry FTMS

Fourier transform mass spectrometers are instrument with the highest resolution power. Via Fourier transformation the signals from the ions are converted into m/z. Two types of instruments are available i) Fourier transformation ion cyclotron resonance mass spectrometers FTICR and ii) Orbitrap.

i) The principle of the FTICR is based ion cyclotron resonance; the ions are trapped in a cyclotron cell and a strong magnetic field is applied. The magnetic field forces the ions to follow distinct trajectories inside the cell directly related to the m/z of the ions and the strength of the magnetic field. The detection of the ions follows after extitation of the ions via the cyclotron frequencies (typical for each m/z in a cyclotron cell) and the recording of the ion induce current the so called image current. The image current is individual for each ion and consequently conclusions on the m/z are possible. The FTICR have mass limits up to 30000 Th and resolution of 500000 and more [20,23,48].

ii)The Orbitrap was first introduced and described in patents 1996 [49] and 2004 [50] by Makarov. The core of the instrument is the so called spindle electrode surrounded by a further electrode. A potential of several kV is applied to the spindle electrode while the surrounding electrode is on ground potential. After the ions are injected into the Orbitrap they start to oscillate around the spindle electrode under the influence of an electrostatic field. The frequencies of the oscillation is typically for each ions with certain m/z. Via furrier transformation those frequencies are transformed into m/z. The orbitrap has mass limits at 50000 Th and a resolution of 100000 [20].

1.2.16 Detectors

In mass spectrometry several types of detectors presently exist. The choice of detector depends on the type of instrument and the analytical application. The possibilities to detect ions are always based on the mass, their charge or their velocity exceptionally the detectors for FTMS instruments. Widely used detectors are faraday cups and electron multipliers.

The principle of a faraday cup is that ions hit the wall of the cup and are neutralized via either accepting or donating electrons. Subsequently this so called discharge current is amplified and detected. The disadvantages of that simple but robust detector are the low sensitivity and the slow response time.

Electron multipliers are presently the most widely used detectors in mass spectrometry. The principle of an electron multiplier is that accelerated ions hit a conversion dynode and secondary particles (ions, neutrals or electrons) are produced. On the first dynode the secondary particles are converted into electrons and these electrons are amplified via a cascade effect in the electron multiplier and finally an electric current is provided. Many mass spectrometers use electron multipliers in various versions e.g. multi channel plate MCP for TOF instruments or channeltrons for quadrupole-, ion trap- and triple quadrupole instruments.

The detectors for the FTMS instruments are generally different from all others the ions produce an image current which is subsequently detected and transformed [20,23].

	Quadrupole	Ion trap	TOF	Orbitrap	FTICR
Mass range / Th	4000	6000	10000	50000	30000
Resolution	2000	4000	20000	100000	500000
Mass accuracy / ppm	100	100	2-10	< 5	< 5

Table 1: Summary of the available parameters for the characterization of mass analyzers [20]

1.3 Analysis of Phenolic compounds in wine

1.3.1 Phenolic compounds

Wine is a composition of the must or the juice of grapes from vitis vinifera chemically altered via yeast fermentation. The initiation of wine making started around 6000 years ago in the present Iran respectively around 9000 years ago in China [51]. Wine is not simply the must of vitis vinifera with alcohol added but the fermentation alters and changes the composition and inter linking of organic acids and phenols and the formation of co-pigments. Studies have shown that wine has beneficial effect on health associated with the presence of polyphenols. These polyphenols are absorbable for human bodies only from wine because due to the formation of copigments and they show an anaerobic and protective redox potential [52,53,54]. Phenols and polyphenolic compounds belong to the most important groups and attributes of wine. They are responsible for the wine's color, astringency, bitterness and partly for the taste or mouth feel respectively. The phenol´s composition in wine varies with wine production, storage and age although the changes in the phenolic pattern are not entirely understood however the antioxidant activity of red vine is already evident. To demonstrate the antioxidant activity of one glass of red wine it would need 12 glasses of white wine, 2 cups of tea, approximately 5 apples or 7 glasses of orange juice. The benefits on health of red wine consumption are only apart from alcohol consumption. The toxicity of alcohol is a fact and the consumption shows a typically "J" shaped response curve. An alternative to "normal" commercially available wine could be dealcoholized wines. Via a combination of reversed osmosis and distillation it is possible to remove the main part of the alcohol. That technology offers the health benefits of the polyphenols of red wines without the negative aspects of the simultaneous intake of alcohol [52].

Subsequently after harvesting the processing of the grapes start and that has a big impact on the polyphenolic content of the final product. The major part of of polyphenols are present in skins and stems of the the grapes hence, for the red wine production, the must is kept in longer contact with the skins and stems for the polyphenol extraction, but wine producers have to balance between the extraction rate of polyphenols (the longer, the more, the better) and the increasing astringency and bitterness which is an implication due to longer extraction rates. In contrary for white wine production the skins and stems are rapidly separated from the must hence the content of polyphenols is approximately 10-12 times lower.

1.3.2 Phenols in Wine

The simplest phenols occurring in wine are mono-, di-, triphenols and phenolic aldehydes e.g. phenol, pyrocatechol, resorcinol, pyroglucinol, vanillin and syringic acidHydroxycinnamic acids like caffeic-, ferulic- and cis and trans p- coumaric acids are present in the grapes and consequently also in wine. More complex phenolic structures in wine contain at least two ore more aromatic rings e.g. the groups of flavanols, flavonols and anthocyanins [51,55,56].

In grapes those groups are generally found in their monoglycoside forms with the sugar residue linked to the oxygen containing ring. The glucosidic forms of kaempferol, quercetin and myricetin are responsible for the formation of copigments with anthocyanins. Penzopyrilium is the base molecule for the structure of anthocyanins.

1.4 Analysis of soil and root related products

The investigations of research focused on soil, plants, roots and root exudates is done to understand and explore processes of plant growth and nutrient uptake. A very particular space surrounding the root is the rhizosphere. By definition the rhizosphere is the volume of soil affected by the presence of the roots of growing plants [57,58]. The volume is defined as approximately 3 mm surrounding the root and its spatial extension depends on the soil structure, particle size, water content and the buffering capacity [59,60,61]. Numerous compounds are released into the rhizosphere of soil grown plants (Table 1), most of them are organic compounds derived from photosynthesis and other plant processes. The exudated amounts of the organic compounds vary with the plant species, the type of soil, the plant's age, the environmental conditions and the physical, biological and chemical stress. Although a lot of compounds have been explored and identified the processes themselves and the interactions are insufficiently understood and sometimes even falsely interpreted.

"*Root exudation cannot be simply explained by a single mechanism but is moreover a combination of complex multidirectional fluxes operating simultaneously. While we currently possess a basic understanding of root exudation, its overall importance in plant nutrition, pathogen responses, etc., still remains largely unknown. Future research should therefore be directed at quantifying the significance of root exudates in realistic plant-soil systems"* [62]

The problems arise from the differences of the processes between the test arrangements e.g. in solution cultures and the cultures grown in soils.

- The surface are for sorption in soil is much greater compared to the in solution cultures
- In solution cultures are mixed constantly
- The microbial activity differs between soil and solution
- The status of water and O_2 is different in soil respectively solution

However in solution cultures are still widely spread and their importance due to the understanding of plant growth is enormous.

Root products are differentiated between 1) diffusates 2) excretions and 3) secretions. Diffusates e.g.: sugars, organic acids, amino acids, water inorganic ions and oxygen have a non functional functions for plants, whereas excretions e.g.: CO_2, bicarbonate ions, protons, electrons and ethylene support the internal metabolism such as the respiration and secretions e.g.: enzymes, siderophores and allelochemicals support the external processes such as nutrient acquisition of plants.

The main part of the compounds released by roots contains carbon except H^+, e^-, inorganic ions, water etc. The protons and electrons are hypothesized to be released in

form of undissociated organic acids and reducing agents [63] even though plasma membranes are believed to be the main source of e^- and H^+ [64,65,66]. The amounts of diffusates, excretions and secretions released depend on the plant type, the age of plants and the environmental conditions [67].

1.4.1 Types of root products and their functions selected for our studies

Root exudates contain almost every substance produced by plants (Table 2) except compounds in connection with photosynthesis. The capability to detect and quantify the compounds is increasing with the sensitivity of the analytical equipment and novel analytical applications.

1.4.2 Organic acids

Organic acids (listed in table 1) released from roots into the rhizosphere supply a wide range of functions e.g. metal detoxification, enhancement of nutrient uptake and the mobilization of P. The form organic acids are released into the rhizosphere can be either dissociated or undissociated although due to the pH in the soil respectively in the cytoplasm it is more likely the dissociated form is secreted. The anions may form complexes with metal ions e.g. citric acid with iron [$C_6H_5O_7^{3-}$- Fe^{3+}] and diffuse back into the root where the complex is absorbed respectively the Fe is separated from the organic acid ligand and then absorbed. Another fate of exudated organic acid is that they are absorbed at the soil surface and rapidly degradated via microbial activity.

Sugars and polysaccharides	Arabinose, desoxyribose, fructose, galactose, glucose, maltose, mannose,raffinose, rhamnose, ribose, sucrose, xylose
Amino acids	Arginine, asparagines, aspartic, citrulline, cystathionine, cysteine, glutamine, glutamic, glycine, homoserine, histidine, leucine, isoleucine, lysine, methionine, mugineic, deoxymugineic, ornithine, phenylalanine, proline, serine, threonine, Tryptophan, tyrosine, valine
Organic acids	Acetic, aconitic, aldonic, ascorbic, benzoic, butyric, caffeic, citric, p-coumaric, erythonoc, ferulic, formic, fumaric, glutaric, glycolic, lactic, malic, malonic, oxalacetic, oxalic, p-hydroxy benzoic, piscidic, propionic, pyruvic, succinic, syringic, tartaric, tetronic, valeric, vanillic
Fatty acids	Linoleic, oleic, palmitic, stearic
Sterols	Campesterol, cholesterol, sitosterol, stigmasterol
Growth factors	p-amino benzoic acid, biotin, choline, n-methyl nicotinic acid, niacin, pantothenic, vitamins B_1, B_2, B_6
Enzymes	Amylase, invertase, peroxidase, phenolase, phosphatases, polygalacturonase, protease
Flavones and nucleotides	Adenine, flavone, guanine, uridine or cytidine
Miscellaneous	Auxins, p-benzoquinone, scopoletin, hydrocyanic acid, 8-hydroxyquinoline, glucosides, hydroxamic acids, luteolin, unidentified ninhydrin positive compounds, unidentified soluble proteins, reducing compounds, ethanol, glycinebetaine, inositol and myo-inositol like compounds, Al-induced polypeptides, dihydroxyquinone, quercetin, sorgoleone

Table 2: organic compounds released by plant roots [68]

1.4.3 Techniques for root exudate exploitation

The first technique for the root exudate collection is the hydroponic system. The plant is grown in a nutrient solution and for the exploitation of the released compounds it is

dipped into a so called trap solution. The advantages are that it is easy to perform, this technique allows kinetic studies using the same plants for several times and via the hydroponics it is possible to get impressions about the qualitative and quantitative exudation pattern. However, this application is restricted to nutrient grown plants because the removal of the plants from solid material is usually connected with a partial damage of the root cells which leads to overestimation of the concentrations of the various exudated compounds. A further drawback is that nutrient grown plants show differences in their root morphology leading to stimulated root exudation compared to solid media grown plants [69].

Root products from solid media grown plants can be obtained via percolation of the culture vessel with the trap solution for a certain time period. Disadvantages for that collection setup are that certain amounts of root exudates might be adsorbed by the utilized material and incomplete elution of the root products result in underestimations of the root product concentrations. Hence a comparison of the exudation pattern from the nutrient grown plants and the solid material grown plants is essential to exclude previously mentioned unwished drawbacks [69].

1.4.4 Sampling techniques with spatial resolution

Root exudation is not equally distributed over the root system. Those spatial variations have been reported considering the exudation of carboxylates, protons, phenols etc. in dicotyledonous plant species and seed rape due to P or Fe deficiency. The exudation of high amounts of carboxylates and protons is highly connected with nutrient and mineral uptake.

Spatial separated sampling the root exudates from solution systems is performed via separating root zones of plants grown in hydroponic systems. The exudates are trapped into a trap solution inside of a plastic ring sealed with agar [70] or vacuum grease. Further applications are to place agarose sheets or other sorption media as filter paper, cellulose acetate filters or ion exchange resin onto the root surface [71,72,73,74,75]. The choice of sorption media depends on the type of root exudates that will be collected (phenols, LMW organic compounds with acidic groups etc.).

Spatial separation of root exudates of plants grown in soil is performed via rhizoboxes with removable front lids, the application of micro suction cups or resin bags or resin agar sheets [76] are placed onto the surface of various root zones. The rhizoboxes can be also equipped with an adapted micro suction cups to obtain soil solution with an exudate collector system (ECS) to sample organic compounds directly at the root monolayer.

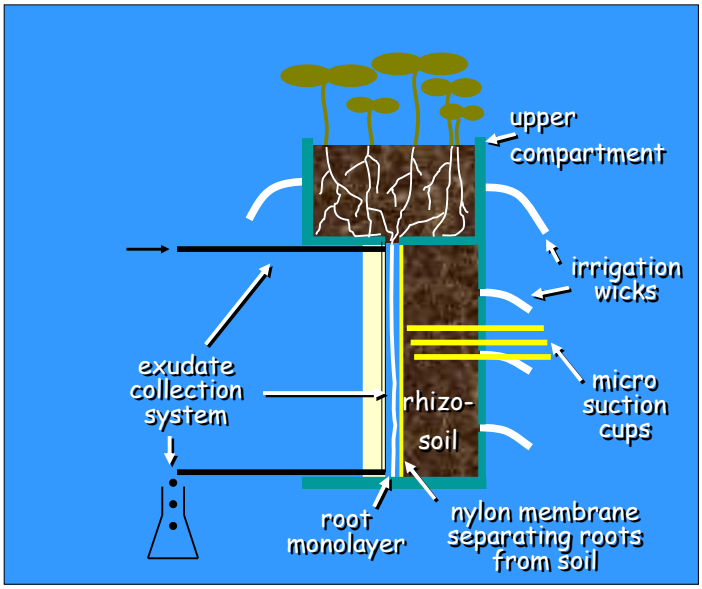

Figure 1: Rhizobox system of Wenzel et al. [77], equipped with micro suction cups [78] and a novel exudate collection system (ECS).

1.4.5 Factors of root exudate recoveries

Factors of the variation of root exudate recoveries can be (i) microbial activity, (ii) sorption at soil matrix and (iii) root injury.

(i) Organic compounds released from roots are continuously metabolized and degraded via microbial activity. Hence there is the possibility to work under sterile conditions, but experiments have shown that root exudation can be stimulated by the presence of metabolites originating from microbes. Working under non sterile conditions as well as working under sterile conditions lead to underestimation of the organic compound root exudation pattern. Experiments have shown that under non sterile conditions the collection time is a very important factor. Recovery tests with amino acids illustrate, the concentration remained stable over a period of 2.5 h collection time but over a period of the next 3.5 h 90 % were degraded via microbial activity. Almost similar was observer for organic acids and sugars in soil. Isotopically labelled citric acid and malic acid were added to soil and the concentrations remained almost constant during 3 h but the organic acids were completely metabolized over a time period of 20-48 h. Studies have shown that microbial activity is mostly present in soils with a high organic fraction but it was found that organic compounds are less affected from microbial activity when they are attached to sorption materials such as ion exchange resins or reversed phase materials. Another way to prohibit microbial degradation of organic compounds is adding antibiotics or bacteriostatic compounds to the collection media. Depending on the plant species and dosage those compounds can show phytotoxic effects and stimulate the release of

organic compounds of dead or damaged microbes leading to a false determination of the root exudate concentrations [69].

(ii) Adsorption of root exudates at the soil material is affected by the pH, the functional groups of the organic compounds and the ion exchange properties of the soil. For instance the lack of charges of various sugars results in almost no interactions with the soil material or metal ions however charged compounds as organic acids or amino acids tend to show high affinity to the soil matrix and to metal ion complexation. Oxalic, malic and citric acid are, due to their number of carboxylic groups, very effective chelators for metal ions such as P and Fe. As previously mentioned this character of those species is important for the detoxification and the nutrient uptake of plants. Compounds with more hydrophobic character such as flavonoids or phenols are affected by humic substances but interactions with metal ions are reported as well.

