TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

M A S T E R A R B E I T

Method development for the analysis of phenolic and flavonoid compounds from red wines and non-alcoholic beverages with one- and two-dimensional high performance liquid chromatography

Ausgeführt am Institut für

Chemische Technologien und Analytik

der Technischen Universität Wien

unter der Anleitung von

Prof. Dr. Erwin Rosenberg

durch

Lamprini Ramopoulou

Chemikerin

Mat. Nr. 230096

Pfeilgasse 3a

1080 Wien

Wien, im Januar 2013 _____

Unterschrift

Abstract

Phenolic compounds in food have attracted considerable interest since the 1990s due to their said beneficial effect on human health. This has triggered research interest towards the characterization of wines and other non-alcoholic beverages, such as tea, and juices with respect to their polyphenol contents. The analytical method of choice is reverse phase liquid chromatography (RP-LC) with diode array (DAD) - and mass spectrometric (MS) detection. However, the complexity of natural samples often precludes the comprehensive characterization and unambiguous identification of phenolic compounds based on one-dimensional separation. For this reason, two-dimensional liquid chromatography (2D-LC) separations have been proposed.

(Poly) phenolic compounds in wines have been studied by two dimensional high performance liquid chromatography (2D-HPLC) with diode-array and ion trap-time of flight-mass spectrometric (IT-TOF-MS) detection. The 2D-HPLC system employed for this task is based on a set-up using two 6-port valves and a single 2D column. It requires careful optimization of operational conditions to overcome the experimental difficulties related to the large difference in mobile phase composition of the two chromatographic dimensions, and to maintain and increase the chromatographic resolution achieved already in the first chromatographic dimension. In this thesis, the design considerations of the 2D-HPLC system, the optimization of the system and the particular considerations that have to be made when running 2D-HPLC with MS detection are described. The merits of this 2D system *versus* one dimensional separation are critically discussed.

SUBJECT AREA: Two-Dimensional Liquid Chromatography

KEYWORDS: polyphenols, RPLC, two-dimensional separation, two x 6-port valve

Zusammenfassung

Phenolische Verbindungen in Lebensmitteln haben seit den 90er Jahren beträchtliche Aufmerksamkeit erregt, da ihnen eine positive Wirkung auf die menschliche Gesundheit nachgesagt wird. Daraus erwuchs das Interesse, Wein und alkoholfreie Getränke, wie z.B. Tee und Säfte, entsprechend ihres Polyphenolgehalts zu charakterisieren. Das hierfür gewählte Analyseverfahren ist Umkehrphasen (RP)-Flüssigchromatographie mit Diodenarray- (DAD) und massenspektrometrischer (MS) Detektion. Allerdings verhindert oft die Komplexität natürlicher Proben die umfassende Charakterisierung und eindeutige Identifikation von phenolischen Verbindungen, basierend auf einer eindimensionalen Trennung. Aus diesem Grund wurden zweidimensionale Flüssigkeitschromatographie (2D-LC)-Trennungen vorgeschlagen.

In der vorliegenden Arbeit werden (poly)phenolische Verbindungen in Wein mit Hilfe der zweidimensionalen Hochleistungs-Flüssigkeitschromatographie (HPLC) mit DAD- und Ionenfallen-Flugzeitmassenspektrometer- (IT-TOF-MS) Detektion untersucht. Für diese Untersuchung wird ein 2D-HPLC-System benutzt, das auf der Verwendung von zwei 6-Port-Ventilen und einer 2D-Säule basiert. Es erfordert eine sorgfältige Optimierung der Betriebsbedingungen, um die experimentellen Schwierigkeiten, die durch die großen Unterschiede der Zusammensetzung der mobilen Phase der beiden chromatographischen Dimensionen, sowie der Erhaltung und Verbesserung der bereits erreichten chromatographischen Auflösung in der ersten chromatographischen Dimension, entstehen. In dieser Arbeit werden die Überlegungen zum Aufbau des 2D-HPLC-Systems, die Optimierung des Systems und die besonderen Aspekte, die beim Betrieb der 2D-HPLC mit massenspektrometrischer Detektion berücksichtigt werden müssen, beschrieben. Die Vorteile dieses 2D-Systems im Vergleich zur eindimensionalen Trennung werden kritisch erörtert.

FACHGEBIET: Zweidimensionale Flüssigchromatographie

SCHLAGWORTE: Polyphenole, RPLC, zweidimensionalen Trennung, zwei x 6-Port-Ventil

Acknowledgement

This master thesis would not have been possible without the guidance and help of several individuals who in one way or another contributed significantly towards the preparation and completion of this study.

First, it is with immense gratitude that I acknowledge the support and scientific guidance of my supervisor; Prof. Erwin Rosenberg gave me the enormous opportunity to be part of his research group. At beginning, he accepted me as an Erasmus undergraduate student and then he inspired me to do my master's thesis in analytical chemistry. I am also thankful to him for his support during my whole graduate program. Whenever I met a difficultly, Prof. Rosenberg had always a positive approach to support and encourage me.

I would also like to express my thanks to all my former professors at the National and Kapodistrian University of Athens for their continuous guidance and encouragement. I am grateful to Prof. Nikolaos Thomaidis who instructed me to come to Vienna as an Erasmus student. In this connection, I would also like to thank Prof. George Zachariadis from the Aristotle University of Thessaloniki.

I would never omit to acknowledge the overall academic atmosphere at the Technical University of Vienna and in particular express my most sincere thanks to all my professors therein; their guidance and ever-present assistance provided a background conducive to fruitful research and of decisive value for all of my research accomplishments.

Most importantly, I would like to express my most sincere gratitude to my thesis committee members: Prof. Erwin Rosenberg, who agreed to become the chair of the committee, Prof. Ingrid Steiner, and Prof. Marko Mihovilovic. They all accepted the challenges of getting involved in this research, kept me focused on the big picture, yet encouraged me to pursue all of my ideas.

I was really lucky because in the research group I met real friends and fellow students Marta Krzysztoch, Olga Lanaridi, Chryssa Kanakaki, Christos Vogiatzis and all the others for their constant support and encouragement throughout my project and during my entire studies.

I cannot find words to express my gratitude to Dr. Angelos Kolokouris for his guidance, support, and encouragement.

Last, but not least, my parents, Elissavet and Nikos, receive my deepest gratitude and love for their dedication and the many years of support during my under and postgraduate studies that provided the foundation for this work. I would like to thank my brother Vasilis for his support.

Table of Contents

List of Figures

List of Tables

Abreviations – Acronyms

1-D	One Dimensional
2-D	Two Dimensional
2D-LC	Two Dimensional Liquid Chromatography
BHT	Butylated Hydroxytoluene
CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
DAD	Diode Array Detector
ECD	Electron Capture Detection
ESI	Electro Spray Ionization
FID	Flame Ionization Detection
FL	Fluorescence
GC	Gas Chromatography
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
NPLC	Normal Phase Liquid Chromatography
PDA	Photo Diode Array
PEG	Polyethylene Glycol
PFP	Pentafluorophenylpropyl

RPLC	Reversed Phase Liquid Chromatography
RSD	Relative Standard Deviation
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SPME	Solid Phase Micro-Extraction
TLC	Thin Layer Chromatography
UFLC	Ultra-Fast Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
UV	Ultra Violet

I. Introduction

1. General Introduction

This project was conducted at the Institute of Chemical Technologies and Analytics (Institut für Chemische Technologien und Analytik) of the Vienna University of Technology (Technische Universität Wien) in Vienna, Austria. The study was a part of the master's degree program of the Vienna University of Technology with the title "Technical Chemistry - Synthesis". The supervisor of the project was Prof. Erwin Rosenberg of the Technical University of Vienna.

1.1. The aim of this study

This study aims to explore appropriate methods for the analysis of phenolic and flavonoid compounds in red wines and other non-alcoholic beverages. This requires careful optimization of operation conditions to overcome the experimental difficulties related to the considerable difference in mobile phase composition of the two chromatographic dimensions, and to maintain and increase the chromatographic resolution achieved already in the first chromatographic dimension. In this thesis, the design considerations of the 2D-HPLC system, the optimization of the system and the particular considerations that have to be made when running 2D-HPLC with MS detection are described.

II. Theoretical Part

2. Polyphenols

Phenolics, natural phenols, bioavailable phenols, low molecular weight phenols or phenoloids [1 - 3] are a class of natural organic compounds. They are small molecules, since they contain one, two or more ring systems. These molecules are smaller in size than polyphenols, containing less than 12 phenolic groups. They can be classified as simple phenols (monophenols), with only one phenolic group, or di- (bis-), tri- and oligophenols, with two, three or several phenolic groups, respectively. They can be found in plants and are known to have antioxidant activity [3]. They constitute the most widely abundant class of plant secondary metabolites and several thousand different compounds have been identified [4].

Polyphenols [5] on the other hand, are a structural class of natural, synthetic, and semi synthetic organic chemicals, characterized by the presence of large multiples of phenol structural units. The number and characteristics of these phenol structures underlie the unique physical, chemical, and biological (metabolic, toxic, therapeutic, etc.) properties of particular members of the polyphenol class. Polyphenols are natural compounds widely found in fruits, vegetables and beverages, such as tea, wine and orange juice [6]. Some of them exhibit strong antioxidant properties, with beneficial physiological and anticarcinogenic properties for human health.