(iii) Root exudate collection is often associated with root injury e.g. during transfer into the trap solutions or the mounting of root exudate collection systems onto the root surface. Due to the damage of hair roots hairs or epidermal cells an overestimation of the exudate concentration is the result.

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2 LC-MS/MS analysis of phenols for classification of red wine according to geographic origin, grape variety and vintage

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A rapid LC-MS/MS method for quantification of phenols and polyphenols in authentic wine samples with unrivalled sensitivity was developed. Excellent limits of detection in the low µg L^{-1} range were achieved. Reversed phase HPLC employing sub-2 µm particles as stationary phase allowed high-throughput analysis with analysis times of 10 min for 11 compounds. The phenolic pattern was assessed in 97 authentic wine samples (stored under identical conditions without further treatment or modification) comprising eleven geographical Austrian regions, six grape varieties and five vintages. Canonical discriminant analysis was applied to the data set showing the suitability of the (poly)phenolic spectrum for classification of the wine samples. The method allowed a geographic discrimination of several grape varieties and a grape variety based discrimination of 4 regions. As a novel finding excellent (poly)phenol-based differentiation of the 5 investigated vintages (2003-2007) was achieved.

Keywords: Phenols, polyphenols, authentic red wine, LC-MS/MS, canonical discriminant analysis

Introduction

Both, red and white wine contain high abundant phenols. In the past, these compounds have been intensively studied (Modun et al., 2008; Serafini, Laranjinha; Almeida and Maiani; 2000; Flamini, 2003) because of their antioxidant and bactericide effects thought to be beneficial for human health. In wine, the phenolic pattern significantly influences colour and important sensory characteristics such as the taste of fullness and body, bitterness and astringency. Major sources of phenols are grape stems, seeds and skins. During maturation in barrels, also wood derived tannins can present an uptake route. Generally, the content of phenolic substances in red wine is more than 10 fold higher as in white wines (Fuhrmann, Volkova, Suraski and Aviram, 2001), since vinification implies maceration and fermentation of the mash leading to extraction of the compounds from the berries. Phenolic compounds include many different molecules, ranging from phenolic acids to complex polyphenols, which are investigated in studies aiming at structure/function based research (Flamini, 2003). The class of phenolic acids contains the series of benzoic acids and the series of hydroxy cinnamic acids (Lee and Jaworski, 1989) Resveratrols belong to the class of stilbenes and are known for their positive effect on cardiovascular diseases (Pour-Nikfardjam, Lásló and Dietrich, 2006).They are generated in grapes in response of fungal parasites and their content varies significantly between different countries and cultivars (Goldberg, Karumanchiri, Soleas and Tsang, 1999). The flavonoid structure forms the base of a big class of phenols, which determine yellow, red and blue colours in plant kingdom. Tannins are divided into two major classes. Gallic tannins are not found in wine naturally but get there by external addition for wine stabilization or by aging in wooden casks. Catechic tannins are polymers of condensed (+)-catechin and (-)-epicatechin. They occur naturally in wine as a consequence of extraction from the grape stems and seeds (Dumon, Michaud and Masquelier, 1991). Many phenolic compounds occur in wine esterified with sugars and

acids. If the free form is found this is a hint on the vinification procedure and the age of the analyzed wine (Vrhovsek, Wendelin and Eder, 1997).

As a matter of fact, the finally present phenol quantity and distribution in wine is not only a question of grape selection and vinification but also pH-value, SO_2 content of the wine, the contact with molecular oxygen, storage temperature and light exposure affect the phenols (Zimman, William, Lyon; Meier and Waterhouse, 2002). Several research groups quantified phenols in the context of grape and wine chemistry, the phenolic content of Vitis vinifera during ageing in bottles, the effect of red grapes co-winemaking in polyphenols and color of wines and the exploration of the nature, the occurrence and dietary burden of flavonols, flavones and flavanols (Flamini, 2003; Monagas, Gomez-Cordoves and Bartolome, 2006; Hollman and Arts, 2000). Numerous analytical techniques including HPLC- UV, HPLC-MS and HPLC-MS/MS were applied for the separation and detection of phenolic compounds in wine samples (Kallithraka, Mamalos and Makris, 2007; Sun, Leandro; de Freitas and Spranger, 2006; Rodriguez-Delgado, Gonzalez-Hernandez; Conde-Gonzalez and Perez-Trujillo, 2002; Puessa, Floren; Kuldkepp, and Raal., 2006; Prosen, Strlic, Kocar and Rusjan, 2007). Ho et al quantified and identified phenolic compounds and furans in fortified wines (Ho, Hogg and Silva, 1989). Malovana et al focused their studies on sample preparation and especially the determination of resveratrol and also other phenolic compounds (Malovana, Garcia Montelongo; Perez and Rodriguez-Delgado, 2001).

Recently, the potential of phenolic compound analysis for the differentiation of wines by grape variety, age or geographical origin in the context of food authentication, food security and anti-fraud has been recognized and first studies have been published (Otteneder, Marx and Zimmer, 2004). Makris et al. performed a differentiation of young red wines based on cultivars and geographical origin (Makris, Kallithraka and Mamalos, 2006). Kallithraka et al.

determined principal phenolic compounds in Greek red wines (Kallithraka, Tsoutsouras; Tzourou and Lanaridis, 2006) as well as La Torre et al. published a similar study for Sicilian wines (La Torre, Saitta, Vilasi, Pellicano and Dugo, 2005).

Our study aimed at the development of an analytical method for separation and accurate quantification of selected phenolic compounds in red wines from Austria. 11 analytes that belong to different classes of phenols were chosen as leading substances for analysis by liquid chromatography combined with tandem mass spectrometry detection (LC-MS/MS). The eleven compounds were selected according to recently published data on the concentration of (poly)phenols in red wine (Flamini, 2003; Makris, Kallithraka and Mamalos, 2006) and to their commercial availability as standard substances. As a novelty, our study has focused on the separation of the structural isomers of catechin/ epicatechin, cis-/ trans-resveratrol and cis-/ trans-para-coumaric acid within one run. The potential of the phenolic spectrum emphasizing the significance of isomeric ratios for classification of the investigated wines according to geographic origin, grape variety and vintage was evaluated.

Materials and Methods

Chemicals and standards

HPLC-MS grade methanol and acetonitrile were obtained from Fisher Scientific, formic acid, Suprapur™, 98-100% and ammonia, Suprapur™ 25% from Merck, Darmstadt, Germany. Ferulic acid, gallic acid hydrate and caffeic acid were purchased from Carl Roth, Karlsruhe, Germany, trans p-coumaric acid, myricetin, kaempferol, trans-resveratrol, (+)-catechin hydrate, (-)-epicatechin from Fluka, Buchs, Switzerland, quercetin dihydrate and naringenin from Sigma-Aldrich, Vienna, Austria. Cis resveratrol and cis p-coumaric acid were not available as standards.

Sample preparation

Authentic wine samples were obtained from the Federal College and Research Institute for Viticulture and Pomology in Klosterneuburg (HBLAuBA). The samples used for the study were 97 authentically vinificated red wines from six different grape varieties. The grapes were obtained from eleven different wine regions in Austria including five vintages from 2003 to 2007. According to a standard protocol approximately 50 kg of grapes were destemmed, crushed and 30 mg L^{-1} SO_2 were added. A quick alcoholic fermentation was achieved by addition of 20 g hl^{-1} of a selected dry yeast preparation (Oenoferm Klosterneuburg, Fa. Erbsloeh, Geisenheim, Germany). The end of alcoholic fermentation was detected by analysis of residual sugars with FT-IR with a Winescan 120 (Foss, Rellingen, Germany). Malolactic fermentation was induced by addition of a culture of Oenococcus oeni. After a natural settling period of two weeks at 4°C the wines were filtered through K 150 (Seitz, Bad Kreuznach, Germany) filter sheets. The wines were stored in darkness at 4° C.

Prior to analysis the wine samples were filtrated, (Iso-Disc™, N-4-4, Nylon, 4 mm * 0.45 µm, Supelco, Bellefonte, PA, United States) and diluted 1:10 using 10 mM ammonium formate buffer pH 3.75, with 10 Vol % methanol. Subsequently, 20 µL of internal standard (50 mg L^{-1} naringenin in methanol) were added.

For each substance a 1 mg mL^{-1} solution in methanol was prepared and a multi compound standard solution was prepared in 10 mM ammonium formate buffer pH 3.75 with 10 Vol % methanol for external calibration of the LC-MS/MS system. The working range of the different analytes was adapted to the concentration of the investigated compounds in the diluted wine samples (Table 1).

Instrumental

Quantification of the selected phenols was performed via LC-MS/MS in negative ionization mode on a 6410 triple quadrupole mass spectrometer from Agilent Technologies (Palo Alto, CA, United States) equipped with an electrospray ionization (ESI) interface. Nitrogen was used as desolvation- and collision gas. The mass spectrometer was connected to a liquid chromatography system (1200 series, Agilent). The system consists of a quaternary gradient pump, an autosampler, a degasser and a column thermostat. C-18 reversed phase separation was performed on a Rapid Resolution HT 2.1 x 50 mm column with 1.8 µm particle diameter (Agilent). Mass Hunter software (version B.01.03) was used for instrument control and data processing. Solvent A was 99 % water, 1 % acetonitrile and 0.1 % formic acid and solvent B consisted of 99 % acetonitrile, 1 % water and 0.1 % formic acid. The flow rate was set to 0.4 mL min^{-1}, column temperature was at 20° C and the injection volume was 3 µL. The gradient profile was 0.0-1.5 min 2% B, from 1.5-11.25 min 45% B, from 11.25-12.75 min 70 and at 12.82 min back to the initial conditions of 2% B.

Statistical data evaluation

The concentration of the phenolic compounds, the geographical origin, the grape variety and the vintage (Pazourek, Gajdošová, Spanilá, Farková, Novotná and Havel, 2005; Giaccio and Del Signore, 2004; Boselli, Boulton, Thorngate and Frega, 2004) of the investigated wine samples were used as input variables for canonical discriminant analysis. All data were processed via SPSS 15 (SPSS, Chicago, United States).

Results and discussion

Selection of authentic wine samples

Wine samples were taken from the Austrian collection of authentic wines at the Federal College and Research Institute for Viticulture and Pomology in Klosterneuburg, HBLAuBA, where these wines were produced in technical scale for the wine data base of the European Union. The selection of wines is representative for the Austrian viticulture, comprising the most important grape varieties from all major Austrian wine regions (see Table 1). Production of authentic wines was performed with healthy grapes (approximately 50 kg per sample) from selected vineyards, which were harvested from the wine control at optimum ripeness. Vinificated was carried out at HBLAuBA Klosterneuburg with a standard procedure without addition of any agents except selected dry yeast and malolactic bacteria (see experimental section).

LC-MS/MS analysis of selected phenols in wine

LC-MS/MS was selected as analytical method for analysis of phenols in wine because of its high selectivity and sensitivity. Compared to the studies published so far we have improved the efficiency of chromatographic separation of phenols using a stationary phase with sub-2 µm particles (Gruz, Novak and Strnad, 2008; Klejdus, Vacek, Lojkova, Benesova and Kuban, 2008; Saenz-Navajas, Tena and Fernandez-Zurbano, 2009). As a consequence and novelty it was possible to separate the isomeric structures of the analytes catechin - epicatechin, cis-/ trans-para-coumaric acid and cis-/ trans-resveratrol within one chromatographic run. The chromatograms depicted in Figure 1 A-C show the separations of the respective isomeric structures. The isomeric structures of cis-p-coumaric acid and cis-resveratrol were produced via ageing of trans-p-coumaric acid and trans-resveratrol,

respectively. Separation of all analytes of interest was performed within 10 minutes leading to a total chromatographic runtime of 15 minutes including post-conditioning of the column. Mass spectrometric detection was performed utilizing the multiple reaction monitoring of the triple quadrupole MS. Table 1 lists the selected transitions for the 11 investigated substances.

Ideal fragmentation voltages were obtained via optimization for each substance (see Table 1). For the isomeric structures of cis-p-coumaric acid and cis-resveratrol the fragmentation voltages of the commercially available trans-standards were applied assuming similar optimum fragmentation conditions of the cis/trans isomers. The over-all working range for analysis of the 1:10 diluted wine samples was between the lower working range of 0.0036 mg L-1 for quercetin (LOQ of the lowest concentrated analyte) and the upper working range of 72 mg L-1 for gallic acid, catechin and epicatechin (see Table 1). LODs and LOQs were calculated according to the 3 sigma and 10 sigma criterion, i.e. the 3-fold and 10-fold standard deviation of the noise quantified via single point calibration. Quantification of the analytes was performed via external calibration with internal standardization (naringenin). The isomers cis-p-coumaric acid und cis-resveratrol were quantified employing the commercially available standards of of trans-p-coumaric acid und trans-resveratrol respectively.

The trueness of results obtained from external calibration was validated by standard addition experiments performed with selected wine samples. The concentrations of the analytes obtained for all 97 investigated wines are available as supplementary data.

Classification of investigated wines

The concentrations of the phenolic compounds were subjected to canonical discriminant analysis, in order to evaluate the potential of the phenolic pattern for classification of the

samples according to the geographical origin, the grape variety and the vintage. It is noteworthy that the inclusion of the isomeric pairs catechin/ epicatechin, cis-/ trans-resveratrol and cis-/ trans-para-coumaric acid led to a substantial increase of the statistical significance of the data.

Geographical origin based classification

Evaluation of the phenol based differentiation of wines according to their geographical origin revealed that the two major Austrian grape varieties i.e. Zweigelt and Blaufränkisch delivered the most significant results. The investigated datasets contained the phenol concentrations obtained for Zweigelt from the regions Südsteiermark, Kamptal, Donauland and Thermenregion, and for Blaufränkisch from the regions Carnuntum, Neusiedlersee Hügelland and Mittelburgenland. Thereby, the according geographical region was set as grouping variable and the evaluated concentrations of the analytes of the considered wines were defined as independent variables. The canonical discriminant analysis for this data set resulted in three discriminant functions (DF 1_1 - DF 1_3; Table 2). The statistical calculation was controlled by a *leave one out test* and 100 % of all grouped cases were classified correctly. Figure 2A illustrates the high canonical correlation and consequent discrimination of regions Südsteiermark, Kamptal, Donauland and Thermenregion for the Zweigelt grape variety.

For the second geographical origin based analysis the grape variety Blaufränkisch from the geographical regions Carnuntum, Neusiedlersee Hügelland and Mittelburgenland were selected. The canonical discriminant analysis resulted in two discriminant functions DF 2_1 and DF 2_2 (Table 2). The statistical calculation was tested and 84 % of the grouped cases were correctly classified.

This results is of lower statistical significance and quality (Table 2, Figure 2B) because the eigenvalues and also the canonical correlation are lower. This is also visible by the result

obtained for the *leave one out test*. A possible reason for this result is the geographical closeness (radius of approximately 50 km) of the wine regions Carnuntum, Neusiedlersee Hügelland and Mittelburgenland.

Determination of the geographical origin of a selected grape variety is predominantly triggered by economical reasons, as wines from different regions do also show differences in quality and price. Another reason is the Austrian DAC classification (Districtus Austriae Controllatus), which defines four high quality regions in Austria. Our results indicate an analytical differentiation of those wines by means of the phenol pattern could support their protection against fraud in the context of geographical origin.

Grape variety based classification

The investigated datasets for the grape variety based discriminant analysis include the results obtained for the grape varieties, Blauer Portugieser, Blauer Wildbacher and Sankt Laurent, and Blauer Zweigelt, Blaufränkisch and Blauburger from the geographical region Weinviertel. For statistical analysis the concentrations of the analytes in the authentic wines were set as independent variables and grape varieties were set as grouping variables. The preliminary results of the statistical evaluation indicated that the data had to be separated into two datasets in order to get significant results. The first dataset consists of three different grape varieties, Blauer Portugieser, Blauer Wildbacher and Sankt Laurent, whereas the second set includes the grape varieties Blauer Zweigelt, Blaufränkisch and Blauburger. The canonical discriminant analysis of the two different datasets resulted in two discriminant functions DF 3_1and DF 3_2 and DF 4_1and DF 4_2 (Table 2). Additionally, the two datasets were tested by a *leave one out test* resulting in 100 % correctly classified cases including the grape varieties Blauer Portugieser, Blauer Wildbacher and Sankt Laurent and 65 % for the grape varieties Blauer Zweigelt, Blaufränkisch and Blauburger. The results obtained for the two datasets are illustrated in Figure 3A and 3B respectively.

The quality of the variety based discriminant analysis for the grape varieties, Blauer Portugieser, Blauer Wildbacher and Sankt Laurent (Table 2, Figure 3A) is satisfying because of the high eigenvalues and canonical correlation. However, the variety based analysis of Blauer Zweigelt, Blaufränkisch and Blauburger (Table 2, Figure 3B) gave non acceptable results because of the low eigenvalues and low canonical correlation. This result is also supported out by the *leave one out* test, where just 65 % of the grouped cases were classified correctly.

Generally, reliable analytical results for a grape based classification of (Austrian) wines would be desirable in order to protect the consumer from fraud related to adulteration of false labelling. Obviously, the data obtained from phenolic pattern lacks significance, but could increase the significance of the data, which is currently obtained from isotope ratio or multi-element based classifications for wine authentication. Moreover, the situation will be much more difficult regarding cuvees or other commercially available wines, as those wines can be blended with 15 % of wine originating from other regions or grape varieties. This fact is challenging in terms of wine authentication and indicates that new chemical analytical strategies are necessary.

Vintage based classification

The investigated datasets for the vintage based discriminant analysis included 3 different datasets, i.e. (A) the results of all available wine samples, (B) the results of the grape variety Blauer Zweigelt and (C) the results of the grape variety Blaufränkisch. For statistical analysis the concentrations of the analytes of the authentic wines were set as independent variables and the five vintages (2003 - 2007) were set as grouping variables. The grape varieties Blaufränkisch and Blauer Zweigelt were chosen in addition to the analysis of the complete

data as they are the main representatives in the sample list of authentic wines and these are also the main representatives of Austrian commercially available red wines.

The canonical discriminant analysis of the data sets resulted in four discriminant functions DF 5_1. - DF 5_4, DF 6_1. - DF 6_4 and DF 7_1. - DF 7_4, respectively (Table 2). The *leave one out* test was processed for each discriminant analysis and all grouped cases were classified correctly for 95 % of A and 100 % of B and C, respectively. The vintage based canonical discriminant analysis shows significant statistical results for all three processed data sets. Figure 4A clearly shows that the discriminant analysis of the concentration of the selected phenols allowed an unambiguous classification of the five vintage years from 2003 until 2007 taking all geographical origins and grape varieties into account. In Fig. 4B and 4C this is also demonstrated for the grape varieties Blaufränkisch and Blauer Zweigelt.

As a novelty the proposed mass spectrometry based methodology could be applied in case of potential vintage fraud. The reason for differentiating vintages is an issue of trademark and consumer protection and clearly economically motivated. In some cases the storage duration in oak barrels and the time span since the harvest of the wine is directly related to the wine quality and consequently to the prize (e. g. the Hispanic Reserva versus the Gran Reserva; Reserva can be sold 4 years after harvesting the berries, the Gran Reserva can be sold only after five years).

Conclusion

The employment of sub-2 µm particles as liquid chromatographic stationary phase for analysis of phenols in red wine allows the separation of several isomeric structures. Statistical evaluation of the data obtained by the LC-MS/MS method showed that phenolic pattern including 3 isomeric pairs can support the origin- and grape variety based classification of authentic wines. In future the phenol distributions will be assessed along with isotope ratio measurements and elemental patterns. We see a great potential of this

data combination for the application in food authenticity. A major finding of our study is the high potential of phenol based analysis for unambiguous classification of Austrian red wines according to different vintages (2003-2007). Future studies will aim at the application of the method to commercially available wines.

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Figure captions

Figure 1: Chromatographic separation and measured quantifiers of isomeric structures via sub-2 µm stationary phase (A trans- / cis - p -coumaric acid; B trans-/ cis-resveratrol; C catechin/epicatechin in Blauer Zweigelt, Weinviertel, 2005).

Figure 2: Geographic origin based discriminant analysis of (A) the grape variety Blauer Zweigelt originating from the regions Südsteiermark, Kamptal, Donauland and Thermenregion. (B) shows the statistical results obtained for the grape variety Blaufränkisch from the regions Carnuntum, Neusiedlersee Hügelland and Mittelburgenland.

Figure 3 Grape variety discriminant analysis of (A) the grape varieties Blauer Portugieser, Blauer Wildbacher and Sankt Laurent stemming from all investigated regions and (B) the grape varieties Blauer Zweigelt and Blaufränkisch originating from the geographical region Weinviertel The third group in the graph is not representative because only two samples were obtained from the grape variety Blauburger.

Figure 4: Vintage based discriminant analysis of (A) all investigated authentic wines obtained from the vintages 2003-2007. (B) and (C) show the reduced data for the two major Austrian grape varieties Blauer Zweigelt and Blaufränkisch, respectively.

Figures

Figure 1:

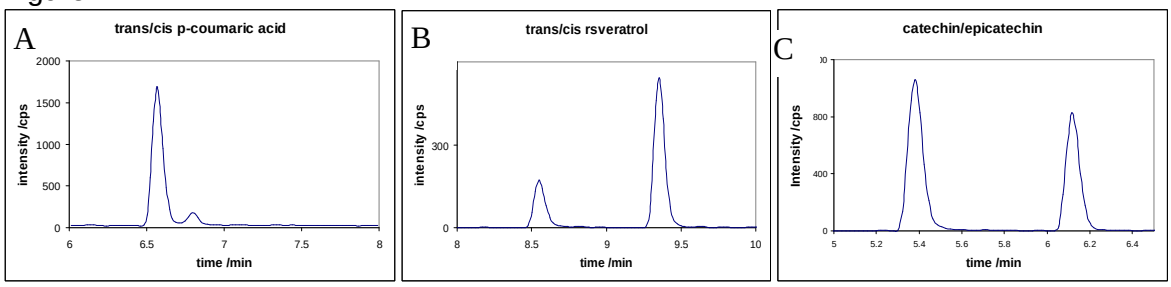


Figure 2A:

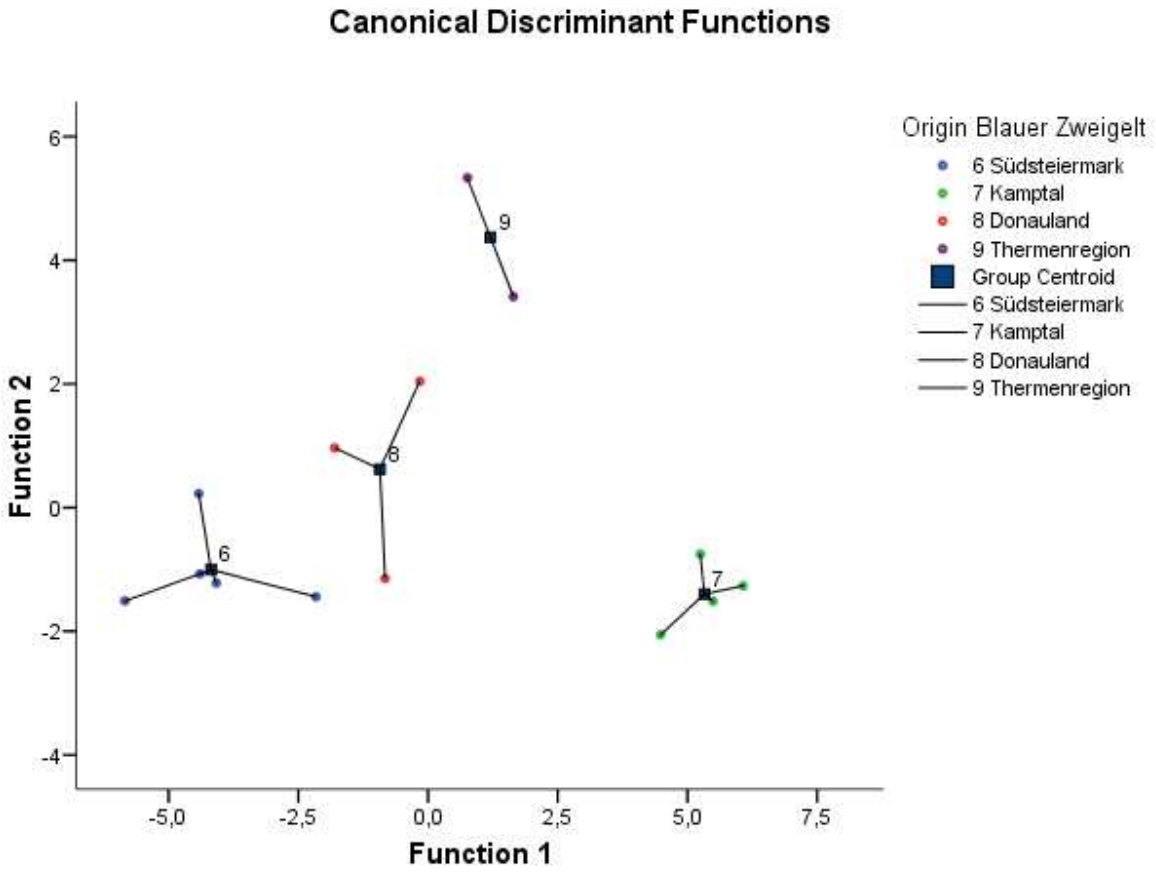

Canonical Discriminant Functions
Origin Blauer Zweigelt
6 Südsteiermark
7 Kamptal
8 Donauland
9 Thermenregion
Group Centroid
6 Südsteiermark
7 Kamptal
8 Donauland
9 Thermenregion
Function 2
Function 1
6
4
2
0
-2
-4
-5,0
-2,5
0,0
2,5
5,0
7,5
9
8
6
7

Figure 2B:

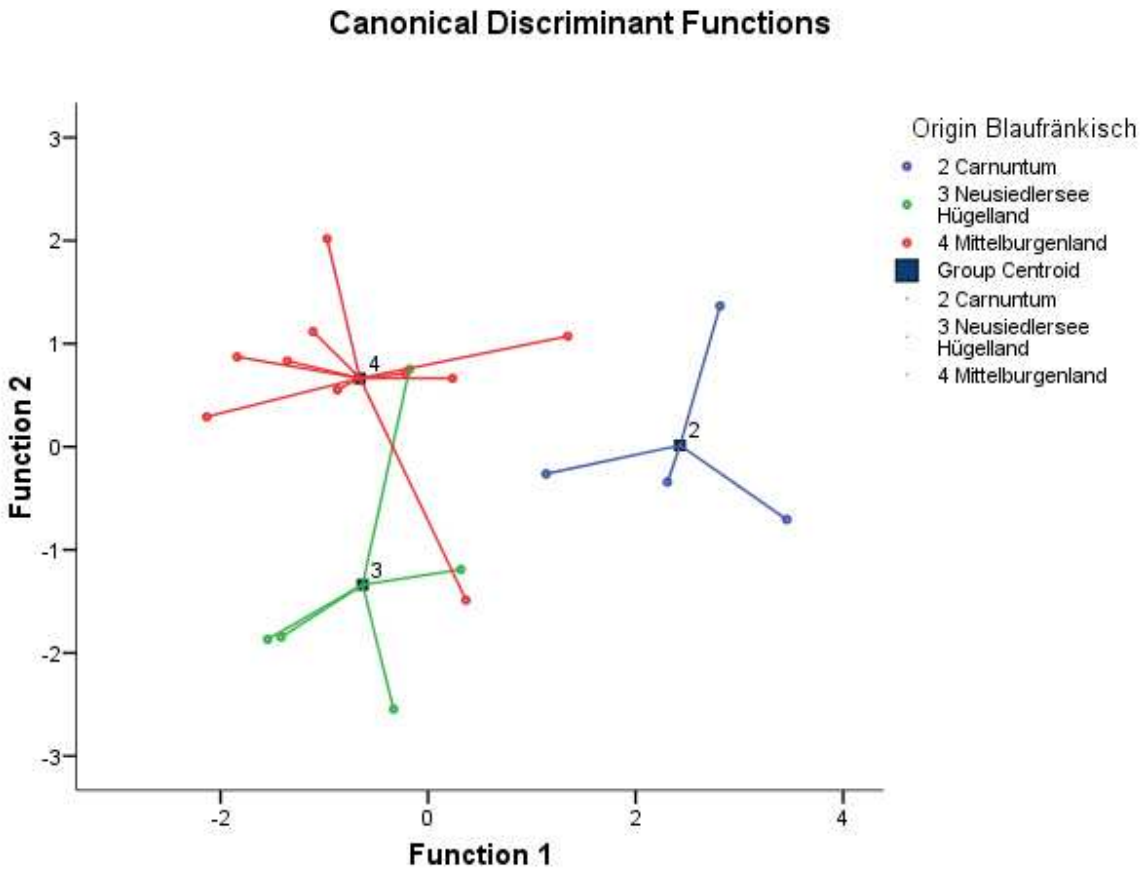

Canonical Discriminant Functions
Origin Blaufränkisch
2 Carnuntum
3 Neusiedlersee Hügelland
4 Mittelburgenland
Group Centroid
2 Carnuntum
3 Neusiedlersee Hügelland
4 Mittelburgenland
Function 2
Function 1
3
2
1
0
-1
-2
-3
-2
0
2
4

Figure 3A:

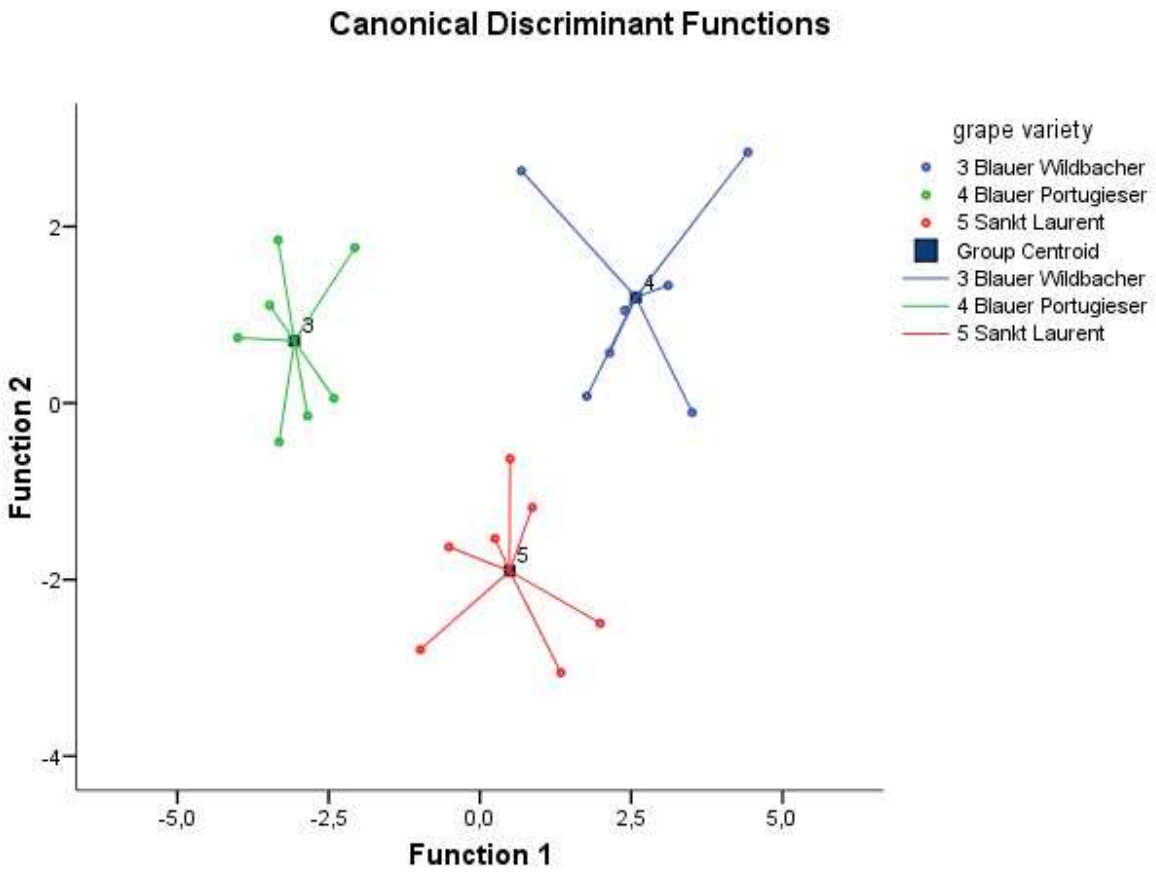

Canonical Discriminant Functions
grape variety
3 Blauer Wildbacher
4 Blauer Portugieser
5 Sankt Laurent
Group Centroid
3 Blauer Wildbacher
4 Blauer Portugieser
5 Sankt Laurent
Function 2
2
0
-2
-4
-5,0
-2,5
0,0
2,5
5,0
Function 1
3
4
5