The polyphenolic compounds in food play a significant role not only for nutritional properties, but also for sensorial properties, such as colour, astringency, bitterness and flavour. Due to the increasing interest in biologically-active compounds in food, many research studies have been devoted to the development of analytical methods for the analysis of natural antioxidants. The profiles of phenolic and flavonoids in real-world samples may vary significantly, and can be too complex to be resolved in a monodimensional HPLC analysis [7]. Therefore, comprehensive multidimensional LC, mainly RPLCxRPLC (reverse phase LC x reverse phase LC) systems comprised of columns of different selectivity in the two dimensions, has proved to be a powerful tool for the separation of such compounds.

Flavonoids (or bioflavonoids) (from the Latin word *flavus* meaning yellow due to their colour in nature), are a class of plant secondary metabolites. Flavonoids were originally referred to as Vitamin P, probably due to the effect they had on the permeability of vascular capillaries, but

this term is rarely used now. [8] According to the IUPAC nomenclature, [9] they can be classified into:

1) Flavonoids, derived from 2-phenylchromen-4-one (2-phenyl-1, 4-benzopyrone) structure (examples: quercetin, rutin).

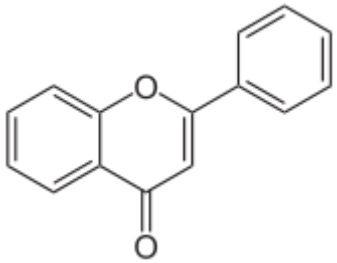


Figure 1: Molecular structure of the flavone backbone (2-phenyl-1, 4-benzopyrone)

2) Isoflavonoids, derived from 3-phenylchromen-4-one (3-phenyl-1, 4-benzopyrone) structure

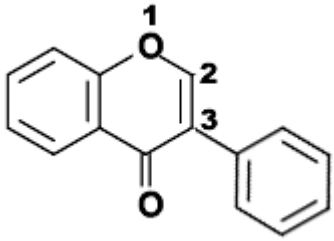


Figure 2: Isoflavon structure

3) Neoflavonoids, derived from 4-phenylcoumarine (4-phenyl-1, 2-benzopyrone) structure.

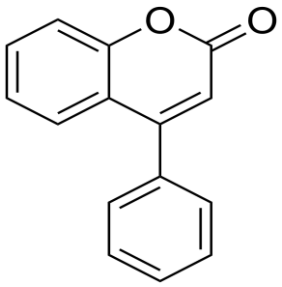


Figure 3: Neoflavonoid structure

The above mentioned flavonoid classes are all ketone-containing compounds, and as such, are flavonoids. This class was the first to be termed "bioflavonoids." The terms flavonoid and bioflavonoid have also been more loosely used to describe non-ketone polyhydroxy polyphenol compounds which are more specifically termed flavonoids, flavan-3-ols (or catechins). [10]

Most flavones are conjugated to a carbohydrate moiety, differing in hydroxylation, methoxylation, glycosylation, or acylation patterns [11]. Practical interest in flavones and related phenolic compounds is due to their properties, contributing not only to the taste and color of plants, but also to their positive role in a variety of biological activities, such as antioxidative [12], radical scavenging [13–14], anti-inflammatory [15], anti-depressant [16], and assumed cancer-preventive effects [17]

2.1. Antioxidant activity and the French Paradox

2.1.1. Antioxidant Activity

Secondary metabolites, such as polyphenols, are produced by plants to protect themselves from other organisms or against adverse environmental effects. It has been proven that the dietary polyphenols play significant roles in human health. The consumption of dietary products which are rich in polyphenols, such as fruits, vegetables and whole grains, has been associated with lowered risks of many chronic diseases including cancer, cardiovascular disease, chronic inflammation and many degenerative diseases [18, 19]. Recently it has been proven that many of these diseases are linked to oxidative stress from reactive oxygen and nitrogen species. Polyphenols have the main role in the total antioxidant activities of fruits, even more than the well-known vitamin C [20].

To define a polyphenol as an antioxidant it must satisfy two basic conditions: [21]

i) "When present in low concentration relative to the substrate to be oxidized it can delay, retard, or prevent the autoxidation or free radical-mediated oxidation.
ii) The resulting radical formed after scavenging must be stable through intramolecular hydrogen bonding on further oxidation."

It has been proven that polyphenols are strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. The highly coupled system and number of hydroxylation patterns, for example the 3-hydroxy group in flavones, are significant in the antioxidant activities. The generation of free radicals is suppressed by polyphenols, therefore, reducing the rate of oxidation by deactivating the active species and precursors of or inhibiting the formation of free radicals. Polyphenols commonly act as direct radical scavengers of the lipid peroxidation chain reactions. Scavengers donate an electron to the free radical and as a result the radicals are neutralized and the scavengers becoming stable (less

reactive) radicals, therefore, the chain reactions stop [21,22]. It has been proven that polyphenols do not act alone. They can be used as a co-antioxidant, and are involved in the regeneration of essential vitamins [23].

2.1.2. The French Paradox

Renaud and De Lorgeril introduced the term “French paradox” in order to describe the paradoxical situation in France, where although the French consume products with high levels of saturated fats, they suffer for relative low mortality from coronary heart disease (CHD) [24]. This term could be used for other countries as well, Greece, Japan, Spain, and other situations, where people traditionally consume a diet rich in saturated fat but show lower than expected CHD mortality rate [25]. This paradox may be attributable in part to high wine consumption and especially to red wine [24].

2.2. Separation, Identification and Analysis of Polyphenols

Although polyphenols have in common the phenolic feature, their physicochemical properties differ because of their structural diversity. Despite the recent advances in new instrumentation, the extraction, separation, identification and analysis of polyphenols remain as challenging as ever, due to the complex glycosylation, polymerization patterns and the various food matrices. Even though it is hard to develop a protocol for all polyphenols, there are some general approaches that can be used in the research of polyphenol.

The collection, the preservation and the preparation of each sample should be prior to the extraction of polyphenols. It is clearly understood that the collected samples must be representative of the real pool.

During the transportation and the preservation of the samples, the researcher must be very careful so that the losses of the target compounds are minimized.

Due to the fact the high moisture or water content increases the enzyme activities and as a consequence this can lead to the degradation of native polyphenols, very often, before extraction, samples are dried, frozen or lyophilized. [26]. High-temperature drying should be avoided because in many cases the polyphenolic composition may be affected from the heat,

light or oxygen. Antioxidants are often used to avoid the oxidation of the polyphenols. Some common antioxidants are butylated hydroxytoluene (BHT) and ascorbic acid. The pre-treatment of the samples can also be done by filtration and centrifugation as well. [26] In (Figure 4) are summarized the strategies for the determination of phenolic acids and flavonoids.

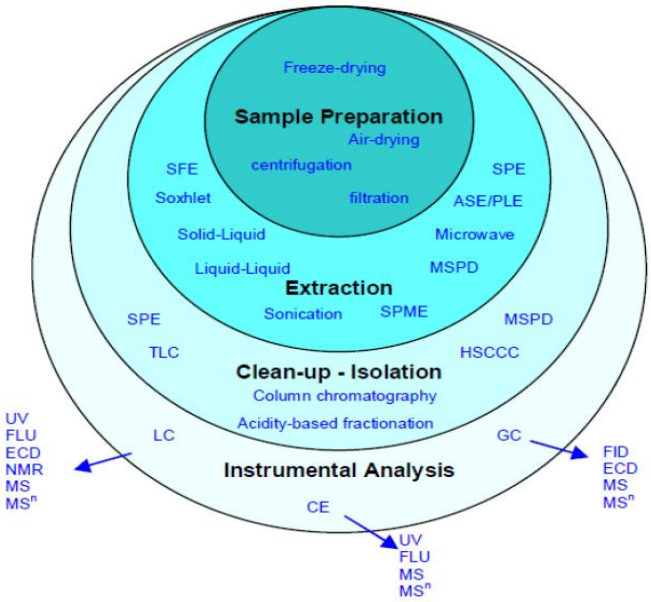


Figure 4: Schematic of strategies for the determination of phenolic acids and flavonoids in biological fluids, beverages, plants and food. Abbreviations: SFE, supercritical fluid extraction, MSPD, matrix solid phase dispersion, SPME, solid phase micro extraction, ASE/PLE, accelerated solvent extraction/pressurized liquid extraction, HSCCC, high speed counter current chromatography, TLC, thin layer chromatography, FL, fluorescence, FID, flame ionization detection, ECD, electron capture detection, CE, capillary electrophoresis [29]

According to the variety of the samples types several extraction methods exist [26 - 28]. The most common solvent extractions, for plant originated food samples, are liquid/liquid partitioning and solid/liquid extraction. The polyphenols are relatively hydrophilic, though free polyphenols, including aglycones, glycosides, and oligomers, are extracted using water, polar organic solvents such as methanol, ethanol, acetonitrile and acetone. Another important factor is the pH of the extraction solvent. Polyphenols are more stable at low pH and due to the fact that polyphenols remain neutral under acidic conditions and that make them readily extractable into organic solvents. The acidification should be done with the use of weak acid or low concentrations of a strong acid, otherwise high acid concentration can cause hydrolysis of glycosides or acylglycosides and this can lead to false conclusions because of the different pictures of native polyphenol profiles. Since not all polyphenols exist in the free form, the

hydrolysis under acid or alkaline conditions can release the phenolics which are partitioned into ethyl acetate or n-butanol. This procedure can simplify the chromatographic separation and aid the quantification and the structural identification of the polyphenols [29].