Figure 3B:

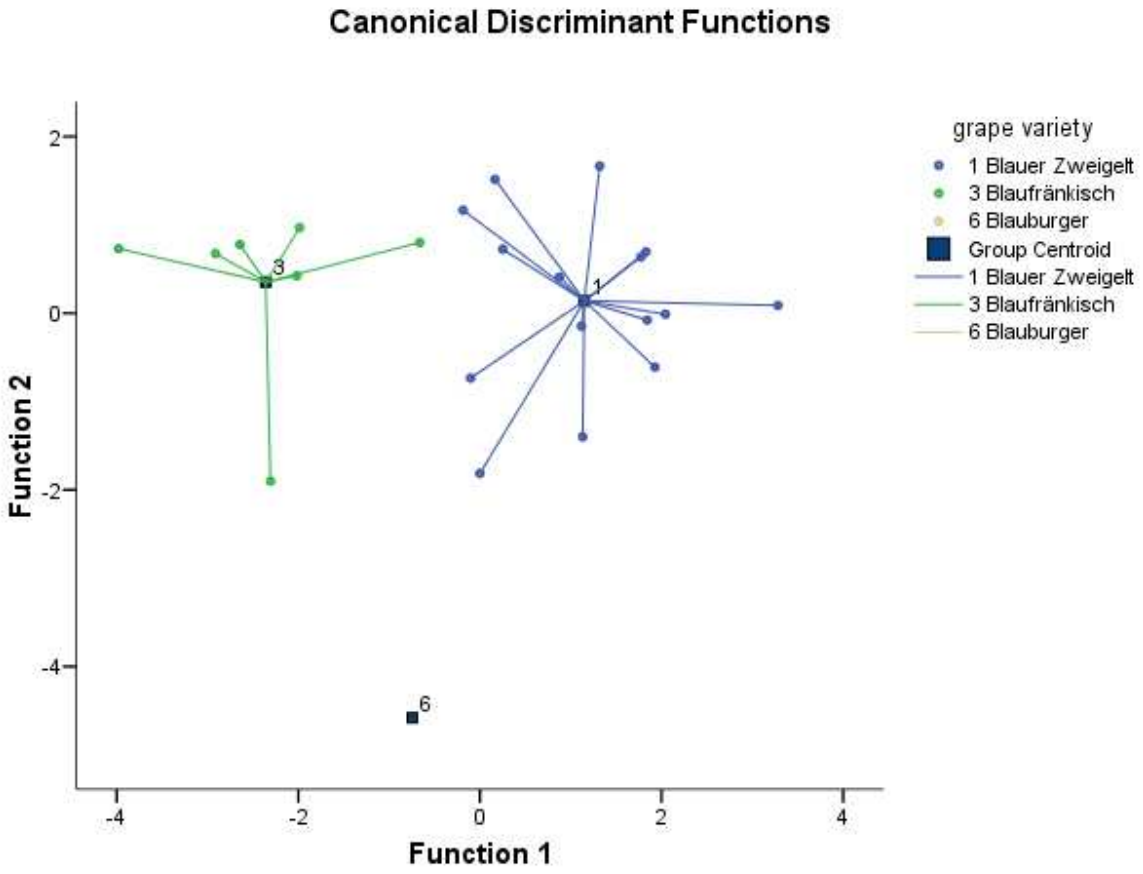

Canonical Discriminant Functions
grape variety
1 Blauer Zweigelt
3 Blaufränkisch
6 Blauburger
Group Centroid
1 Blauer Zweigelt
3 Blaufränkisch
6 Blauburger
Function 2
Function 1
2
0
-2
-4
-4
-2
0
2
4
3
1
6

Figure 4A:

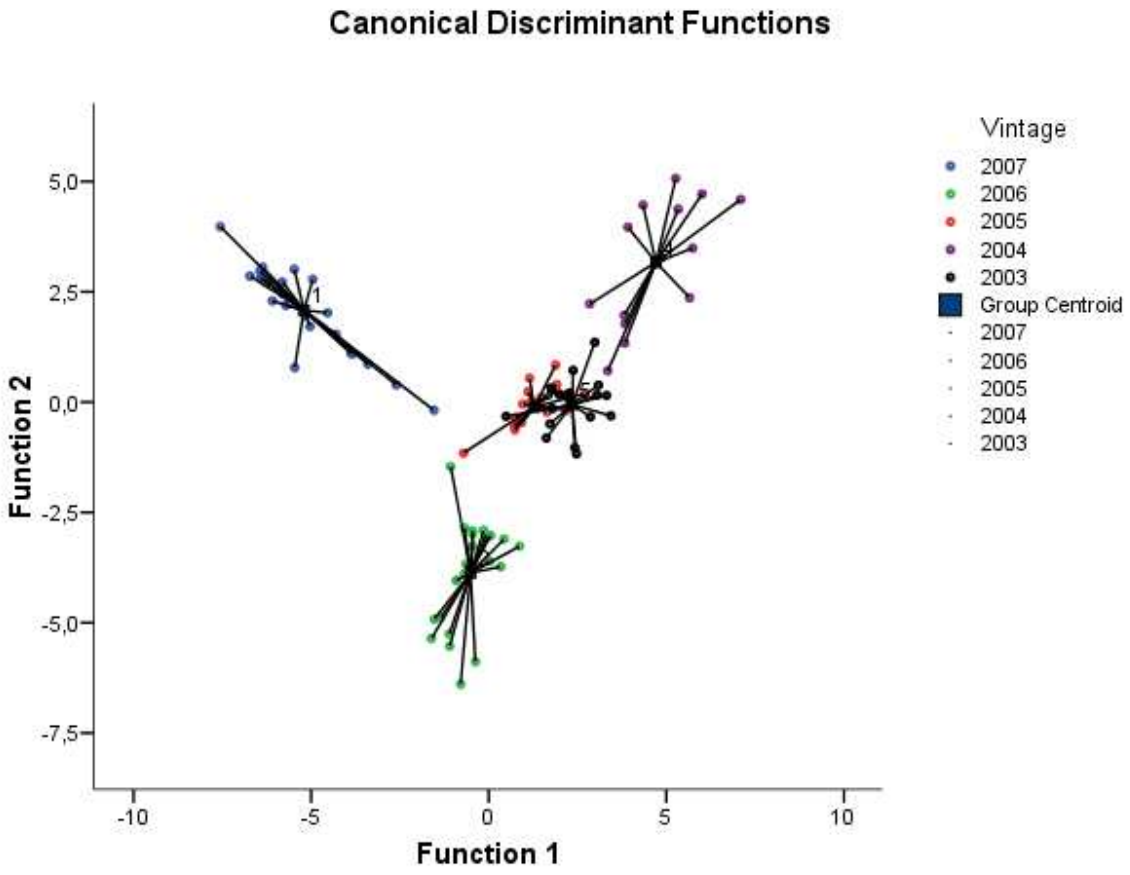

Canonical Discriminant Functions
Function 2
5,0
2,5
0,0
-2,5
-5,0
-7,5
-10
-5
0
5
10
Function 1
Vintage
2007
2006
2005
2004
2003
Group Centroid
2007
2006
2005
2004
2003

Figure 4B:

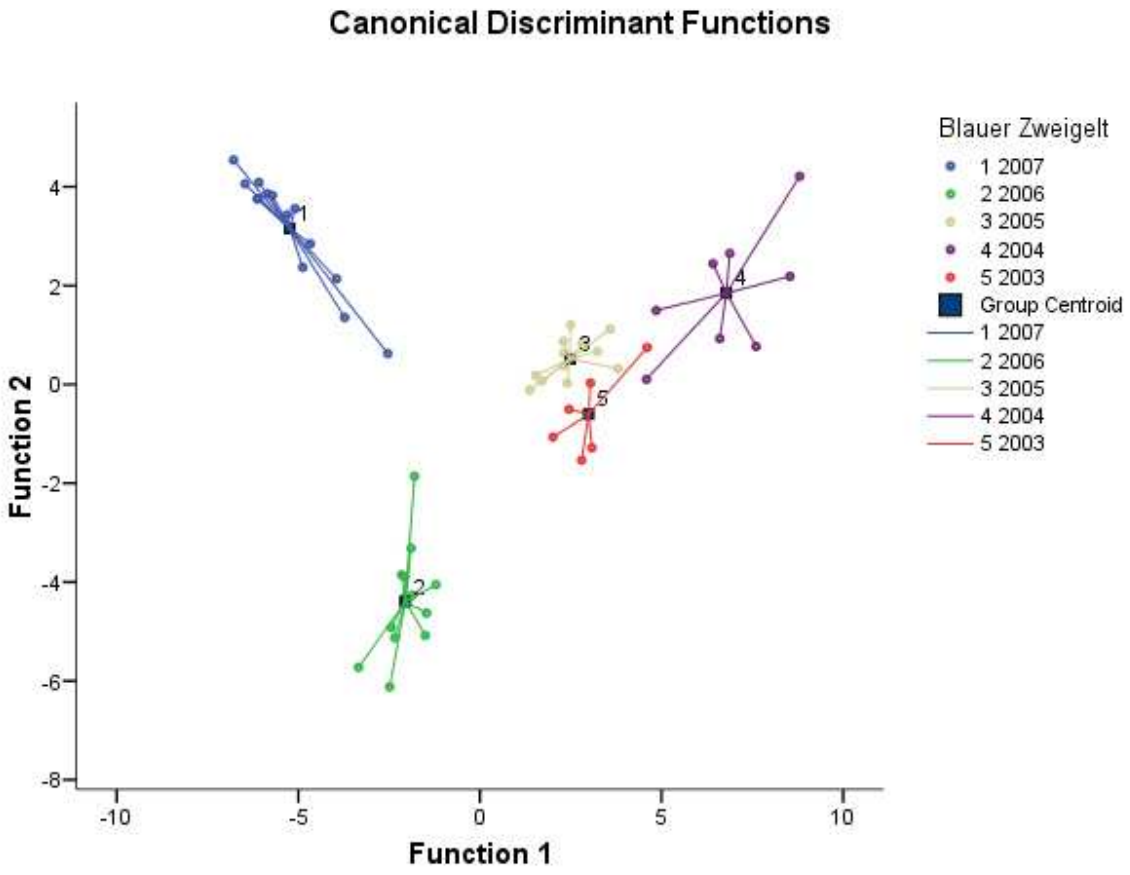
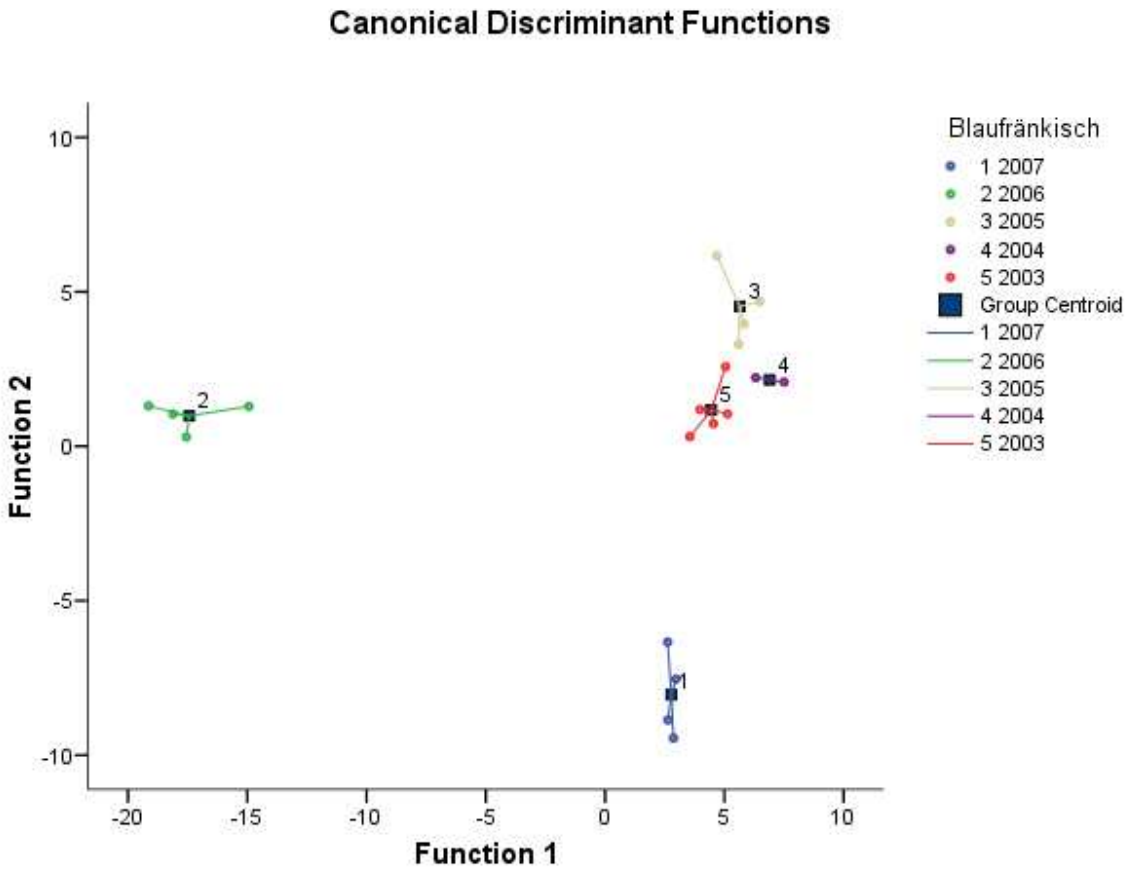

Canonical Discriminant Functions
Function 2
Function 1
4
2
0
-2
-4
-6
-8
-10
-5
0
5
10
1
2
3
4
5
Blauer Zweigelt
1 2007
2 2006
3 2005
4 2004
5 2003
Group Centroid
1 2007
2 2006
3 2005
4 2004
5 2003

Figure 4C:


Canonical Discriminant Functions
Function 2
Function 1
10
5
0
-5
-10
-20
-15
-10
-5
0
5
10
1
2
3
4
5
Blaufränkisch
1 2007
2 2006
3 2005
4 2004
5 2003
Group Centroid
1 2007
2 2006
3 2005
4 2004
5 2003

Tables

Table 1: Basic parameters of the analytes including the retention time, limit of detection (LOD), limit of quantification (LOQ), working range, monitored masses (m/z) and MS/MS parameters applied for analysis of the selected compounds. Only one transition was available for gallic acid, catechin, caffeic acid and epicatechin. The identity of these analytes was confirmed via one transition and the retention time. Due to their structural similarity, the validation parameters for cis-p-coumaric acid and cis-resveratrol were adopted from trans-p-coumaric acid and trans-resveratrol respectively, as no standards were commercially available.

	Retention time [min]	LOD [µg L^{-1}]	LOQ [µgL^{-1}]	working range [µgL^{-1}]	M Da	[M-H] m/z	Quantifier m/z	Qualifier m/z	Fragmentor voltage /V	Collision energy /V
gallic acid	0.9	30	90	90-7500	170	169	125	-	110	10
catechin	5.4	25	83.2	83.2-5000	290	289	203	-	110	10
caffeic acid	5.5	3.1	10.3	10.3-900	180	179	135	-	110	15
epicatechin	6.1	20.6	68.8	68.8-5000	290	289	203	-	110	10
cis p-coumaric acid	6.5	1.5	4.9	4.9-1500	164	163	119	93	110	15
trans p-coumaric acid	6.8	1.5	4.9	4.9-1500	164	163	119	93	110	15
ferulic acid	6.9	0.4	1.3	1.3-180	194	193	134	178	110	15
myricetin	8.1	0.3	0.9	0.9-1350	318	317	151	179	110	20
cis- resveratrol	9.4	0.05	0.2	0.2-4500	228	227	143	159	110	20
trans- resveratrol	8.6	0.05	0.2	0.2-4500	228	227	143	159	110	20
quercetin	9.2	1.1	3.6	3.6-630	302	301	151	178	110	20

Table 2: Results of the statistical evaluation with canonical discriminant analysis. A detailed discussion of the values is given in the text.

Function	Eigenvalue	% of Variance	Canonical correlation
DF 1_1	20.633	78.9	0.977
DF 1_2	5.221	20.0	0.916
DF 1_3	0.308	1.1	0.485
DF 2_1	1.868	69.1	0.807
DF 2_2	0.837	30.9	0.675
DF 3_1	6.327	74.5	0.929
DF 3_2	2.160	25.5	0.827
DF 4_1	2.398	93.9	0.840
DF 4_2	0.157	6.1	0.368
DF 5_1	11.582	54.6	0.959
DF 5_2	6.356	30.0	0.930
DF 5_3	2.086	9.8	0.822
DF 5_4	1.174	5.5	0.735
DF 6_1	19.173	53.7	0.975
DF 6_2	9.034	25.3	0.949
DF 6_3	3.969	11.1	0.894
DF 6_4	3.549	9.9	0.883
DF 7_1	112.020	71.2	0.996
DF 7_2	25.782	16.4	0.981
DF 7_3	11.224	7.1	0.958
DF 7_4	8.219	5.2	0.944

Supplementary data: Concentrations [mg L^{-1}] of the analytes in the authentic wine samples. Measurement uncertainty is given in terms of the relative standard deviation obtained for 3 repetitive measurements.

Vintage	Grape variety	Region	gallic acid	RSD %	caffeic acid	RSD %	catechin	RSD %	epicatechin	RSD %	cis resveratrol	RSD %	trans resveratrol	RSD %
2003	BF	NsHl	5.1	0.4	7.8	2.9	7.5	1.1	2.3	6.2	1.9	4.8	1.4	1.3
	BF	MB	12.5	1.1	13.7	1.1	16.7	2.0	8.2	4.3	2.3	2.1	1.8	1.7
	BF	MB	13.8	9.0	12.6	6.5	14.2	7.8	4.7	1.1	3.8	9.8	3.3	3.3
	BF	MB	9.3	3.8	7.8	1.3	14.0	2.0	7.5	2.4	4.0	3.6	2.8	2.5
	BF	MB	8.2	2.8	14.8	3.7	16.4	6.7	11.2	5.4	4.4	2.0	4.1	1.3
	BP	WV	4.9	6.5	17.1	6.2	10.8	1.9	2.6	7.7	2.8	7.4	2.1	8.4
	BP	WV	5.1	6.4	6.0	6.1	15.5	6.6	4.5	5.8	0.5	1.9	0.7	8.5
	BW	WSt	17.2	4.5	7.2	4.1	22.9	3.0	6.8	2.8	4.6	4.1	2.4	1.6
	BW	WSt	9.5	3.4	5.5	4.4	19.0	7.1	6.1	1.7	6.3	5.1	3.2	2.8
	BW	WSt	10.8	6.8	5.9	4.2	27.8	4.4	8.3	8.8	0.5	6.1	0.6	5.4
	BZ	Car	12.7	3.9	12.4	5.0	16.1	7.0	7.4	3.1	1.9	6.5	1.9	0.4
	BZ	MB	11.2	4.8	9.0	3.0	6.5	7.2	2.9	3.5	1.5	5.1	1.3	3.1
	BZ	SSt	9.2	5.4	9.0	7.9	8.0	9.3	4.3	5.3	2.3	7.5	1.5	7.6
	BZ	SoSt	12.2	5.2	10.7	6.7	11.0	6.3	4.1	0.8	0.3	5.5	0.5	7.0
	BZ	MB	5.8	4.9	8.3	4.4	7.5	2.4	3.9	8.0	1.4	9.8	1.0	4.6
	CS	MB	18.5	3.7	4.2	2.5	17.7	2.7	4.2	5.9	2.3	7.2	1.4	0.4
2004	BF	NsHl	7.1	3.5	8.5	2.4	13.8	2.4	3.9	2.3	7.0	3.2	4.5	0.6
	BF	Car	11.6	3.1	8.3	2.2	21.9	3.1	6.0	5.2	2.7	3.7	3.8	2.4
	BP	WV	5.3	6.3	25.0	6.2	16.6	3.8	4.6	6.2	5.0	8.6	4.4	7.4
	BP	WV	6.2	0.8	26.6	0.9	2.3	2.6	1.4	4.9	1.1	5.5	0.9	5.7
	BW	WSt	71.5	3.4	11.1	2.6	47.0	4.0	29.3	4.4	1.9	4.3	3.4	3.1
	BZ	WV	53.4	5.4	38.2	5.8	53.4	5.3	29.9	3.5	6.0	7.0	8.1	2.7
	BZ	WV	12.7	5.9	8.6	5.7	15.9	4.3	6.3	9.2	4.2	5.8	5.5	1.7
	BZ	WV	51.0	3.5	9.3	3.0	45.4	3.5	25.7	2.5	4.4	1.6	7.7	2.3
	BZ	Car	12.7	1.9	20.2	1.8	12.9	3.6	5.1	2.4	4.6	2.7	6.5	0.2
	BZ	SSt	41.5	4.0	26.0	2.8	25.7	1.6	30.0	4.9	1.4	2.2	2.9	1.9
	BZ	SoSt	28.9	0.9	31.7	1.0	23.1	4.0	18.4	1.1	1.9	3.6	2.5	1.8
	BZ	NsHl	12.3	4.0	5.9	3.3	9.8	2.7	5.4	2.8	11.6	3.0	7.5	1.7
	BZ	KT	28.3	1.4	6.4	1.3	14.6	2.8	6.2	3.7	2.6	3.4	4.3	1.4
	SL	TR	12.0	0.7	36.2	1.6	12.6	1.5	4.5	6.6	7.3	3.3	7.1	2.2
2005	BF	NsHl	1.7	0.9	7.8	1.8	14.1	3.9	8.6	3.9	9.1	0.9	6.7	2.6
	BF	MB	0.9	7.4	9.3	5.5	17.8	5.9	7.4	7.8	5.0	8.5	4.5	3.8

	BF	MB	2.3	7.7	10.1	13.3	25.6	12.0	10.2	7.0	1.2	6.8	0.5	9.0
	BF	NsHl	1.7	8.6	5.3	9.6	13.3	4.2	4.9	8.2	2.9	4.5	2.8	9.4
	BF	Car	1.2	5.3	5.0	3.4	7.2	1.5	5.0	9.2	7.1	5.7	3.6	4.9
	BP	WV	0.9	1.5	9.9	2.8	1.4	5.5	1.1	7.1	1.2	2.9	0.3	6.9
	BZ	Car	0.8	3.9	5.9	3.0	5.4	4.4	2.1	4.4	3.9	4.0	1.5	3.4
	BZ	MB	0.9	2.3	6.2	2.1	11.3	2.9	3.1	5.1	4.5	3.8	1.8	2.9
	BZ	MB	1.6	5.1	5.7	4.1	6.1	1.4	4.5	4.6	3.8	6.3	1.5	7.3
	BZ	KT	1.9	0.8	4.9	2.0	13.9	4.2	11.5	2.6	3.9	1.8	2.7	5.2
	BZ	WV	1.2	4.6	9.3	4.5	5.9	6.8	1.4	13.3	6.8	7.9	2.0	4.7
	BZ	NsHl	0.5	2.1	8.2	2.2	11.5	5.1	4.4	2.0	3.1	5.2	1.7	2.4
	BZ	Wv	1.2	6.1	6.2	5.4	10.2	9.5	2.1	12.3	3.6	7.0	0.7	8.0
	BZ	Dl	2.5	5.7	3.6	2.6	18.4	6.5	13.3	7.4	3.0	3.2	1.1	7.7
	BZ	WV	1.4	5.0	6.2	4.6	11.3	7.4	7.8	7.2	4.9	6.2	1.6	8.1
	SL	TR	1.1	3.2	8.8	3.0	10.3	3.8	4.6	1.6	6.1	2.2	2.3	4.3
	SL	NsHl	2.2	2.5	6.1	2.9	16.2	1.3	11.4	2.1	3.9	5.6	4.2	0.9
2006	BB	WV	13.9	0.5	9.1	0.4	33.5	1.7	15.8	0.9	1.5	2.0	0.8	3.9
	BF	NsHl	23.0	6.4	7.1	4.4	43.4	6.8	27.3	8.1	5.8	4.2	4.6	5.6
	BF	MB	25.2	9.4	10.9	8.4	42.2	7.8	25.4	5.1	4.6	6.1	3.5	6.7
	BF	MB	21.4	6.6	6.2	6.3	33.2	5.7	12.7	3.2	4.2	7.6	2.9	7.2
	BF	Car	18.7	8.2	10.0	8.0	33.8	7.4	12.8	7.2	11.2	5.7	8.2	7.3
	BP	WV	17.9	3.7	2.8	3.8	38.6	1.7	11.7	2.1	0.5	8.6	0.9	3.4
	BW	WSt	117.9	8.6	9.9	8.1	65.4	5.3	43.9	5.3	0.2	14.7	0.1	13.0
	BZ	BZ	18.4	8.7	5.0	8.3	23.9	4.3	13.5	7.9	5.7	8.7	2.3	7.7
	BZ	MB	15.0	8.0	4.0	6.5	14.7	8.2	5.7	3.8	3.0	8.3	0.9	0.8
	BZ	SSt	15.8	6.2	4.9	5.3	15.6	3.0	7.4	7.3	1.6	6.5	0.9	9.3
	BZ	MB	21.2	7.5	26.9	8.5	25.0	7.5	17.3	7.9	2.5	3.9	1.6	1.6
	BZ	NsHl	14.7	6.3	5.8	6.5	15.4	6.1	10.6	7.6	7.3	8.6	3.8	6.9
	BZ	KT	8.7	1.2	3.6	1.1	12.4	5.1	6.4	3.4	5.6	2.4	3.0	6.2
	BZ	WV	19.4	8.8	6.6	7.8	18.8	6.8	16.7	6.4	1.4	4.3	0.8	1.5
	BZ	WV	29.2	1.4	5.6	0.7	29.1	2.0	28.8	2.1	2.9	3.9	2.4	1.7
	BZ	DL	18.0	0.7	3.4	1.8	17.1	2.0	11.0	4.9	3.0	2.2	1.9	5.2
	BZ	WV	15.9	0.5	3.2	0.7	19.4	1.4	16.7	0.6	1.2	1.8	1.3	3.4
	BZ	TT	22.8	0.6	4.6	2.5	29.7	1.9	23.7	1.9	1.8	1.8	1.7	2.8
	BZ	NsHl	8.9	0.7	3.2	1.4	6.1	1.0	2.0	6.0	3.1	3.5	0.8	5.1
	BZ	TR	23.4	2.8	4.3	1.6	16.6	1.8	7.7	3.6	8.2	1.7	3.3	2.1
	BZ	WV	11.5	1.5	4.8	0.7	13.9	2.7	5.3	2.2	1.9	0.6	1.8	5.6
	SL	TR	23.9	8.6	8.9	9.1	29.8	7.2	10.0	7.7	7.3	7.6	3.1	8.3
	SL	NsHl	21.6	6.3	8.5	6.4	28.4	4.9	8.2	5.7	2.6	7.2	0.9	8.6
2007	BB	WV	8.1	7.8	1.3	2.8	7.4	7.2	5.0	8.4	4.9	4.8	2.5	6.3

BF	NsHl	13.1	4.6	4.4	6.6	22.6	2.9	11.8	5.0	2.7	5.9	1.5	4.8
BF	MB	3.7	4.6	5.4	6.7	11.7	5.7	4.7	0.4	8.3	3.3	4.6	4.9
BF	MB	6.0	4.3	3.0	7.0	9.5	6.5	4.2	4.9	3.8	7.3	2.5	9.3
BF	Car	4.9	4.3	2.0	5.8	14.8	6.4	6.2	5.2	2.2	3.6	1.2	4.3
BP	WV	3.6	4.4	1.4	6.8	6.5	7.4	1.5	1.1	0.9	5.6	0.4	5.5
BW	WSt	15.0	4.5	4.7	5.9	47.3	5.1	22.8	5.3	0.9	0.9	0.3	7.2
BZ	BZ	4.3	4.0	4.1	6.2	4.9	8.4	2.3	7.4	1.8	0.4	1.0	4.1
BZ	MB	8.9	5.4	3.4	5.7	7.1	1.8	6.0	5.8	4.7	6.7	2.0	12.4
BZ	SSt	7.0	3.6	2.0	5.3	10.7	1.6	6.0	5.7	1.6	8.3	1.2	6.1
BZ	MB	7.9	4.2	2.7	5.3	12.8	1.9	11.6	4.0	3.3	4.4	1.9	7.1
BZ	NsHl	8.6	4.3	34.4	4.4	6.9	2.9	6.2	3.6	9.5	4.6	3.2	6.9
BZ	KT	5.2	4.7	1.7	5.4	9.4	1.4	7.1	1.9	2.9	4.2	1.4	5.0
BZ	WV	17.6	4.4	1.7	3.7	20.7	3.2	16.2	2.1	1.5	14.0	0.8	5.1
BZ	WV	6.6	7.6	1.2	1.2	9.7	8.2	6.0	7.8	1.6	5.0	1.1	4.2
BZ	DL	9.5	8.4	2.1	1.8	10.3	5.8	6.0	5.3	4.0	6.7	1.8	3.6
BZ	WV	10.4	8.1	1.6	1.0	10.4	7.8	6.7	3.3	2.1	8.1	1.6	1.4
BZ	TT	7.3	6.3	2.0	1.0	10.9	7.8	6.5	7.5	3.1	3.4	2.7	2.3
BZ	NsHl	4.7	8.4	2.5	0.7	9.2	7.3	3.4	2.9	11.1	4.5	4.2	5.5
BZ	TR	6.9	6.9	3.3	3.5	6.1	4.3	3.2	6.3	7.0	4.9	2.2	5.8
BZ	WV	4.9	6.8	1.9	3.1	13.4	2.8	5.3	4.2	5.0	5.3	3.3	4.5
SL	TR	9.6	3.7	7.5	4.0	7.1	0.8	2.3	4.0	12.0	3.3	3.3	6.1
SL	NsHl	9.4	3.5	24.2	5.0	12.5	5.2	3.7	3.0	8.3	4.4	1.2	4.6