2.3. Analysis of Polyphenols

2.3.1. General Methods

For the analysis of polyphenols have been used several methods: Spectrophotometric Methods [29, 30]. Thin Layer Chromatography (TLC) [31, 32], Capillary Electrophoresis (CE) [33, 34],. Gas Chromatography (GC) [35, 36], High Speed Counter Current Chromatography (HSCCC) [37, 38]. Ultra Performance Liquid Chromatography (UPLC) [30, 39], High Performance Liquid Chromatography (HPLC) and Two Dimensional Liquid Chromatography (2DLC). The two last methods are going to be discussed in more detail in the following.

2-(p-Hydroxyphenyl) ethanol is antioxidant which can be found in olive oil red wine and tea. [40]

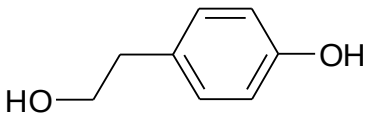
Ethyl gallate is added to food as an antioxidant. However it can be found naturally in wines and in a variety of plant sources. It is produced from gallic acid and ethanol [41].

Gallic acid is a water soluble phenolic acid present in grapes and in the leaves of many plants. Gallic acid esters, such as tannins, catechin gallates and aliphatic gallates are potent antioxidants in vitro. However, gallic acid itself also appears to have antioxidant, anticarcinogenic and antiangiogenic activity in vitro. [42]

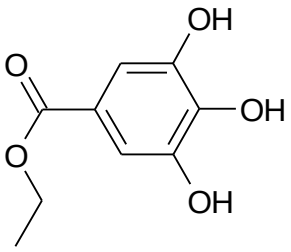
(-) epicatechin Is an antioxidant mainly found in chocolate [43].

Caffeic acid is an organic compound that is classified as hydroxycinnamic acid. It is a yellow solid which consists of phenolic and acrylic functional groups. It is found in all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal sources of biomass [44]

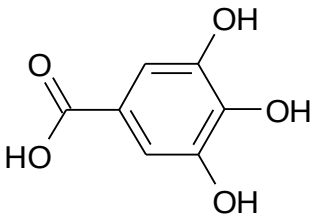
Rutin trihydrate is one of the phenolic compounds found in the invasive plant species *Carpobrotus edulis* and contributes to the antibacterial and antioxidant properties of the plant [45].



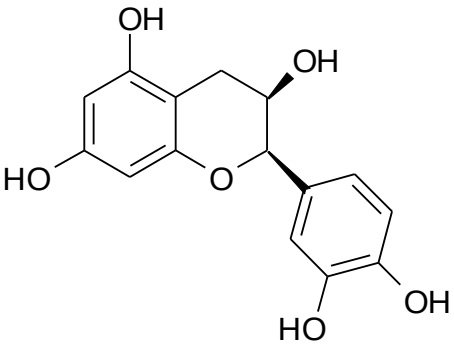
(1)



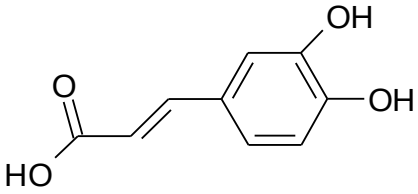
(2)



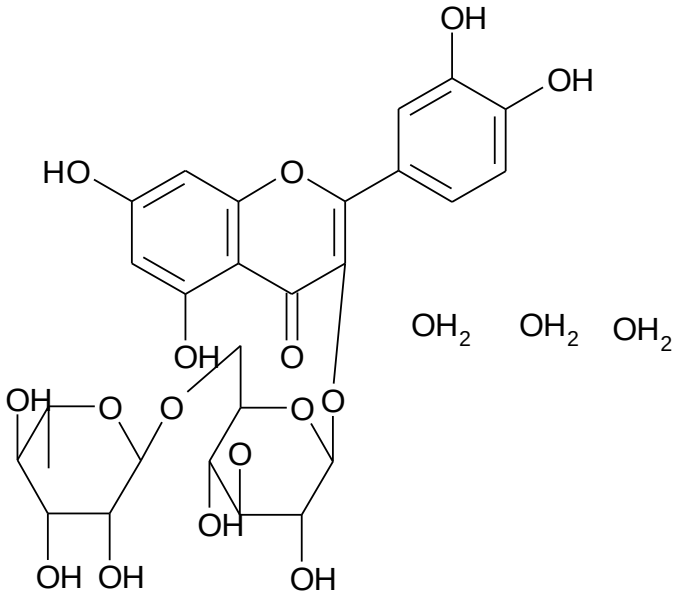
(3)



(4)



(5)



(6)

Figure 5: Chemical structures of the phenolic and flavonoid compounds. (1) 2-(p-Hydroxyphenyl) ethanol, (2) Ethyl gallate, (3) Gallic acid, (4) (-) epicatechin, (5) Caffeic acid, (6) Rutin trihydrate

3. High Performance Liquid Chromatography

3.1. Chromatographic separation

Chromatography is a separation method by which organic, organometallic and inorganic compounds can be separated. This is done based on distribution between two phases, the stationary and the mobile phases, according to their different physicochemical properties [46,47]. The stationary phase is immobilized, typically in a column, while the mobile phase flows through the stationary phase. The stationary phase commonly consists of either solid particles or a highly viscous liquid (only in the case of gas chromatography) which is coated on the surface of solid particles or on the wall of a capillary tube. The mobile phase can either be a liquid (liquid chromatography and capillary electrochromatography, CEC) a supercritical fluid (supercritical fluid chromatography, SFC) or a gas (gas chromatography, GC) [48].

In a chromatographic process, under ideal conditions, should be avoided the peak-broadening effects (such as diffusion, slow mass transfer, etc.) [49]. The aim of an efficient chromatography is to achieve sufficient separation of the relevant substances in the shortest possible time.

3.2. High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography, also known as high pressure liquid chromatography, is a chromatographic technique which is used in analytical chemistry and biochemistry to separate mixtures of compounds. The purpose of this technique is to identify, quantify and purify the individual components of the mixture. HPLC uses a pump, which provides high pressure to force the mobile phase through a packed bed of very small particles in order to achieve separation [50]. HPLC is a preferable choice for environmental, agricultural, pharmaceutical, food chemistry, biotechnological samples etc. It is suitable to analyse non-volatile, thermally labile and high molecular weight compounds for instance, carbohydrates, proteins, nucleic acids, polymers, etc.

3.2.1. Separation modes in HPLC

3.2.1.1. Normal phase liquid chromatography (NP-LC)

Normal phase (NP) chromatography is also known as normal-phase high performance liquid chromatography (NP-HPLC), or adsorption chromatography. This method is used to separate analyses compounds with different polar affinity. The polar stationary phase can be bare silica or support-bonded amino (NH_2), diol or cyano (CN) phases. The mobile phase can be either non-polar or weakly polar organic solvents such as hexane, dichloromethane, ethyl acetate and isopropanol. The polar molecules are retained longer on the column than the non polar molecules, which leave faster the column. By increasing the polarity of the mobile phase the retention time is decreased.

3.2.1.2. Hydrophilic interaction chromatography (HILIC)

*H*ydroph*il*ic ***i***nteraction ***c***hromatography (HILIC) is a variation of Normal Phase -LC, [51] with the main advantage being the use of solvents that are miscible with water. This separation is also known as "reverse reversed-phase" or "aqueous normal phase" Chromatography. This chromatographic separation mode was already known from 1950, however also under the name NP-LC. This term was used until 1990 when Alpert [51] proposed "HILIC" to distinguish it from the classical NP-LC which uses non-aqueous mobile phases. The stationary phase of a HILIC column is polar material such as silica, cyano, amino, diol, etc. The mobile phase is highly organic, usually higher than 80%, with a small amount of aqueous or polar solvent, for example water, methanol etc.

The advantages of HILIC are:

"• Retention of highly polar analytes that would be un-retained by reversed-phase chromatography

• Complementary selectivity to reversed-phase

• Enhanced sensitivity with mass spectrometric detection

• Shortens the sample preparation procedure

– Final SPE, protein precipitation, or liquid/liquid extraction step is usually an organic solvent (*e.g.*, acetonitrile, isopropanol, etc.)

– This organic solvent must then be evaporated to dryness and reconstituted in mobile phase before injecting onto reversed-phase HPLC column, therefore

– Elimination of evaporation/reconstitution step by directly injecting this organic eluent" [52]

Hydrophilic interaction chromatography has been developed to separate polar or ionised analytes with limited retention in RPLC [53,54]. Until 1990 HILIC has been used to analyse sugars and oligosaccharides [55,56]. In this period Alpert [51] proved that HILIC can be used for other compounds as well, for instance amino acids, proteins, peptides, organic acids and oligonucleotides.

Subsequently, the application of HILIC has been extended to other compounds including cosmetics [57], pharmaceuticals [58, 59] and flavonoids [60, 61].

3.2.1.3. Reversed phase liquid chromatography (RP-LC)

Reversed-phase liquid chromatography (RP-LC) is a chromatographic method which uses a non-polar or weakly polar stationary phase and for mobile phases use relatively polar, normally aqueous phases [62]. Commonly used stationary phases are octadecyl (C18), octyl (C8), hexyl phenyl (C6-Ph) and cyano (CN) phases combined with mobile phases consisting of water, methanol, acetonitrile, tetrahydrofuran or mixtures.

In RP-LC, polar compounds are eluted first while non-polar compounds are retained. Nowadays, reversed-phase column chromatography accounts for the majority of analyses are performed by liquid chromatography [63].

Reversed phase chromatography is also known as hydrophobic chromatography. Lipophilic groups are attached to the stationary phase of the column. For instance when a solution of proteins or molecules is passed through the column, lipophilic proteins will remain in the column, while hydrophilic proteins will flow through the column [64]. The elution order may be different because of the following factors: solvent properties, pH and temperature.