vintage	Grape variety	Region	Cis p-coumaric acid	RSD %	trans p-coumaric acid	RSD	Ferulic acid	RSD	quercetin	RSD	myricetin	RSD
2003	BF	NsHl	0.4	4.7	2.9	1.9	0.2	6.8	2.7	2.9	3.6	4.3
	BF	MB	1.1	6.0	14.6	0.6	0.5	6.0	1.6	3.2	4.1	4.1
	BF	MB	0.6	0.0	9.4	6.1	0.6	2.1	3.4	8.9	5.0	2.1
	BF	MB	0.8	6.3	7.9	0.7	0.3	4.0	1.8	5.2	3.9	0.2
	BF	MB	1.0	9.8	11.6	2.5	0.3	0.2	0.4	10.3	3.4	8.3
	BP	WV	0.8	7.1	31.1	6.8	0.4	6.1	5.6	7.1	6.7	1.1
	BP	WV	0.5	8.0	6.8	5.0	0.2	8.3	2.4	7.5	4.6	5.1
	BW	WSt	1.1	6.6	8.0	3.8	0.2	5.7	1.8	5.0	4.2	1.5
	BW	WSt	1.0	4.9	9.3	3.7	0.2	6.3	1.8	7.9	4.2	1.2
	BW	WSt	0.5	8.8	3.2	7.1	0.2	0.8	4.0	6.3	3.7	0.5
	BZ	Car	0.7	8.0	9.1	3.7	0.3	1.3	4.1	6.5	5.3	3.2
	BZ	MB	1.2	3.4	18.4	3.9	0.3	6.2	1.3	6.4	4.9	1.8
	BZ	SSt	0.6	7.4	14.2	4.4	0.2	6.3	1.2	6.4	3.9	1.2
	BZ	SoSt	0.8	3.0	8.2	6.8	0.3	6.2	2.0	7.1	4.1	0.7
	BZ	MB	1.2	2.3	17.5	5.5	0.3	7.7	0.5	4.9	4.2	4.0
	CS	MB	1.4	7.2	19.2	1.9	0.2	4.1	3.8	5.0	6.0	2.1
2004	BF	NsHl	0.8	6.8	15.7	1.0	0.3	7.9	6.6	3.7	6.7	0.2
	BF	Car	0.9	5.5	34.3	0.4	0.4	4.5	1.4	4.6	6.7	5.4
	BP	WV	1.3	7.0	22.1	5.9	0.4	5.1	8.1	4.9	8.9	0.7
	BP	WV	0.9	4.5	14.7	1.6	0.4	7.0	2.2	3.5	5.3	0.1
	BW	WSt	0.6	6.2	5.1	3.9	0.2	1.4	0.1	9.3	3.5	0.4
	BZ	WV	0.3	3.4	21.8	7.6	0.6	0.7	1.3	3.3	8.3	2.1
	BZ	WV	1.0	5.1	9.8	5.4	0.6	2.5	6.4	4.4	5.6	3.5
	BZ	WV	0.7	3.1	14.3	5.1	0.1	1.3	0.0	4.1	4.5	7.2
	BZ	Car	0.4	2.6	5.1	2.8	0.2	6.8	7.5	3.2	4.5	5.6
	BZ	SSt	0.4	12.5	4.7	3.3	0.1	5.2	4.7	4.8	3.6	0.5
	BZ	SoSt	0.4	1.7	13.5	4.6	0.2	6.5	2.9	5.5	4.1	0.2
	BZ	NsHl	0.5	5.6	16.5	1.8	0.5	6.6	-	6.4	3.2	0.6
	BZ	KT	0.7	5.1	17.7	3.4	0.2	8.9	4.9	3.6	6.3	2.4

	SL	TR	0.5	3.7	4.0	2.6	0.1	6.8	8.7	3.2	7.2	3.1
2005	BF	NsHl	0.6	2.3	5.2	4.0	0.25	2.8	0.444	5.2	2.11	6.7
	BF	MB	0.8	6.4	9.2	1.7	0.55	4.9	0.014	4.7	1.77	5.6
	BF	MB	0.6	9.4	7.9	5.2	0.21	8.5	0.750	6.1	1.89	4.9
	BF	NsHl	0.5	9.6	5.6	7.7	0.21	9.4	0.118	6.9	0.17	2.0
	BF	Car	0.4	6.1	9.0	3.1	0.37	10.3	0.106	2.2	1.09	4.4
	BP	WV	0.5	7.5	4.6	1.7	0.2	6.0	8.1	3.6	7.8	4.3
	BZ	Car	0.2	2.2	22.9	2.4	0.61	2.2	0.011	6.8	0.04	2.7
	BZ	MB	0.6	1.0	8.2	4.3	0.36	2.7	0.035	0.2	2.82	8.6
	BZ	MB	0.3	5.3	1.9	2.6	0.14	2.5	0.031	0.2	1.25	0.2
	BZ	KT	0.3	8.8	3.0	7.4	0.13	2.0	1.194	12.2	3.91	6.8
	BZ	WV	0.6	4.8	8.5	0.5	0.22	7.4	0.557	1.6	5.33	3.7
	BZ	NsHl	0.8	3.9	21.5	6.4	0.47	4.0	0.006	10.1	0.39	9.1
	BZ	Wv	0.6	9.5	4.6	2.7	0.25	4.1	0.007	9.4	0.30	4.8
	BZ	Dl	0.5	2.0	24.4	6.0	0.41	1.0	-	7.5	0.14	10.9
	BZ	WV	0.4	4.1	3.6	10.8	0.15	2.6	0.006	3.3	0.32	10.9
	SL	TR	0.6	4.3	10.6	0.7	0.11	10.5	0.187	1.3	1.97	5.8
	SL	NsHl	0.8	2.5	17.6	5.3	0.39	3.2	0.041	1.5	0.98	6.2
2006	BB	WV	0.40	5.5	5.9	2.6	0.10	5.7	0.02	9.6	1.84	1.2
	BF	NsHl	0.57	4.0	7.7	7.7	0.15	0.6	0.03	8.2	3.78	6.1
	BF	MB	0.61	9.5	5.0	3.4	0.57	0.5	0.01	9.4	1.20	6.6
	BF	MB	0.44	6.4	5.1	4.2	0.36	3.3	0.01	8.7	2.20	5.6
	BF	Car	0.34	7.3	26.8	6.0	0.65	6.2	0.01	8.2	0.66	14.3
	BP	WV	0.5	7.6	6.7	6.2	0.14	4.2	0.009	9.6	0.79	2.5
	BW	WSt	0.65	11.3	4.3	7.6	0.15	5.3	0.01	3.6	1.29	8.6
	BZ	BZ	0.10	15.1	7.1	3.7	0.39	6.1	0.02	4.7	6.81	5.4
	BZ	MB	0.29	5.3	3.2	7.0	0.25	3.3	0.01	1.0	1.79	7.9
	BZ	SSt	0.31	4.2	5.8	8.1	0.45	6.4	0.08	5.2	0.14	0.2
	BZ	MB	0.28	5.7	1.5	7.6	0.12	6.0	0.04	7.9	4.68	2.5
	BZ	NsHl	0.47	7.9	12.0	7.9	0.29	6.6	-	5.1	0.14	10.5
	BZ	KT	1.39	1.4	17.8	6.5	0.23	3.6	0.01	11.5	0.02	6.9
	BZ	WV	0.39	0.2	19.0	6.9	0.22	4.7	0.01	5.2	0.54	12.6
	BZ	WV	0.61	3.8	5.3	7.1	0.14	3.8	0.05	6.5	3.10	3.0
	BZ	DL	0.60	7.5	3.7	1.8	0.10	1.4	0.01	6.3	2.91	1.8
	BZ	WV	0.48	4.8	4.2	1.9	0.09	1.8	0.15	1.7	3.43	4.4
	BZ	TT	0.27	0.0	6.7	1.0	0.17	2.3	0.01	7.4	0.28	13.6
	BZ	NsHl	0.31	10.2	1.4	2.0	0.17	1.2	0.06	2.2	1.26	5.8
	BZ	TR	0.05	6.5	2.0	2.2	0.13	4.2	0.26	2.7	0.52	1.7
	BZ	WV	1.00	1.0	12.5	2.4	0.21	11.0	0.01	9.4	5.77	6.3

	SL	TR	0.83	2.1	6.0	0.8	0.17	12.3	0.01	9.8	0.55	2.0
	SL	NsHl	0.93	6.5	4.1	5.3	0.28	11.0	0.02	1.3	2.83	7.8
2007	BB	WV	0.10	4.3	2.0	5.6	0.05	4.5	0.034	6.7	2.0	2.0
	BF	NsHl	0.06	8.9	1.1	6.2	0.06	5.2	0.615	4.1	1.6	3.3
	BF	MB	0.08	5.3	1.1	5.9	0.10	3.7	0.033	3.5	0.3	5.9
	BF	MB	0.08	1.7	1.5	6.3	0.17	4.2	0.045	0.4	0.5	3.2
	BF	Car	0.40	8.5	10.1	7.2	0.37	9.1	0.003	5.9	0.0	0.9
	BP	WV	0.36	5.5	2.8	2.3	0.19	8.9	0.01	1.1	3.24	7.6
	BP	WV	0.07	5.4	1.3	5.4	0.10	7.4	5.657	5.6	8.2	2.3
	BW	WSt	0.24	7.1	2.6	5.2	0.19	0.3	-	5.5	0.8	7.8
	BZ	BZ	0.03	2.7	1.2	7.5	0.14	8.7	0.432	2.5	2.0	6.0
	BZ	MB	0.12	3.5	1.2	8.5	0.08	8.2	0.007	2.4	0.3	5.3
	BZ	SSt	0.18	1.8	2.3	8.3	0.10	8.9	0.125	0.5	0.1	8.0
	BZ	MB	0.09	3.3	1.0	5.0	0.04	2.4	0.007	7.1	0.9	4.5
	BZ	NsHl	0.21	3.5	2.5	4.0	0.05	6.3	-	0.0	0.2	5.5
	BZ	KT	0.40	4.0	18.1	7.4	0.70	3.5	0.020	5.8	1.2	1.0
	BZ	WV	1.86	1.9	17.4	5.4	0.75	5.4	0.026	2.2	0.4	3.9
	BZ	WV	0.10	12.7	2.3	6.5	0.05	5.4	0.008	5.3	0.2	7.1
	BZ	DL	0.05	2.1	0.9	5.8	0.10	3.1	0.007	7.3	0.7	9.5
	BZ	WV	0.23	8.8	1.3	7.6	0.06	4.2	0.013	4.3	0.9	3.9
	BZ	TT	0.12	8.6	2.0	6.6	0.06	6.0	0.045	1.7	1.0	6.9
	BZ	NsHl	0.04	3.9	1.0	4.5	0.06	4.8	0.179	6.8	1.3	5.5
	BZ	TR	0.06	8.9	1.1	6.9	0.21	6.8	0.404	2.2	3.7	0.1
	BZ	WV	0.22	4.2	3.3	7.3	0.07	5.4	0.006	9.8	0.5	8.6
	SL	TR	0.17	6.0	1.8	5.9	0.12	2.5	0.007	3.8	0.2	6.3
	SL	NsHl	0.06	7.3	0.7	5.3	0.09	6.8	0.661	5.1	0.4	6.9

3 *Highly sensitive LC-MS analysis of low molecular weight organic acids derived from root exudation*

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A novel method for sensitive quantification of oxalic, citric, fumaric, malic, malonic, succinic, trans aconitic acid, and in soil and root related samples is presented. The method is based on a fast and simple esterification procedure and subsequent analysis via LC-MS. Derivatization comprises the sylilation of the carboxyl groups of the di- and tri-acids (SN_2 mechanism), followed by a nucleophilic substitution utilizing benzyl alcohol (SN_1 mechanism). The esterification with the aromate decreases the polarity of the analytes and improves both the capacity factor on reversed phase material and electrospray ionization efficiency. Quantification of the analytes was performed via external calibration utilizing ^{13}C- labeled oxalic and citric acid as internal standards. With a total sample intake of 500 µL the limits of detection were 1 nM for citric, 47 nM for fumaric, 10 nM for malic, 10 nM for malonic,16 nM for oxalic, 15 nM for succinic, and 2 nM for aconitic acid. The methods working range was 3 nM to 10 µM for citric acid, 158 nM to 10 µM for fumaric acid, 34 nM to 10 µM for malic acid, 33 nM to 10 µM for malonic acid, 53 nM to 10 µM for oxalic acid, 48 nM to 10 µM for succinic acid, and 6 nM to 10 µM for aconitic acid. The robustness of the method was tested for soil extracts and samples from hydroponic experiments. The latter concerned the regulation of phosphorus uptake via plant root exudation of citric-, malic and oxalic acid.

Keywords: low molecular weight organic acids, root exudates, LC-MS, esterification

Introduction

Plant roots have the ability to change the chemical and biochemical properties of the surrounding soil, i.e. the rhizosphere, through the uptake of nutrients and water, and the exudation of various organic (carbohydrates, amino acids, phenols, organic acids) and inorganic (carbon dioxide, water) molecules. Root exudates are substances released by roots triggering several processes as mobilization of poorly soluble nutrients, stimulation of microbial growth and turnover as well as complexation of potentially toxic metals [79,80]. Furthermore they can act as signaling compounds for symbiotic and allellopathic interactions [81]. Differences in exudation between and within plant species may account for differences in adaptations/tolerance to limited nutrient availability, and are therefore of considerable practical interest. Low molecular weight organic acids have been implicated in increasing phosphate availability to plants and enhanced exudation of these compounds has frequently been found under low phosphorus supply [82]. Among a large variety of compounds, oxalate, malate and citrate are the most important organic acids identified in root exudates [83,84,85,86].

The detection of organic acids in root exudates poses a particular analytical challenge due to limitations of sampling techniques. Plants develop very heterogeneous root systems that usually spread through a large volume of soil but the zone of influence of root exudates is generally limited to the closest vicinity of the root surface. Furthermore, in many plants a high spatial variation in root exudation has been observed, with exudation mainly occurring at the apical root zone. Additionally, due to the natural pH range of soils from 3-8, organic acids are present as anions and are therefore easily adsorbed by soil minerals, mainly Fe and Al hydroxides. Also, microbial breakdown might further decrease the concentration of organic acids in the soil solution rendering identification and accurate quantification of actual concentrations released by the roots in soil a difficult task.

Rhizosphere related samples comprise soil extracts (water leachates with defined ionic strength and pH), samples from hydroponic experiments (immersion of the root system into aerated trap solutions containing water or low concentrated $CaCl_2$ solution), and solutions collected via micro suction cups. The latter set-up allows to sample the soil solution in the close vicinity of the root surface [87]. However, to preserve the high spatial resolution, only very low (20 µL and less) sampling volumes can be collected with these miniature soil solution samplers. Analytical requirements in root exudates analysis therefore are high robustness, high sensitivity in case of hydroponic samples and the handling of low sampling volumes i.e. solutions from micro suction cups.

Ion chromatography (IC) is applied for determination of low-molecular-weight organic acids on a routine base. In most cases the method uses suppressed conductivity detection revealing limits of detection (LODs) in the range of 1-3 µM for a wide range of low molecular weight organic acids (LMWOA) [88,89,90,91,92]. Additionally, gas chromatography-mass spectrometry (GC-MS) and capillary zone electrophoresis (CZE) have been employed for analysis of LMWOA in various matrices. Yang et al. performed GC-MS analysis of oxalic acid in aerosol samples after derivatization with N,O-bis (trimethylsilyl)-trifluoracetamide (BSTFA). A LOD of ~27 µM was achieved for oxalic acid [90]. Ohkawa developed a quantitative gas chromatographic (GC) method for the determination of oxalic acid in foods with a LOD of 0.2 µM [93]. For analysis of organic acids CZE generally implies anodic detection [94] and reversal of the electro osmotic flow utilizing quaternary ammonium salts with long alkyl chains [95,96] or permanent coating of the capillaries [97]. Organic acids are detected using either direct [98] or indirect [95] UV absorption and yielded LODs in the range of, 2 µM [99] and 5 µM [100].

For improvement of LODs, several groups have combined liquid chromatography and electrospray mass spectrometry (LC-ESI-MS), as mass spectrometric detection offers high selectivity and sensitivity. Ross et al. performed a profiling of organic acids utilizing reversed phase chromatography in combination with MS. For accurate quantification deuterated and $^{13}C_2$ labeled internal standards were utilized. The LOD for oxalic acid was between 0.6 and 11 µM [101]. However, the employed reversed phase material offered only limited retention and chromatographic resolution of the investigated acids. As a drawback, most of them were quantified in the void volume of the LC-MS system. Chen et al. quantified carboxylic acids from plant root exudation by ion exclusion chromatography with ESI-MS detection. Nine organic acids were separated within 8 min revealing a LOD of 100 nM L^{-1} [102]. Bydlund et al applied the separation of LMWOA with ion exclusion chromatography and detection with electrospray ionization tandem mass spectrometry (LC-MS/MS) for soil solution and stream water samples. The method revealed LODs between 1-50 nM for malic and citric acid and 200 nM for oxalic acid with an injection volume of 100 µL[103]. In general the employment of mass spectrometry lead to an improvement of sensitivity in the case of tricarboxylic acids. However, due to low electrospray ionization efficiency and signal suppression effects no improvement could be achieved in the case of oxalic acid, which is considered as important compound triggering soil-root-plant processes. Consequently, our work aimed at the development of a method offering high selectivity and low nM sensitivity for analysis of LMWOA in rhizosphere samples, with special emphasis on the analytical figures of merit of oxalic acid.

Experimental

Chemicals and standards

Oxalic acid monohydrate and citric acid dihydrate were purchased from Merck, Darmstadt, Germany, chlorotrimethylsilane (TMS-Cl) puriss. 99%, succinic acid, puriss. >99,5%, fumaric acid, puriss. ≥99.5%, malonic acid, puriss >99% and trans aconitic acid, puriss ≥99% were obtained from Fluka, Buchs, Switzerland. The isotope enriched $^{13}C_2$-oxalic acid monohydrate was purchased from Isotech, benzyl alcohol was obtained from Riedel-Dehaen and acetonitrile was purchased from Fisher Scientific, Loughborough Leicestershire, UK. Diethyloxalate 99%, dibutyloxalate 99% and tetramethylammonium fluoride (TMAF) 97% was purchased from Sigma Aldrich, Vienna, Austria. DL malic acid and citric acid p.a. were purchased from Merck, Darmstadt, Germany. The dibenzyloxalate was synthesized at the Division of Organic Chemistry, University of Natural Resources and Applied Life Sciences. The purity of the product was determined by NMR spectroscopy.

Derivatization Procedure

Depending on sample matrix and expected LMWOA concentration 10-500 µL of sample were transferred into a 1.5 mL Eppendorf tube. After addition of the internal standards (solutions containing 50 µM $^{13}C_2$ – oxalate and 50 µM $^{13}C_2$ – citrate) the water phase of the sample was removed utilizing a vacuum evaporation system set to 60 ℃ (Eppendorf Concentrator, 5301 Eppendorf AG, Hamburg, Germany). Afterwards 50 µL of benzyl alcohol and 30 µL of TMS-chloride were added to the dry samples and the closed Eppendorf tube was placed in the ultrasonic bath (room temperature, 45 min) and in an oven (80 ℃, 45 min). The reaction was stopped addi ng 20µL of 0.3 mM TMAF in THF. A final volume of 500 µL was adjusted by adding a solution containing 50 % water and 50 % acetonitrile.

Instrumental

Quantification of the derivatized acids was performed with a LC-ESI-TOFMS system (6210 TOF, Agilent Technologies, Palo Alto, CA, United States) equipped with a dual electrospray ionization (ESI) interface. The mass spectrometer was connected to a liquid chromatography system of the 1100/1200 series from Agilent Technologies, Palo Alto, CA, United States. The system consisted of a 1200SL binary gradient pump, a degasser, column thermostat and a HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). Reversed phase separation of the derivatives was performed on a GraceSmart 2.1*150 mm column from Grace Davison Discovery Sciences, Deerfield, Il, United States, with a silica based C-18 stationary phase (5 µm particle diameter). Mass Hunter software B.02.00 was used to control the instruments and for data processing. The

gradient programme was 0-2 min 50% B, from 2-9 min to 95% B and at 9.1 min back to the initial conditions of 50% B. The injection volume was 3 µL. The LC-separation and the ESI settings of the time of flight instrument were optimized utilizing a dibenzyloxalate standard, which was produced by adding oxalylchloride to the benzylalcohol causing an immediate esterification and a subsequent purification of the dibenzyloxalate. The purity of the standard (> 99%) was determined via NMR. The operation parameters of the LC-ESI-TOFMS method are listed in Table 1. The chromatographic run was divided into two segments in order to direct early eluting derivatization reagents to the waste avoiding contamination of the ion source. In order to correct for mass drifts ant to maintain the mass accuracy of 2 ppm a reference mass mix containing purine and 1H,1H,3H-tetrafluoropropoxy)phosphazine ($M+H^+$ =121.050873 Da and 922.009798 Da, respectively) was continuously introduced into the ESI interface with the reference sprayer.

Quantification of the derivatized OAs was performed via the sodium adducts $[M+Na]^+$ extracted from the total ion chromatograms obtained from the LC-ESI-TOFMS system. The extracted mass ranges are listed in Table 2.

Reference measurements of oxalic acid were performed via ion exclusion chromatography on an Aminex® HP-87H, 300*7.8 mm column from Biorad Laboratories, Hercules, CA, United States. An isocratic flow of 0.6 mL of 4 mM H_2SO_4 was applied with a subsequent detection on a UV-detector (1100 series, Agilent Technologies) at 210 nm.

Preparation of standards and samples:
A stock solution containing 1 mM of LMWOAs was diluted to concentrations of 0.5, 1.0, 2.5, 5.0, 7.5, 10.0 µM using ultrapure water. $^{13}C_2$-oxalic acid and $^{13}C_2$-citric acid was added as internal standard to all samples and standards revealing a final concentration of 2.5 µM.

Soil adsorption experiment
6 g of the investigated soil was oven dried at 105°C. The water content was adjusted to 20%. The soil was incubated with water containing 50, 100, 250, 500, 750 and 1000 µM LMWOA mixture for 72 h at 20℃. After centrifugatio n at 3500 rpm for 15 min at room temperature the supernatant was filtrated utilizing a 0,45 µm cellulose acetate syringe filter and the samples were stored at 4℃ until measuremen t. Prior to measurement all samples were diluted in order to match the working range given above.

Hydroponic plant experiments

To compare different LMWOA exudation pattern of different cultivars at low and high phosphorus supply, two rape cultivars, one efficient (Brassica napus cv. Carracas) and the other inefficient (Brassica napus cv 1886) in phosphorus uptake have been grown in an aerated nutrient solution at 2 different levels of phosphorus (Table 3) for 4 weeks. After 28 days, root exudates were sampled by immersing the plants in 100 mL deionised water (Millipore Elix 3.15, 15 MΩ) for 4 hours. The samples were stabilized with 5 mL methanol (LC-MS grade, Fisher Scientific) and stored at 4℃ until analysis.

Soil solution samples in the rhizosphere of rape (Brassica napus cv. Carracas) were collected with micro-suction cups using the rhizobox setup described by Puschenreiter et al. [87]. Briefly, this setup allows the sampling of a well defined rhizosphere soil due to the establishment of a rootmat along a nylon membrane (mesh size 30µm) that separates the roots from the soil but still allows chemical and biochemical exchange processes to occur. The micro-suction cups were positioned in 1 mm distance from the root matt surface.

Results and Discussion

Our novel strategy aimed at the accurate ultratrace analysis of LMWOA in complex matrices. The developments included (i) the enhancement of sensitivity by pre-concentration via vacuum evaporation, (ii) the enhancement of selectivity utilizing a derivatization procedure targeting carboxyl groups, (iii) the improvement of chromatographic selectivity, as the derivatives are amenable to reversed phase chromatography and (IV) the gain of mass spectrometric sensitivity, as the benzylated acids reveal significantly higher ionization efficiency as underivatized LMWOA measured in negative ionization mode.

Derivatization of LMWOA

The conversion of carboxylic acids into their corresponding methylesters is a well known derivatization technique in HPLC-MS. Methylesters are easy to prepare in a one step procedure via addition of diazomethane [104] or methyl iodide [105] under basic conditions. Especially the commercially available, easy to handle diazomethane solution has the advantage to be a high yielding and traceless reagent at room temperature. However, our first experiments with oxalic acid revealed that the oxalic acid methylester did not provide improved mass spectrometric sensitivity nor a satisfying capacity factor in reversed phase separation.

Higher carboxylic esters are obtained *via* classical Fischer esterification, where the acids are stirred with an excess of alcohol and catalytic amounts of sulphuric acid. However, oxalic acid, in contrast to most other carbon acids, easily decarboxylizes under strong

acidic conditions and/or elevated temperatures, which explains the low yields of this procedure. Hence, larger amounts of oxalic acid esters are commonly prepared *via* the acid chloride. As a matter of fact, the acid chloride preparation itself requires conditions we wanted to circumvent due to our matrix.

Consequently, milder methods have been investigated. Among them, 2-halopyridinium salts benzotriazoles or (immobilized)-lipases, were successfully applied. Our attention was drawn towards a more recently developed protocol by Nakao [106] utilizing trimethyl cholorsilan. According to Figure 1, this reagent generates HCl *in situ*. Trimethyl cholorsilan is commercially available, easy to handle although moisture sensitive, and gives rapid conversion to the corresponding esters in one step. The developed derivatisation method consists of (i) the sylilation of the di- respectively triacids (SN_2 mechanism), which is the time determining step in the method, followed by (ii) the nucleophilic substitution utilizing benzyl alcohol (SN_1 mechanism). As a result the carboxylic acid residues are esterified with the benzyl group, which leads to the decrease of polarity required for reliable LC-MS analysis. The structures of the obtained benzyl esters are depicted in Figure 2.

For the derivatization procedure benzyl alcohol was added to the dried samples and standards followed by trimethylsilyl (TMS-Cl) chloride was added and mixed. For input of energy and the desolvation of the analytes as well as for elimination of wall adsorption effects the samples were ultra-sonicated and subsequently heated to 80℃. The reaction was stopped by addition of tetramethylammonium fluoride (TMAF). The excess of the derivatization reagents TMS-Cl and benzyl alcohol react under the formation of the benzyltrimethyl-silyl ether. The unpolar ether would interfere with the derivated LMWOAs during the chromatographic separation. The addition of TMAF induces the conversion of the benzyltrimethyl-silyl ether into benzyl alcohol and trimethylsilyl-fluoride, which can be easily separated from the analytes employing the developed reversed phase chromatographic separation.

The derivatization method was optimized considering the total reaction time (ultra-sonication followed by heating to 80 ℃ under reflu x conditions) and amount of TMS-Cl. Two independent experimental series varying reaction time and TMS-Cl concentration were performed over a working range of 0.5 – 10 µM oxalic acid. The experiments showed that a reaction time between 1 -2 hours and an amount of 30 µL of TMS–Cl give the highest product yields. The original sample volume in these experiments comprised 500 µL of sample and an amount of 50 µL of benzyl alcohol. In order to standardize the method and improve repeatability the total reaction time was set to 1.5 h (45 min in an ultrasonic bath and a subsequent closed vessel heating for 45 min at 80° C in an oven). To

compensate for analyte losses during sample preparation as well as humidity effects $^{13}C_2$ labeled oxalic and citric acid were implemented as internal standards.

Chromatographic separation of LMWOA-derivatives and by-products

The LMWOA-benzylesters were separated by reversed phase chromatography employing the conditions given in Table 1. As can be seen in Figure 2 all investigated analytes were eluting in the time range of 3-9 minutes (retention times see Table 2). The high selectivity of the TOF-MS allowed an unambiguous identification and quantification of all analytes. The high rate of acetonitrile at the end of the gradient was necessary to remove unpolar by-products originating from the derivatization procedure. The excess of TMAF and TMS-Cl respectively are irrelevant for the chromatographic separation due to their high polarity leading to elution in the void volume of the chromatographic system. Under the applied chromatographic conditions the huge excess of benzylalcohol is clearly separated from the benzylesters, eluting at a retention time of 2-3 min. In order to avoid contamination of the ion source the eluent was bypassed into the waste for three minutes.

Analytical figures of merit

Limits of detection and working range

For procedural standard solutions linear calibration curves with correlation coefficients R^2> 0.99 were obtained for all seven acids within the range 0.5 – 10 µM utilizing an initial and final sample volume of 500 µL. Limits of detection calculated according to the 3 sigma criterion were in the range 1 - 47 nM and limits of quantification (10 sigma) were 4 - 158 nM (see Table 4). It is noteworthy that the working range of the method can be significantly extended via increasing the sample volume (maximum 1.5 mL) and/or reducing the final volume i.e. the volume of the solution containing 50% water and 50% acetonitrile (minimum 100 µL). Moreover the sensitivity of the method could be increased via increasing the injection volume of the chromatographic system or the implementation of an on-line enrichment step (trapping column).

Repeatability

The repeatability of the method was assessed via measurement of three sets of samples containing 5 µmol L^{-1} of the LMWOA and 2 µmol L^{-1} of the internal standards. Generally, the peak area obtained for ^{13}C labeled oxalic acid was employed for correction of the oxalic acid signals, whereas ^{13}C labeled citric acid was used for correction of all other LMWOA signals. Table 5 lists the results of the repeatability experiments. The first data set (set 1) represents the repeatability of 6 repetitive injections of a derivatized standard containing a

5 µM LMWOA mixture. The data clearly demonstrate the excellent repeatability of the LC-ESI-TOFMS system. Set 2 and set 3 were performed in order to assess the repeatability and uncertainty contribution of the derivatization procedure.

Set 2 was obtained for a low volume of 6 independently derivatized LMWOA-mixtures simulating sample volumes obtained from micro suction cup experiments.

25 µL of a 100 µM standard solution including each LMWOA as well as the internal standards were immediately derivatized after removal of the water phase (45 min at 60℃). The samples were filled to a final volume of 500 µL after derivatization.

The data reveals that the precision of the derivatization procedure is in the range of 10% relative standard deviation.

Set 3 aimed at the determination of repeatability for aqueous samples at a higher sample intake: 25 µL containing 100 µM standard solution of the OAs were filled to a final volume of 500 µL utilizing ultrapure water. Prior to derivatization the water phase of the sample was removed utilizing a vacuum evaporation system (2 h at 60℃). The experiment demonstrates the stability and the repeatability of the method for the exclusion of undesired effects e.g. loss or degradation of organic acids within the sample preparation even though a preconcentration step prior derivatization is necessary.

Accuracy and robustness

The recovery of the sample preparation procedure was determined for oxalic acid via quantification by purified dibenzyloxalate. For the oxalate a purity of 99.9% was determined via NMR-spectroscopy. Comparison of the areas obtained for a 5 µM dibenzyloxalate standard from the external calibration and a 5 µM standard of the synthetic dibenzyloxalate revealed a recovery of 60 ± 5%. As a matter of fact this recovery is corrected via internal standardization utilizing $13C_2$-oxalic acid and $13C_2$-citric acid. Due to a lack of a suitable reference material we have obtained the trueness of oxalic acid quantification via method intercomparison utilizing ion exclusion chromatography. The Aminex® HPX-87H, 300*7.8 mm column under isocratic conditions with 4 mM sulfuric acid as eluent and a subsequent detection at 210 nm. Succinic acid was utilized for internal standardization. The result of the comparison between the two methods was between 91 and 104%.

The robustness of the developed method in the context of soil related samples was assessed via comparison of the slopes of an external calibration curve and the corresponding results of a standard addition experiment. The standards in the range of 0.5-10 µM were added to a “blank” sample obtained from a soil adsorption experiment. The

results presented in Table 6 demonstrate the method is highly robust and suitable for quantification of LMWOA in the investigated samples.

Analysis of samples from hydroponic plant experiment

The novel method was applied to samples from a hydroponic plant experiment. Figure 4 shows the measured concentration of oxalate, malate and citrate in the hydroponic samples expressed as exudation rate per minute per g root fresh weight. The exudation of all 3 organic acids increased for both cultivars under low P supply, supporting our finding that organic anions can solubilize P from the mineral surfaces via ligand exchange or via the ligand promoted dissolution of the mineral on which P is adsorbed to. High concentrations of organic acids in the rhizosphere can therefore increase phosphate availability in the soil solution resulting in a better P nutrition of the plant. The higher exudation rate of *B.napus Caracas* might account for the higher P uptake capacity of this cultivar compared to *B. napus* 1886 (data not shown).

Conclusion

A highly sensitive method for quantification of LMWOA in samples related to rhizosphere research has been developed. The high matrix tolerance of the method is promising concerning its applications to samples from other research fields e.g. food and biological samples. Future developments will include the implementation of additional ^{13}C labeled internal standards as well as the full automatization of the method.