The RP mode is the most widely employed liquid chromatographic separation mode because of its ability to separate a wide range of compounds (e.g. acids, bases and neutrals). It is a relatively straightforward method since separation principles are well-known. RP-LC

provides faster column equilibration than non-aqueous adsorption separations and the fact that water, commonly used as mobile phase, is cheap and freely available.

3.3. HPLC instrumentation

HPLC instrumentation includes a pump, injector, column, detector and data system. The most important part of the equipment is the column where separation occurs. A high pressure pump is required to move the mobile phase through the column, because the stationary phase is composed of micrometre size porous particles. [Figure 6]

The chromatographic process begins by injecting the solute onto the head of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Finally, each component elutes from the column as a narrow peak on the control instrument.

The detection of the eluting components can be either selective or universal; this depends on the applied detector. A chromatogram is the response of the detector to each component which displayed on a chart recorder or computer screen. The following equipment are used to collect, store and analyse the chromatographic data: a computer, an integrator, and other data processing equipment [65].

Figure 6: Simadzu HPLC instrument [66]

3.3.1. Solvent delivery system

The solvent delivery unit is composed of the solvent reservoirs and the pumps.

The samples should be filtered before using them in the HPLC system so that any particle will be removed. The presence of particles can lead to damage or clogging of the pumps, injector or column.

Solvents play an essential role in an LC separation. For reliable analytical results it is necessary to use highly pure solvents.

It is important that the solvents should be degassed before their use. The dissolved gases should be removed or bubbles will exist in the system. The existence of bubbles in the system can lead to unsteady and irreproducible flow rates, erratic gradient profiles or an increased baseline noise. Degassing can be achieved by ultrasonication, sparging of the mobile phase with an inert gas of low solubility to force any dissolved gases out of solution, or by use of an on-line degasser consisting of a vacuum pumping system and membrane.

The pump is needed in HPLC instrument as to force the solvent through the lines and more importantly to control the column for the build-up of the backpressure. The HPLC pump is used to generate a highly reproducible and pulse-free flow in the range of 0.001 to 10 mL/min the pressure typically limited up to 400 bar, more recently up to 600 bar. [67].

There are three types of pumps which are used in HPLC instrumentation: reciprocating pumps, syringe or displacement pumps. Approximately 90% of the commercial HPLC instruments are equipped with the reciprocating pump. The pump consists of a small cylindrical chamber that is alternately filled and emptied with the mobile phase during the reciprocating motion of the piston. As a result, a pulsed flow is produced. This pulsation should be decreased to avoid excessive baseline noise. In order to control the flow direction the pump head is equipped with inlet and outlet check valves. The inlet check valve prevents backward flow of the mobile phase into the solvent reservoir, while the outlet check valve prevents backward flow from the column into the pump. The advantages of reciprocating pump are: high output pressure, small internal volume, constant flow rates, and the compatibility with gradient elution.

According to Csaba Horvath [68], **isocratic** is the separation mode in which the mobile phase composition remains constant throughout the whole procedure.

In many researches the composition of the mobile phase does not remain stable. So, **gradient elution** is the separation mode in which the mobile phase composition is changed stepwise or continuously during the separation process.

Gradient elution is generally preferred to analyse compounds with a wide retention range, but also isocratic elution could be used [69,70]. Generally gradient elution increases the solvent strength and, as a result, the values of the retention factor (k) of the later eluting compounds are reduced. Most of the compounds which are separated with HPLC are analysed with gradient elution. This is mainly due to the advantages of gradient elution, which are: improved resolution, better detection and quantitation of strongly retained compounds and shorter analysis times [71, 69].

To establish the gradient elution, the earlier HPLC instruments were equipped with a gradient programmer, which was used for programming the solvent composition related to the time. Modern HPLC instruments are equipped with a computer to control the whole system and offer easier data analysis.

Another important part of the pumping unit is the mixing chamber. The use of the mixing chamber is to mix solvents in order to get a uniform composition. There are two possibilities to do the mixing. The first possibility is when the mixing is done before the solvent reaches the pump (low-pressure mixing) or the second one is after passing through the pump (high-pressure mixing). Due to the fact that in high-pressure mixing, the mixing takes place in a small chamber under high pressure and the presence of gases in the mobile phase at this stage can lead to bubble formation from decompression, it is notable to use a solvent degasser.

3.3.2. Injection system

In the earlier HPLC the user used to inject the sample onto the column. The modern HPLC instruments are equipped with an auto sampler and the samples are injected automatically onto the column.

3.3.3. The HPLC column

An HPLC column is the heart of the system due to the fact that the whole separation takes place in it. The columns are typically made out of stainless steel to prevent the damaging of the column because of the generated high pressure which is obtained when there is a flow of mobile phase through a packed bed of small particles. It is important that the material of the columns should be chemically inert.

The typical length of HPLC columns is between 10-30 cm and the particle sizes are between 3-10 μm. For analytical purposes are commonly used columns with internal diameters between 1-4.6 mm [71]. Columns with internal diameters between 4-10 mm are used for semi-preparative, while for preparative applications; columns with 10-25.4 mm internal diameters are employed [55,72].

The optimal flow rate is proportional to the column cross-sectional area (cm^2) which means that an increase in column cross-sectional area has a consequence of increasing flow rate and resulting to high solvent consumption [55]. Moreover, the sample dilution factor is higher in larger than in smaller-bore columns, and as a consequence, an increase in column diameter should be followed by proportional increase in injection volume if sensitivity should not be lost. Smaller-bore columns are commonly used for small amount of samples with low concentration of analytes and low solvent consumption is achieved [47, 55].

The broadness of the peak determines the efficiency of a chromatographic separation [50]. Further, the number of theoretical plates (N) measures the separation efficiency of a chromatographic column:

$$N = 16\left(\frac{t_R}{w_b}\right)^2 = 5.54\left(\frac{t_R}{w_h}\right)^2 = \frac{L}{H} \qquad \textbf{(3.1)}$$

Where:

t_R = the analyte retention time (min),

w_b = the base peak width (min),

w_h = the peak width at half height (min),

L = the column length (mm) and

H = the theoretical plate height (mm).

The Van Deemter equation in chromatography relates the variance per unit length of a separation column to the linear mobile phase velocity by considering physical, kinetic, and thermodynamic properties of a separation: [73]

$$H = A + \frac{B}{u_0} + C \cdot u_0 \qquad (3.2)$$

where:

H = theoretical plate height, which relates the variance of a band to the distance travelled through the column (mm)

u_0 = mobile phase flow linear velocity (mm/sec)

A = the multiple path term, which is independent of flow rate (Eddy-diffusion)

B = the longitudinal diffusion term, which is inversely proportional to flow rate

C = the finite equilibration time between the mobile and stationary phases

The A, B and C terms contribute to band broadening in packed columns.

Operating under optimum conditions, it is obtained from the van Deemter curve (H-u_0 plot) that $H \approx 2d_p$. In this example, **equation 3.1** becomes:

$$N \approx \frac{L}{2d_p} \qquad (3.3)$$

Where:

d_p = is the particle diameter (μm)

On the one hand the efficiency of an HPLC column is increased when the particle size is decreased and the column length is increased. On the other hand, small particles have higher resistance to solvent flow [50], which means that higher operating pressures are needed. For these reason columns longer than 30 cm cannot be used otherwise pressures exceeding the practical instrument pressure range will be obtained. Nowadays, the Ultra high pressure LC (UHPLC) systems are commercially available, which can work at up to 15 000 psi (about 1000 atm), and short columns packed with small particles sizes (< 2 μm) resulting to highly efficient and fast separations.

In order to prolong the lifetime of analytical columns, it is recommended that all the HPLC samples are filtered before the analysis and guard columns packed with a stationary phase similar to that of the analytical columns before the analytical column are used [74].

The ideal characteristics of HPLC columns should be the following: i) in order to gain flexibility for method development the column should be usable in the range of pH between 0.8-12.0; ii) also to achieve maximum versatility the column should be compatible with 100% aqueous mobile phase; iii) furthermore, high surface coverage of the column leads to tolerance to large volume and mass injected; iv) moreover, by using the minimum buffer concentration needed, better sensitivity for LC-MS and column lifetime is obtained; v) furthermore, the wide variety of chemistries available leads to broad selection of polarity. Columns with excellent column efficiency lead to better and faster separation; vi) to reduce the cost of column it should be achieved the maximum lifetime of the column and vii) finally all the columns should have tightest specification so that the user will have better confidence in HPLC results [75].

Also the 1D column should be long with small diameter and should be operated at low flow rates 40µl/min-100µl/min, because the loop has very small capacity and it should be filled with eluent coming out of the 1D column, during the time that the chromatographic separation in the 2D column takes place. The 2D column should be short with big diameter and should be operated at higher flow rates 1-3ml/min so that the 2D chromatographic separation analysis will be over within 1-2 minutes

3.3.4. Detection

Detectors form an essential part of an HPLC system. These allow monitoring of the eluate exiting the column for the presence of analytes [47, 71, 76 and 77]. An ideal detector should provide a linear response, be sensitive to low concentrations of analytes, insensitive to fluctuations in temperature and mobile phase composition, be non-destructive and should not broaden analytes peaks [47, 50, 71, 77 and 78]. Several types of detectors have been developed for HPLC analysis.

3.3.4.1. Ultraviolet-Visible (UV-VIS) detection

The most common detection mode in HPLC is the UV-VIS detection. This detection mode has great sensitivity to a wide range of compounds, are insensitive to temperature changes, and are compatible with gradient elution and with low cost [47, 71]. A UV detector works according to the principle of Beer-Lambert law:

$$Absorbance = \log \frac{I_0}{I} = \varepsilon l c \qquad (3.4)$$

Where:

I_0 = intensity of the incident light

I = intensity of the transmitted light

l = path length of the cell (cm)

ε= molar absorptivity or molar extinction coefficient (M^{-1} cm^{-1})

c = concentration of the light absorbing species in the sample (mol L^{-1}).

According to Beer-Lambert law absorbance is directly proportional to the concentration of the light absorbing species in the sample and the molar absorptivity (ε) of the analyte at the specified wavelength. The UV detector measures the difference between the incident light and the transmitted light. The light from the lamp passes through the flow cell and is transmitted onto a diode that measures the light intensity. An analyte molecule can be detected by UV-VIS. The prerequisite is the existence of a chromophore group, which contains an atom, or a group of atoms, with the valence electrons with low excitation energies, resulting in UV absorption [47, 71]. It is also important that a mobile phase with acceptable UV transmittance at the selected wavelength is used to maximize the detection sensitivity with respect to the analyte molecules [47, 69 and 71].

There are several types of UV detectors which are used in HPLC: some of them are fixed wavelength (FW), variable wavelength (VW) and photodiode array (PDA) detectors.

PDA detectors use deuterium or tungsten lamps. The combination of the two lamps covers the radiation in the full spectral range of 190-800 nm. In the PDA detector, the diffraction grating assembly is placed after the flow cell. So, the entire range of wavelengths transferred from the

source to the flow cell. The transferred light passes through a prism which disperses the light according to different wavelengths, each of which is detected by a diode (Figure 7). During the elution of the analytes the whole spectrum is monitored by the PDA detector [47, 69, and 77]. PDA detector is a powerful qualitative tool and efficient for mixtures of compounds which absorb at various wavelength values [79].

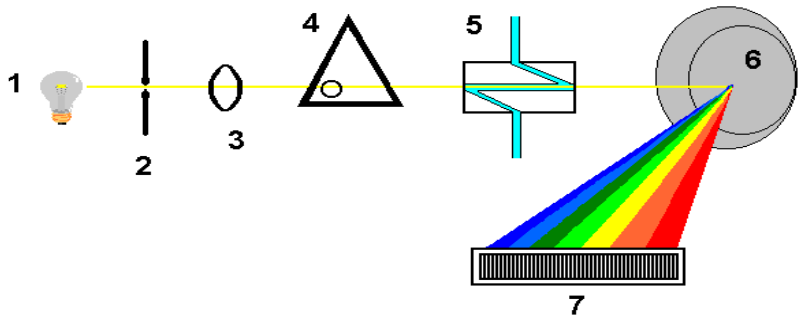


Figure 7: Scheme of a diode array detector (photodiode-array, PDA, DAD). The light from the radiation source (1) after passing through a slit (2), lens (3), shutter (4) and the detection cell (5) is dispersed at the holographic grating (6), so each of the photodiodes (7) turns the radiant flux into an attenuated wavelength absorption in the detector cell [80].

The PDA detector is more suitable for applications where the light level is relatively high and where higher output accuracy is needed [81].

3.3.4.2. Liquid Chromatography Mass Spectrometry (LC-MS)

The LC-MS is an analytical technique that combines the physical separation capabilities of liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry (MS). LC-MS is a really powerful technique with very high sensitivity and selectivity, used in many situations. Generally this technique is oriented towards the potential identification and specific detection of compounds in complex mixtures [82].

3.3.4.3. Mass Spectrometry (MS)

Mass Spectrometry (MS) is an analytical technique used for determining the mass of one ion and also providing accurate mass information of a sample or molecule. Furthermore it may be used for elucidating the chemical structures of molecules, such as polyphenols and other

compounds. This method is based on measuring the mass-to-charge ratio (m/z) of charged molecules. There are several different types of mass analyzers, using statistic or dynamic fields, and magnetic or electric fields. The MS instrument consists of three modules: ion source, mass analyzer and detector. A typical MS procedure consists of five steps. First step is the introduction of the sample into the device. Second is the ionization of the sample by one of the following methods: Electron Ionization (EI), Thermospray (TSP), Chemical Ionization (CI), Fast-Atom Bombardment (FAB), Secondary Ion Mass Spectrometry (SIMS), Laser Desorption (LD), Matrix Assisted Laser Desorption Ionization (MALDI), Electrospray (ESI) and Atmospheric-pressure chemical ionization (APCI) [83]. In this study ESI was used. The next step is when the ionized ions pass into the following part of instrument where they are separated according to their mass-to-charge ratio and the ion beam are formed. The final steps are analysis and detection. Ions are directed to the detector, which converts the signal in a quantitative manner in the form of ionic current into an electrical signal that is recorded by a computer in the form of the spectrum. In this spectrum on the horizontal axis is the mass to charge ratio (1 Da = 1 atomic mass unit / elementary charge), the vertical axis is the intensity (number of ions registered by the spectrometer).

3.4. Limitations of 1-D- HPLC chromatography

For the analysis of complex samples it is needed to improve the separation power. The peak capacity (n_p) measures the resolving power of a chromatographic separation system [84]. Peak capacity is the maximum number of peaks that can be separated at a given resolution within a given separation time [46,85,86]. The resolution can be limited either by the selectivity of the stationary phase or by the attainable efficiency. Selectivity can be improved by changing the stationary phase, mobile phase composition, pH or temperature. However, the peak capacity is limited by the number of theoretical plates (N) and the separation space [85]. The peak capacities for isocratic and gradient analyses [86] are given by equations 3.5 and 3.6, respectively:

$$n_p = 1 + \frac{\sqrt{N}}{4}\ln(k+1) \qquad (3.5)$$

N= the column plate count

k= the retention factor for the last eluting compound.

$$n_p = 1 + \frac{t_g}{w} \quad (3.6)$$

Where t_g is the gradient run time (min) and w is the average peak width (min).

The number of theoretical plates (*N)* of a chromatographic separation can be increased by increasing the column length or by decreasing the particle size. However, both techniques are limited because of a significant increase of the pressure is obtained and HPLC instrumentation and columns are limited in terms of maximum operating pressure (the limit for current commercial instruments is ~ 400 bar). Another approach to increase the peak capacity is the increase of the separation space. This can be achieved by coupling multiple, orthogonal (non-correlated) separation mechanisms and presenting the sample to both separation mechanisms [85].

3.5. Multidimensional LC (MD-LC)

MD-LC techniques were developed to overcome the limit of the resolving power of conventional 1-D LC techniques.

Multidimensional liquid chromatography (MD-LC) is a very powerful separation technique for analyzing exceptionally complex samples [87]. It is a useful technique because the separation space is increased. The eluting zone of the analysed compound is not only through the x-axis, one-dimensional (1-D), but also through the y-axis, two-dimensional (2-D). As a result the 2D-LC has much higher resolution, selectivity and peak capacity than single column separation. Typically the 2D-LC is a slow technique. However, if the time needed to achieve separation in 1D for a complex mixture is compared to the time needed in 2D-LC, then the latter is more favourable.

MDLC methods are typically divided into two main groups: comprehensive separations concerned with the separation and quantitation of large numbers (ca. 10 s to 1000 s) of constituents of a sample , and targeted ‘heartcutting’ or ‘coupled-column’ methods concerned with the analysis of a few (ca. 1–5) constituents of the sample matrix. Due to the fact that wine samples contain large amounts of phenolic and flavonoid compounds, comprehensive LCxLC separation is considered to be the ideal mode of analysis for these particular types of samples. In comprehensive LCxLC the whole eluent from the 1D is subjected to

chromatographic separation in the 2D column therefore the separation power is increased whereas by heartcutting only certain fractions (regions of interest) of the first dimension separation are analysed in the second dimension.

3.5.1. General aspects of MD-LC

Orthogonality: The first fundamental requirement is that the separation mechanisms used in each dimension of a 2D system must subject sample constituents to two separation steps involving mechanisms that are dependent upon different physico-chemical factors. So, the 'orthogonality' denotes the degree of difference between the 1st and the 2nd dimension separation. Secondly, Giddings [88] stated that separation gained in the first dimension of a multi-dimensional separation must not be lost in subsequent separation steps. To achieve an orthogonal separation the 2 columns should have different separation mode.

Peak capacity: is the number of peaks that can be side by side at some stated resolution between two retention times or at a start and finish length, in the case of planar separation system (Giddings [89,90]).

The mathematical equation of peak capacity, n_c, for an isocratic separation was given by Grushka [91]

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln \frac{t_f}{t_1} \tag{3.7}$$

N=the number of plates

t_f =time of final peak

t_1 =time of void peak

In isocratic separations the peak width is not stable whereas in gradient elution the peak width is expected to be constant.

The peak capacity [91,92] for a time-based column system subjected to gradient elution with constant peak width is:

$$n_c = \frac{\sqrt{N}}{4} \left(\frac{t_f}{t_1} - 1 \right) \tag{3.8}$$

Giddings has mentioned that the total peak capacity for multidimensional chromatographic system is the product of individual peak capacity in each dimension:

$n_T = n_1 * n_2 * n_3 * \cdots$

More specific, ideally, the total peak capacity, n2D, is equal to the product of the peak capacities in the first (n1) and in the second (n2) dimensions in fully orthogonal 2D systems with non-correlated selectivity in the first and in the second dimensions [93,94]:

$$n_{2D} = n_1 \times n_2$$

In 2D-LC the peak capacity can be lower than the expected product approximation, if the two media used for separation are correlated with respect to their retention mechanism. For this reason it is difficult to obtain a completely orthogonal system [85,95]. Another reason which can lead to the loss of peak capacity in the two dimensional separations is the extra peak broadening effects and the sampling rate of the first dimension peaks. A lot of statistical and mathematical approaches are being employed in order to provide more accurate practical peak capacities, for instance by Liu *et al* [96] and Li *et al* [97]. Liu *et al* method accounts for orthogonality in the estimation of the 2-D peak capacity calculation. The method of Li *et al* accounts for under-sampling.