Acknowledgement

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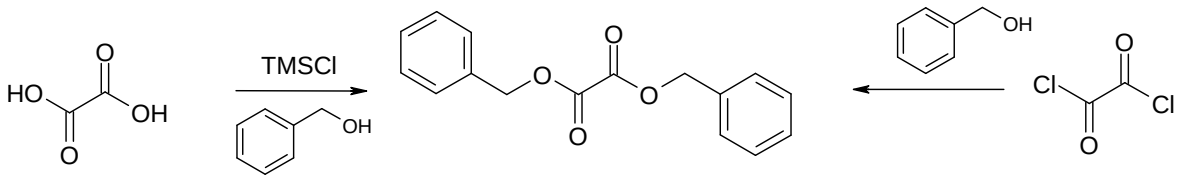
Figures:

Figure 1: Derivatization procedure for esterification of low molecular weight organic acids at ultra-trace concentration levels.



Mechanism:

$BnOH + TMSCl \longrightarrow TMS\text{-}OBn + HCl$ (1)

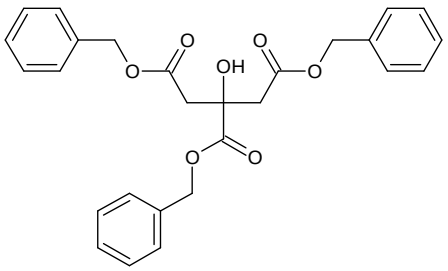
$(COOH)_2 + BnOTMS \longrightarrow (COOBn)_2 + TMS\text{-}OH$ (2)

$TMS\text{-}OH + BnOH \longrightarrow TMS\text{-}OBn + H_2O$ (3)

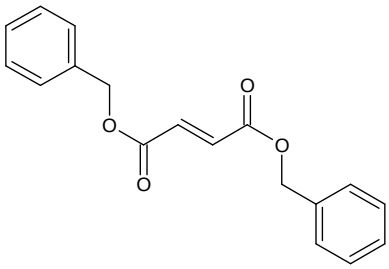
$2\ TMS\text{-}OH \longrightarrow TMS\text{-}O\text{-}TMS + H_2O$ (4)

$TMSCl + H_2O \longrightarrow TMS\text{-}OH + HCl$ (5)

Figure 2: Structures of the investigated LMWOA derivates a) tribenzylcitrate, b) dibenzylfumarate, c) dibenzylmalate, d) dibenzyl-malonate, e) dibenzyloxalate, f) dibenzylsuccinate and g) tribenzylaconitate



a)



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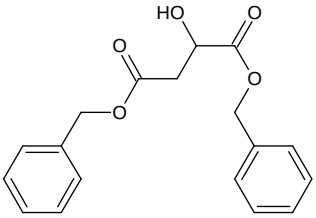

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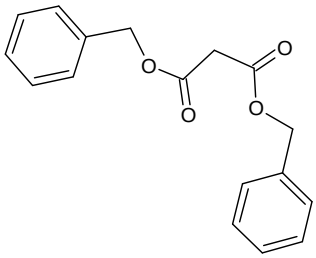

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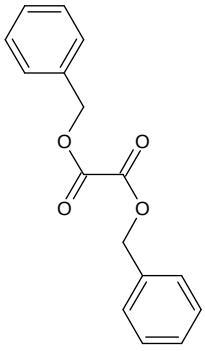

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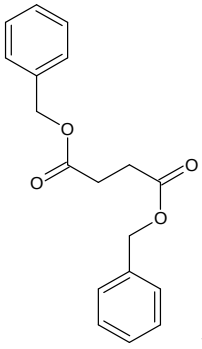

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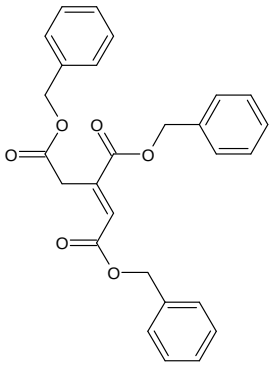


g)

Figure 3: Extracted ion chromatograms (± 0.005 Da) of LMWOA-derivates (5 µM) and the internal standards $^{13}C_2$-dibenzyloxalate and $^{13}C_2$-tribenzylcitrate (2.5 µM).

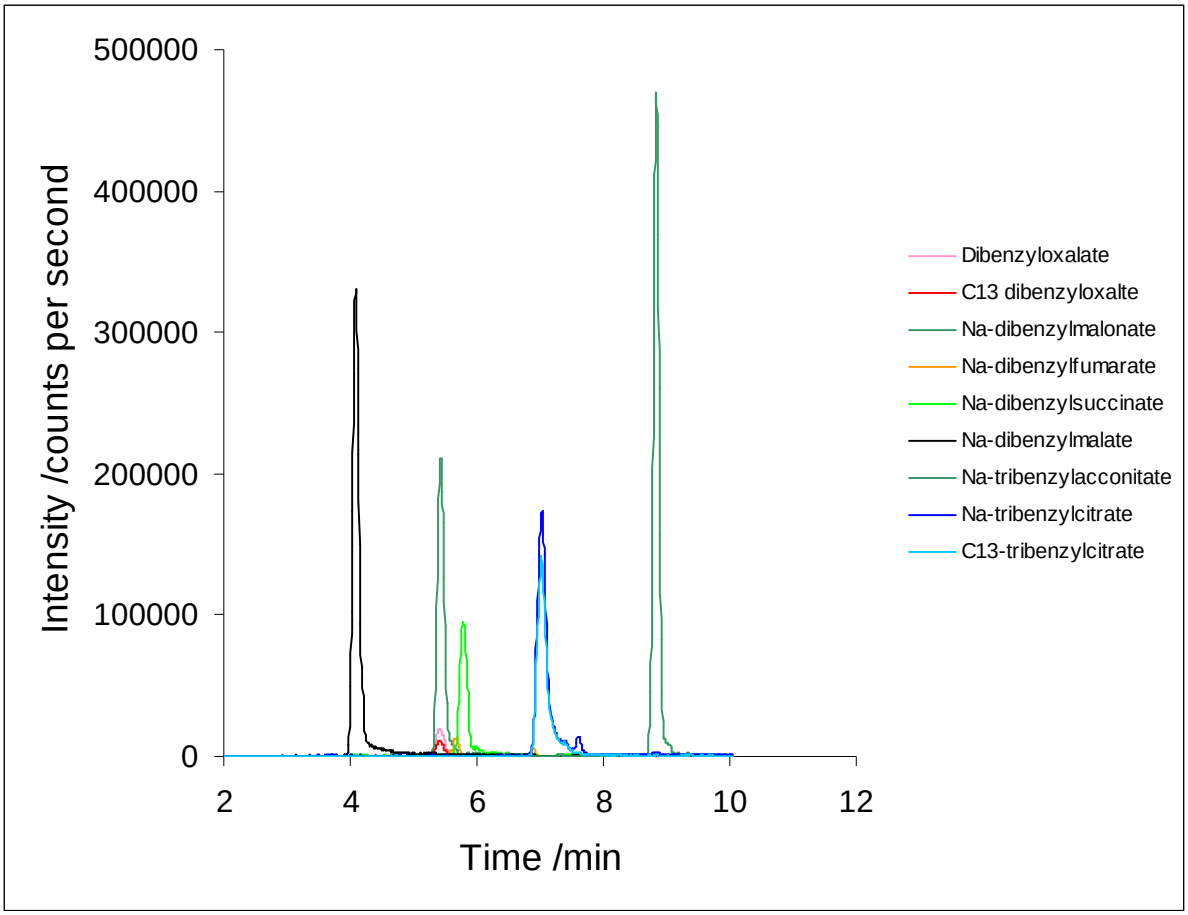
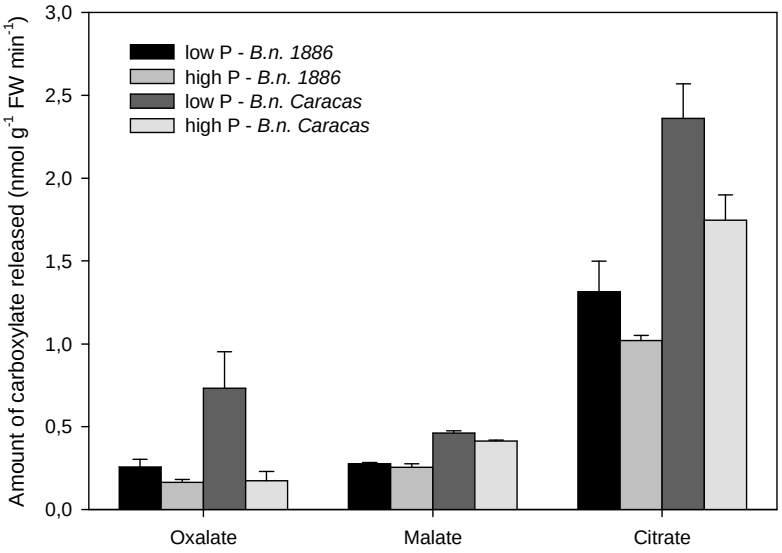


Figure 4: Exudation rates of the two different rape cultivars at low and high phosphorus supply. (Values represent mean ± sem with n = 3)



Tables:

Table 1: LC-ESI-TOFMS operation parameter

HPLC	
Stationary phase	GraceSmart, RP 18, 2.1*150 mm, 5 µm particle size
Phase A	H_2O, 1% acetonitrile, 0.1% formic acid
Phase B	Acetonitrile, 1% H_2O, 0.1% formic acid
Injection volume /µL	3
Flow rate /µL min^{-1}	250
Column temperature /℃	40

TOF-MS	
Gas temp /℃	325
Drying gas flow /L min^{-1}	10
Nebulizer pressure /psig	40
Capillary voltage /kV	4
Fragmentor voltage /V	220
Skimmer voltage /V	85

Table 2: Masses of the sodium adducts of the LMWOA derivatives. A mass range of ± 0.005 Thompson was used for generation of the extracted ion chromatograms.

Analyte	Retention time /min	Adduct	$m/z_{theoretical}$ /Th	$m/z_{experimental}$ /Th
Tribenzylcitrate	6.3	$[M+Na]^+$	485.2016	485.2016
Dibenzylfumarat:	5.6	$[M+Na]^+$	319.0941	319.0988
Dibenzylmalate	3.7	$[M+Na]^+$	337.1049	337.1046
Dibenzylmalonat:	5.0	$[M+Na]^+$	307.0941	307.0959
Dibenzyloxalate	4.9	$[M+Na]^+$	293.0784	293.0784
Tribenzylaconitate	8.1	$[M+Na]^+$	467.1465	467.1500
Dibenzylsuccinat	5.2	$[M+Na]^+$	321.1097	321.1142

Table 3: Composition of the nutrient solution for the hydroponic experiment

Formula		Concentration in the nutrient solution/ mM
KH_2PO_4	99.9% Sigma-Aldrich, Vienna, Austria	0.01
		0.1
$Ca(NO_3)_2$ x 4 H_2O	99-103%, Riedel-de Haen, Vienna Austria	1.25
$MgSO_4$ x 7H2O	≥ 99.0% Fluka, Vienna Austria	2.0
K_2SO_4	≥ 99.0% Fluka, Vienna Austria	0.44
KCl	≥ 99.0% Fluka, Vienna Austria	0.625
Fe(III)-EDDHA	≥ 5.7% Fe, Duchefa Biochemie, Vienna Austria	0.005
H_3BO_3	≥ 99.8% Merck, Vienna Austria	0.00625
$MnSO_4$ x H_2O	≥ 99.0% Fluka, Vienna Austria	0.000375
$ZnSO_4$ x 7H_2O	≥ 99.5-103 % Merck, Vienna Austria	0.000375
$CuSO_4$ x 5H_2O	≥ 99-100.5 % Merck, Vienna Austria	0.000125
$(NH_4)_6Mo_7O_{24}$ x 4H_2O	≥ 99.0% Merck, Vienna Austria	0.000006

Table 4: Limits of detection and limits of quantification of the investigated LMWOA derivatives

	Tribenzyl-citrate	Dibenzyl-fumarate	Dibenzyl-malate	Dibenzyl-malonate	Dibenzyl-oxalate	Dibenzyl-succinate	Tribenzyl-aconitate
LOD /nM	1	47	10	10	16	15	2
$LOD_{absolute}$ /fmol	4	142	30	30	48	44	5
LOQ /nM	4	158	34	33	53	48	6
$LOQ_{absolute}$ /fM	16	473	101	100	160	145	18

Table 5: Precision of derivatization method and precision of measurement of derivatized OAs via LC-ESI-TOFMS. Set 1 represents the derivatization of solid OAs, set 2 includes the includes a drying step necessary for liquid samples. Set 3 gives the precision of 6 repetitive injections of a derivatized standard.

		Citric acid	Fumaric acid	Malic acid	Malonic acid	Oxalic acid	Succinic acid	Aconitic acid
Set 1	Rel. area	135.406	7.956	19.552	7.252	2.433	6.222	36.631
	SD	3.672	0.087	0.376	0.435	0.069	0.065	1.007
	RSD %	2.71	1.09	1.92	6.00	2.85	1.04	2.75
Set 2	Rel. area	138.932	10.127	18.918	5.561	2.592	9.761	37.586
	SD	13.541	1.308	1.680	0.606	0.134	0.980	4.435
	RSD %	9.75	12.91	8.89	10.90	5.15	10.04	11.80
Set 3	Rel. area	146.791	8.975	21.319	6.817	2.866	7.419	38.951
	SD	14.828	1.148	2.510	1.431	0.243	0.953	4.678
	RSD %	10.10	12.80	11.77	20.99	8.48	12.84	12.00

Table 6: Evaluation of method robustness via a standard addition in a soil extract

	Slope external calibration	Correlation coefficient/ R^2	Slope standard addition	Correlation coefficient/ R^2
Tribenzylcitrate	0.99	0.998	1.14	0.995
Dibenzylfumarate	0.97	0.998	1.15	0.997
Dibenzylmalate	1.01	0.996	0.95	0.995
Dibenzylmalonate	0.99	0.996	0.98	0.998
Dibenzyloxalate	1.07	0.997	1.00	0.998
Dibenzylsuccinate	1.07	0.995	1.14	0.999
Tribenzylaconitate	1.12	0.999	1.00	0.999

Curriculum Vitae

Personal information

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Work experience

Dates	1.7.2006- present, Supervisor Assoc. Prof. Dr. Stephan Hann
Occupation or position held	PhD Student
Main activities and responsibilities	Co-worker at the Department of Chemistry, Division Analytical Chemistry at the University of Natural Resources and Applied Life Science
Name and address of employer	University of Natural Resources and Applied Life Science, Department of Chemistry, Division of Analytical Chemistry Muthgasse 18, 1190 Vienna

Type of business or sector	Method development for liquid chromatography combined with molecular mass spectrometry
Education and training	
Dates	12.9. 2009-Sept. 2011
Title of qualification	Master of Business Administration
Principal subjects/occupational skills covered	Biotech and Pharmaceutical Management
Name and type of organization providing education and training	Danube University Krems
Level in national or international classification	ISCED 5A
Dates	1.8.2005-20.6.2006
Title of qualification awarded	Mag. rer. nat. Chemistry
Principal subjects/occupational skills covered	Key course elements: analytical chemistry and spectroscopy Diploma thesis: Affinity binding of Human Rhinovirus to receptor-functionalized surfaces for analyte preconcentration prior to capillary electrophoresis“ Supervisor: Prof. Dr. Ernst Kenndler
Name and type of organization providing education and training	University of Vienna
Level in national or international classification	ISCED 5A
Dates	September 1993 – June 1998
Name and type of organization providing education and training	Secondary school: Gewerbegymnasium Güssing
Principal subjects/occupational skills covered	General qualification for university entrance and additional training as carpenter
Level in national or international classification	ISCED 3
Dates	September 1989 – June1993
Name and type of organization providing education and training	Secondary school: BORG Eisenstadt

Training abroad	
Dates	February- April- 2009 November 2007- February 2008
Name and type of organization providing education and training	Corvinus University Budapest (H), Department of Applied Chemistry Head of the Department: Prof. Dr. Peter Fodor
Dates	February- July 2005
Name and type of organization providing education and training	University of Bologna, Department of Pharmaceutical Sciences of the Faculty of Pharmacy, Laboratory of Pharmaco-Toxicological Analysis Head of the Laboratory: Prof. Dr. Maria Augusta Raggi
Personal skills and competences	
Mother tongue	German
Other languages	English, Italian (basic knowledge)