In the factor analysis, Liu *et al* [96] used a geometrical approach to estimate the orthogonality of a 2-D separation. Consequently, they developed the equations accounting for this parameter in the calculation of the practical peak capacity. It was obtained that in 2-D separations there were two sets of independent data and as a result Liu et al mentioned that each set of retention data can be taken as an independent vector.

Accordingly, in each 2-D separation exist two independent vectors and the correlation between them can be calculated by using the scaled retention factors of analytes separated in each dimension.

The following equation gives the correlation matrix (*C*):

$$C = \left(\frac{1}{N-1} \right) k'^{T} \, k' \qquad (3.9)$$

Where *N* is the number of components in each dimension, *k'* is the matrix of the scaled retention factors, k'^{T} is the transpose of the matrix of the scaled retention factors. The correlation matrix C is an N by N matrix with unity value diagonal elements and is symmetrical *Cij = Cji*, so only the upper and lower triangular terms need to be discussed. The

diagonal elements of this matrix are equal to 1 because these values are completely correlated with themselves. When *Cij* = 1, a totally correlated system is obtained and when *Cij* = 0, a totally orthogonal system is obtained [96].

Most two dimensional separation systems are between perfect orthogonality (when non-correlated separation mechanisms are selected) and perfect correlation (when identical separation mechanisms are used) [96]. Consequently if there is a correlation between the selected separation modes, the available separation space is reduced to a fraction of the orthogonal case [98]. A graphical representation of the 2-D retention space with a peak spreading angle (β) is shown in Figure 8

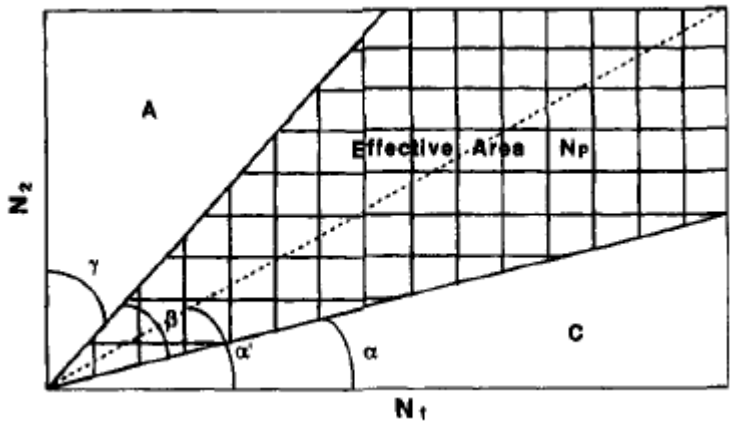


Figure 8: An effective non-orthogonal 2-D retention space when the spreading angle is β [96].

The effective or practical peak capacity, N_p is shown to be the product of peak capacity per each dimension minus the A and C area which are unavailable due to correlation [71]. In this example, angles α, α', β and γ as well as unavailable areas A and C are calculated using the following equations:

$$\alpha' = \tan^{-1}(n_2 / n_1) \qquad (3.10)$$

$\beta = \cos^{-1}(r)$ r is the square root of the correlation coefficient (3.11)

$$\alpha = \alpha'(1 - 2\beta/\pi) \qquad (3.12)$$

$$\gamma = \pi/2 - \alpha - \beta \qquad (3.13)$$

$$A = \tfrac{1}{2} n_2^2 \tan\gamma \qquad (3.14)$$

$$C = \tfrac{1}{2} n_1^2 \tan\alpha \qquad (3.15)$$

The practical peak capacity (n_p) is then calculated using:

$$n_p = n_T - (A + C) \tag{3.16}$$

$$n_p = n_T - \tfrac{1}{2}(n_2^2 \tan\gamma + n_1^2 \tan\alpha) \tag{3.17}$$

In a 2-D separation, in order to avoid loss of resolution already achieved during the first dimension separation, it is required that the eluting peaks from the first dimension column are sampled at least 3 times. [99,100]. However in most of the cases this rule is not obeyed with the consequence of loss of peak capacity [99].

3.5.2. Instrumental aspects of 2D-LC systems

Apart from the two main groups' comprehensive separations and heartcutting which already mention above the 2D-LC can also be performed in either online or off-line mode. In the on-line setup, fractions from the first dimension are directly transferred to the second dimension for further analysis. This can be achieved by using switching valve(s) equipped with sampling loops or trapping columns. Columns and loops trap the fractions from the first dimension at defined time intervals.

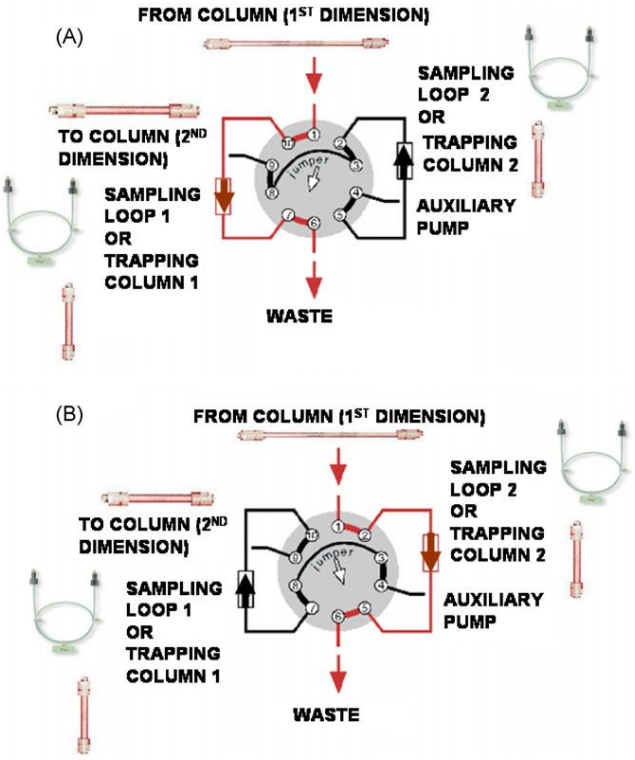


Figure 9: Scheme of the 2D-LC sample transfer interface using a ten-port switching valve with two sampling loops or two trapping columns, A: load position; B: inject position [101].

The Figure 9 shows an on-line configuration of 2D-LC using a 2-position 10-port switching valve. In the position A, a fraction of the effluent is loaded from the first dimension to the sampling loop 1 or the trapping column 1 and back-flushing the previous fraction from the sampling loop 2 or the trapping column 2 to the second dimension. In the position B the effluent fraction is back-flushed from the sampling loop 1 or the trapping column 1 to the second dimension and the next fraction of the effluent is loaded from the first dimension to the sampling loop 2 or the trapping column 2 [101].

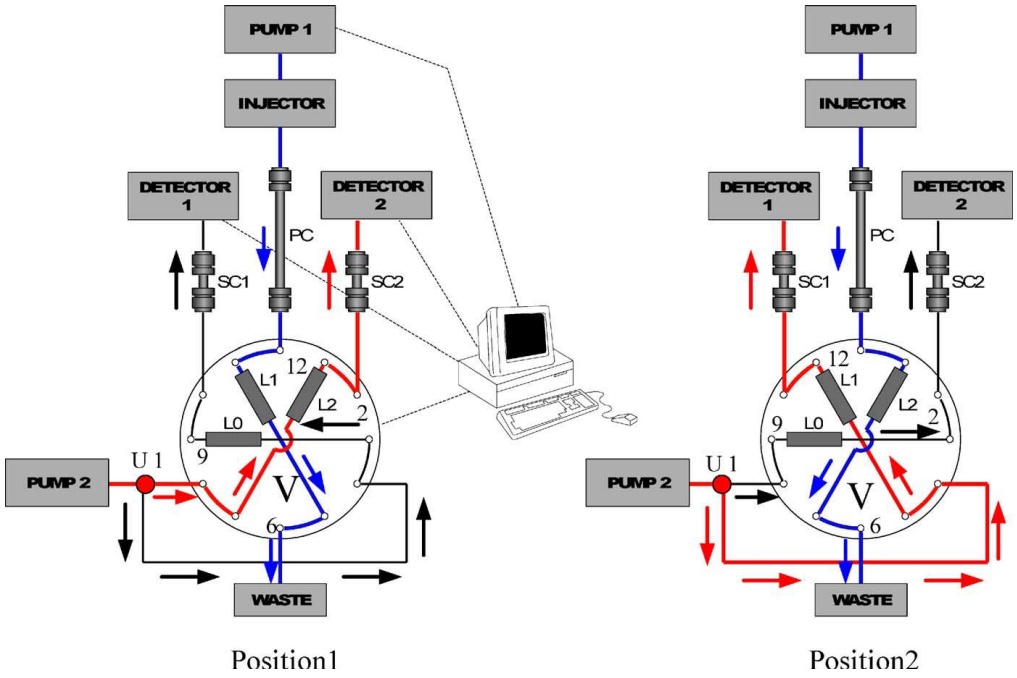


Figure 10: Schematic of a 2D-LC system showing the flow of mobile phase through the two dimensions. [102]

The Figure 10 shows an on-line configuration of 2D-LC using a 2-position 12-port switching valve. In position 1, the eluent from the primary column (PC) flows through the sampling loop L1 to the waste. The mobile phase from pump 2 flows through secondary columns SC1 and SC2 flowing through sampling loops L0 and L2, respectively. When the valve position is switched (right hand side), the primary column eluent flows through sample loop L2 to waste. The mobile phase from pump 2 flows through secondary columns SC1 and SC2 flowing through sampling loops L1 and L0, respectively. The flow through the columns is uninterrupted throughout the chromatographic run with primary column eluent alternatively sampled into the secondary columns SC1 and SC2. It is worth to mention that this approach differs from the previous one in that it requires two 2D columns and two detectors [102].

The fraction which is transferred to the second dimension column, should be fully analysed before the subsequent transfer in order to avoid the “wrap round” effect (where analytes from the previous analysis elute in the separation space of the next transfer), and this results in

chaotic band displacement [103]. This means that the second dimension analysis time should be equal or less than the first dimension analysis time [95, 103]. Monolithic column in the second dimension leads to fast separation time with high flow rates and with low backpressures [104]. The reason why is high permeability of the column and the good mass-transfer properties [105].

The principal advantages and disadvantages associated with on-line and off-line analyses are summarised in the following paragraphs.

The advantages of the On-line 2D-LC are: i) the automation of the method, ii) faster than the Off-line separation and iii) better reproducibility. On the other hand, the Off-line separation has i) higher peak capacities, ii) Ease of operation, iii) Allows combination of non-compatible LC modes with evaporation of solvents and re-dissolution prior to second dimension analysis and iv) The sample can be concentrated before second dimension analysis for better sensitivity

Which technique is more appropriate depends on the problem which is to be solved. Both techniques have their own disadvantages. For instance On-line separation has quite complicated instrumental configuration and requires specific interfaces. For these reasons on-line instrumentation are expensive. On the other hand, the Off-line technique has difficulties in automation, with poor reproducibility. Also it consumes time and solvents. Off-line separation has risk of sample loss, contamination and /or degradation [103, 105].

3.5.3. Two-Dimensional Liquid Chromatography (2D-LC)

The level of complexity of polyphenols and the characteristic limits of regular HPLC procedures restrict the field of study of polyphenols [106]. The two dimensional liquid chromatographic (2D-LC) systems improve the separation power by maximizing the separation space and minimizing the overlap probability. The 2D-LC technique is optimal for the separation of complex mixtures.

According to the method of Liu et al [107] in the analysis of procyanidins in cocoa beans and apples has been used a combination of HILIC column in the first dimension and with RPLC column in the second dimension. The separation has been performed under gradient conditions in both dimensions. In the first dimension the mobile phase consisted of acetonitrile-acetic acid-methanol-water and in the second dimension was acetonitrile-formic

acid-water. It was proved that the combination of HILIC with RPLC had a high degree of orthogonality and the value of the obtained peak capacities was extremely high. In the above system the theoretical maximum values of 4384 and 3024 for cocoa and apple, respectively. And the Practical peak capacities was calculated [107] of 3512 and 2493 respectively [108].

The group of François [109] combined the NPLC with the RPLC for the analysis of citrus oil extracts. They used a microbore diol column for the first dimension and a C18 monolithic column for the second dimension. In the first dimension the mobile phase consisted of hexane-ethyl acetate whereas in the second dimension water-acetonitrile was used. A two-position 10-port switching valve was used. In the first dimension was applied isocratic elution and in the second gradient elution. The separations in the second dimension were achieved within one minute intervals. Flavones were mainly detected and the practical peak capacity achieved was 453, whereas the one dimensional peak capacities were 46 for the normal phase (NP) and 15 for the reverse phase (RP).

François et al in another study went further by increasing the peak capacity for the same sample to 1095. This was achieved by increasing to 2 min the analysis time in the second dimension and another 10-port switching valve was added to the system and an additional second dimension column was used parallel to the previous one. The schematic presentation of this system is in Figure 11 [110].

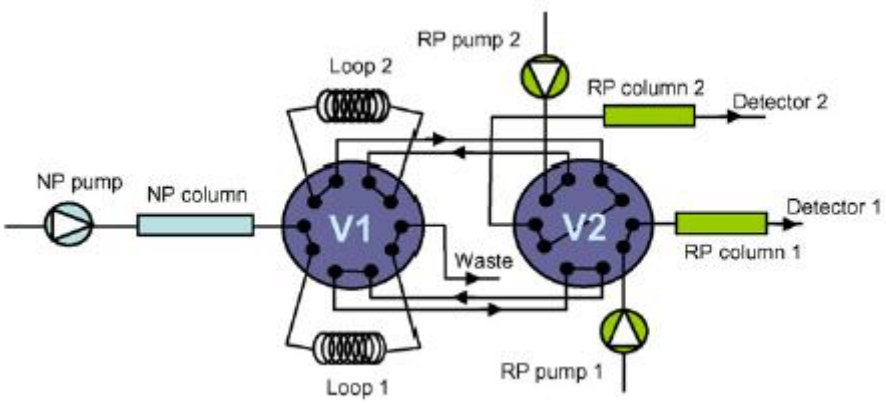


Figure 11: A schematic diagram of the LC×2LC system with two parallel second dimension columns. V1: Position 1, V2: Position 1 [110]

Another research group (T. Hyötyläinen et al) used for the analysis of phenolic antioxidants in wines and juices a combination of RPLC and RPLC involving ion pair chromatography. The system was operated in an on-line mode and the two dimensions were connected via a two-

position 10-port switching valve. Gradient elution was used for the first dimension and isocratic elution for the second. The mobile phase had the same composition: acetic acid-water-acetonitrile and tetrapentylammonium bromide-acetonitrile-acetic acid-water. For the packed second dimension column the practical peak capacity was 572 and for the monolithic was 616 [111, 112].

The same group also worked on the determination of phenolic acids in Lamiaceae herbs. They used an on-line combination of RPLC and NPLC. In the first dimension was used a C18 column whereas in the second dimension was employed a cyano column. For the two dimensional system was used a two-position 10-port switching valve. The first dimension was operated under gradient conditions and the mobile phase was acetonitrile-acetic acid-water. On the other hand an isocratic elution with acetic acid-water-acetonitrile, as mobile phase, was used in the second. They succeed to reach a peak capacity of 375 [113].

In the analysis of phenolics in standard and wine samples, Mondello et al used a microbore phenyl column coupled with a partially porous short column or a monolithic C18 column. The analysis was performed in on-line mode. In both dimensions were used gradient elution and the composition of the mobile phase was acetonitrile and water. A two-position 10-port valve was used and the modulation time was set to 2 min. The range of the theoretically calculated peak capacity was 541-726. The peak capacities, which were practically obtained, were much lower (386-516) because the two mechanisms were correlated. However, the separation plane was used effectively, so the separation of phenolics was successfully achieved [114]. The efficiency of the method in the determination of wine phenolics was proven in a further publication [115].

Another research group of Mondello used parallel gradients in the two dimensions systems combined various columns, such as Polyethylene Glycol (PEG) silica, phenyl silica and C18 columns for the first dimension and these were coupled with two secondary C18 columns for separation of phenolic antioxidants. The application of parallel gradients has as a consequence not only the speeding up of the separation but also in partially correlated systems it yields more regular spacing of the sample zones over the two dimensional plane. The relevance of these columns and their combinations in the two-dimensional comprehensive separations of phenolics was proven by the results of the retention correlation studies [116].The schematic presentation of this system is in Figure 9

Mondello and co-workers coupled C18 with pentafluorophenylpropyl silica (PFP) and C18 with PEG silica column for the separation of natural phenolic antioxidants. They proved that

the separation selectivity of C18 and PFP were strongly correlated, and on the other hand the combination of C18 and PEG columns were useful for phenolic antioxidants that are not separated on single columns [117]. The same group, to improve the resolution of the earlier eluting compounds in the first dimension, coupled a PEG column with two second dimension columns C18 or zirconia columns. They used a two-position 10-port switching valve (Figure 9). The mobile phases consisted of acetonitrile- water buffered with ammonium acetate and formic acid. The first dimension was under isocratic conditions and the second was under gradient conditions. They conclude that both separation systems are fully orthogonal [118].

Mondello et al had analyzed beer and wine samples with a C18 column in the first dimension and two zirconia columns in the second dimension operated at different temperatures. In the second dimension the high temperatures provided fast elution and short switching cycles between the two dimensions [119]. The use of zirconia in the second dimension provided satisfactory separation times resulting in enhanced peak capacity.

3.5.4. Introduction to the Two-Dimensional Liquid Chromatography (2D-LC)

Some of the applications of multidimensional techniques in the separation of real-world samples: Combination of NP-LC and RP-LC has been employed in the separation of a variety of samples in the food and flavours industry such as lemon and citrus oil extracts [120] red orange essential oil [121], carotenoids [122] and polymers [123]. RP-LC × RP-LC has also been used widely in the analysis of various samples such as steroid mixtures and sulphonamide drugs [105], phenolic compounds [124-126] and polymers [127].

3.6. Ionization method used in this study

Electro-spray ionization (ESI) is an ionization method used in mass spectrometry. In this method the ionization occurs in the liquid phase. ESI has good application for heavier compounds, because it excludes the propensity of these molecules to fragment when ionized. The introduced liquid is dispersed by electrospray into a fine aerosol. Large-flow can be obtained from additional inert gas spraying such as nitrogen. Then it is sprayed from a small tube, called capillary, into a strong electric field between 1-5 kV under atmospheric pressure. The droplets are formed by evaporation in a region maintained at a vacuum of few torr

causing the charge to increase on the droplets. In atmospheric-pressure chemical ionization (APCI) samples introduced from a high-performance liquid chromatography are sprayed by an APCI interface and chemically ionized under atmospheric pressure. APCI is effective for the ionization of medium-polarity compounds, which are difficult to ionize with ESI. Solvent gas heated inside the APCI interface to temperature 300-500ºC is ionized by the corona discharge created by applying a high voltage. Voltage is between 3 and 5 kV. The reaction ion $[S+H]^+$ is formed (where S is compound from solvent gas). Molecules are chemically ionized by the following ion-molecule that occurs with the reaction ions.

$$M + [S+H]^+ \rightarrow [M+H]^+ + S \qquad (1)$$

For this reason, a proton-donating solvent is used as mobile phase. As with ESI, nebulizer gas is used to nebulize the liquid. ESI, APCI usually produces singly charged ions with addition or loss of a proton: positive mode [M+H]+ , m/z = M+1 and negative mode [M-H]- , m/z = M-1. It is possible to switch between these two methods by replacing the interface as appropriate. Typically, APCI is less "soft" than ESI, it generates more fragment ions relative to the parent ion. For the tuning files only ESI can be used.

3.7. MS instrument used in this study

Shimadzu's LC-MS-IT-TOF is a novel hybrid mass spectrometer for biomarker discovery, metabolite identification and human health research, and ideal for applications in forensics, drug development, and environmental chemistry. Coupling atmospheric pressure ionization with Ion-Trap (IT) and Time-of-Flight (TOF) technologies, the LC-MS-IT-TOF delivers high mass accuracy and high mass resolution (10,000 at 1000 m/z). Ballistic Ion Extraction is an ion accelerating method to inject ions into the TOF instantly by applying a high voltage with opposite polarity to the end-caps and lowering the ring electrode RF voltage to 0V. The LC-MS-IT-TOF maximizes sensitivity and selectivity by optimizing the ion transport to the TOF analyzer and redefining the capability of the quadrupole ion trap. The ion trap is used to focus ions before ejection into the TOF as well as supporting MS^n analysis with effective precursor ion selection capabilities (resolution > 1,000 at 1,000 m/z) [128].

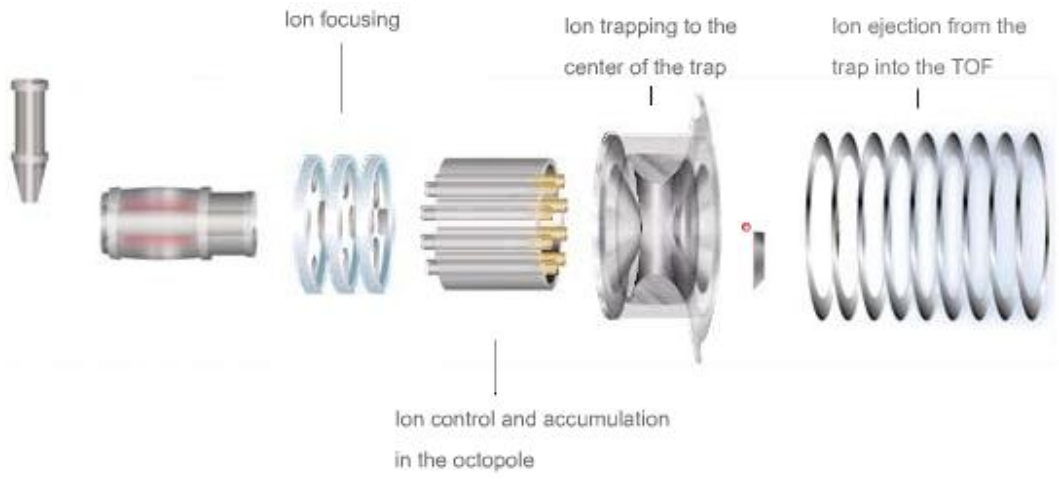


Figure 12: The first high resolution LCMS-ion trap-time-of-flight mass spectrometer [128]

III. Experimental

4. Materials and reagents

Standards of phenolic compounds and flavonoids listed in Table 1 were obtained from Sigma-Aldrich in Germany.

Table 1: Phenolic and flavonoids compounds were purchased from Sigma-Aldrich Germany

	Company	Purity %
2-(p-Hydroxyphenyl)ethanol	Sigma-Aldrich Germany	≥ 98.0%
Ethyl gallate	Sigma-Aldrich Germany	≥ 96.0%
Gallic acid	Sigma-Aldrich Germany	≥ 97.5%
(-) epicatechin	Sigma-Aldrich Germany	≥ 90.0%
Caffeic acid	Sigma-Aldrich Germany	≥ 98.0%
Rutin trihydrate	Sigma-Aldrich Germany	≥ 95.0%

In this study were used the following columns:

The Kinetex 2.6 µm PFP, 100 A 50 x 4,6mm, Kinetex 2.6 µm C18, 100 A 50 x 4,6mm, Kinetex 2.6 µm C18, and 100 A 30 x 4,6mm, were obtained from Phenomenex. The Onyx Monolithic 50 x 4,6 mm and the SecurityGuard C18 4 x 3 mm pre-column were also purchased from Phenomenex. The Hilic column EC 125/2 Nucleodur 100-3 Hilic was obtained from Macherey-Nagel.

4.1. Equipment

Both mono-dimensional analysis and comprehensive analysis were conducted in a Shimadzu Prominence UFLC liquid chromatographic system (Kyoto, Japan), with two flow lines, each of which equipped with an on-line degasser (DGU-20A3), and a binary pump (LC-20AD), then an auto-sampler (SIL-20A), thermostated column compartment (CTO-20AC), diode array detector (SPD-M20A) and coupled with Shimadzu's LC-MS-IT-TOF.

The full UV spectra were recorded for peak identification. The sampling frequency was set to 1.25 Hz .In the two-dimensional LC×LC setup, the first-dimension and the second-dimension columns were connected via an electronically controlled set of two-position 6-port switching valves equipped with a 100 μL sample loop. The modulation times were 1 min and 2 min. Both chromatographic dimensions and the switching valve were controlled by the LC-MSsolution Version 3.50.348 software (Shimadzu). ASCII data files were created by using the export function of the LC-MSsolution software and then converted and visualized by GC Image Version 2.2b0 software for multidimensional chromatography (Lincoln University, Nebraska). The 2D chromatograms were plotted with the coordinates calibrated in the first-dimension elution times on the x-axis and in the second-dimension elution times on the y-axis (with the time scale range corresponding to the fraction transfer modulation frequency).

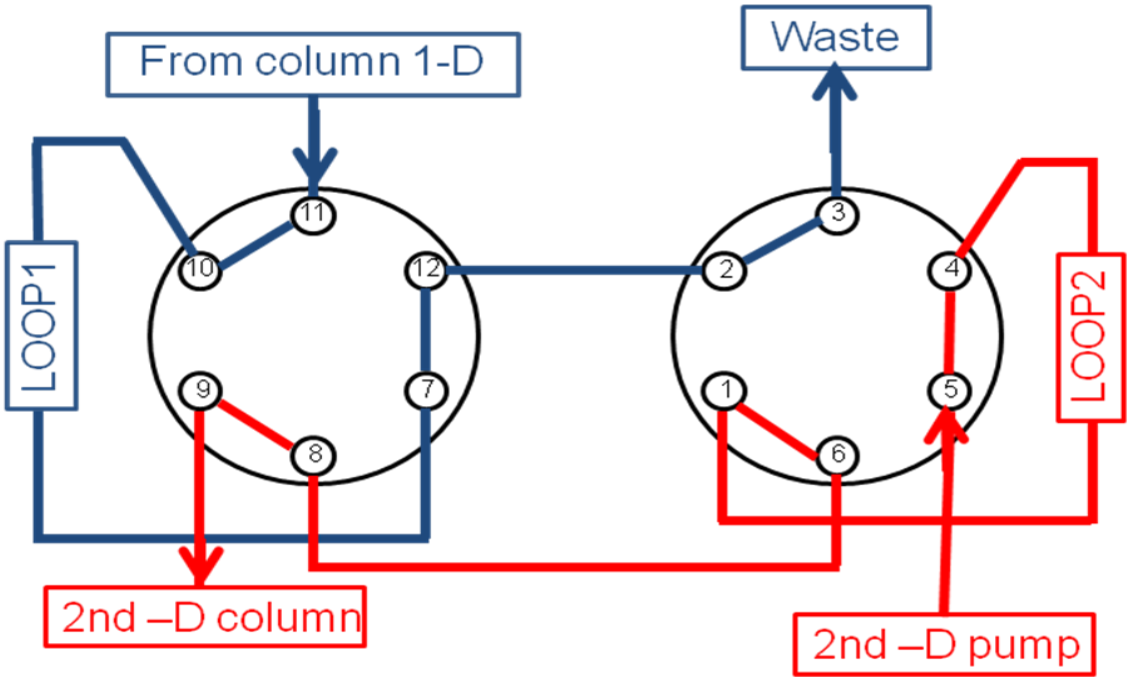
There are two factors that should be considered to adjust the flow rate of the first dimension flow rate:

a) The time need to complete the 2^{nd} dimension separation

b) The capacity of the loop

So, the collection of the fraction eluting from the first dimension will be achieved, without any loss of analytes.

Position A



Position B

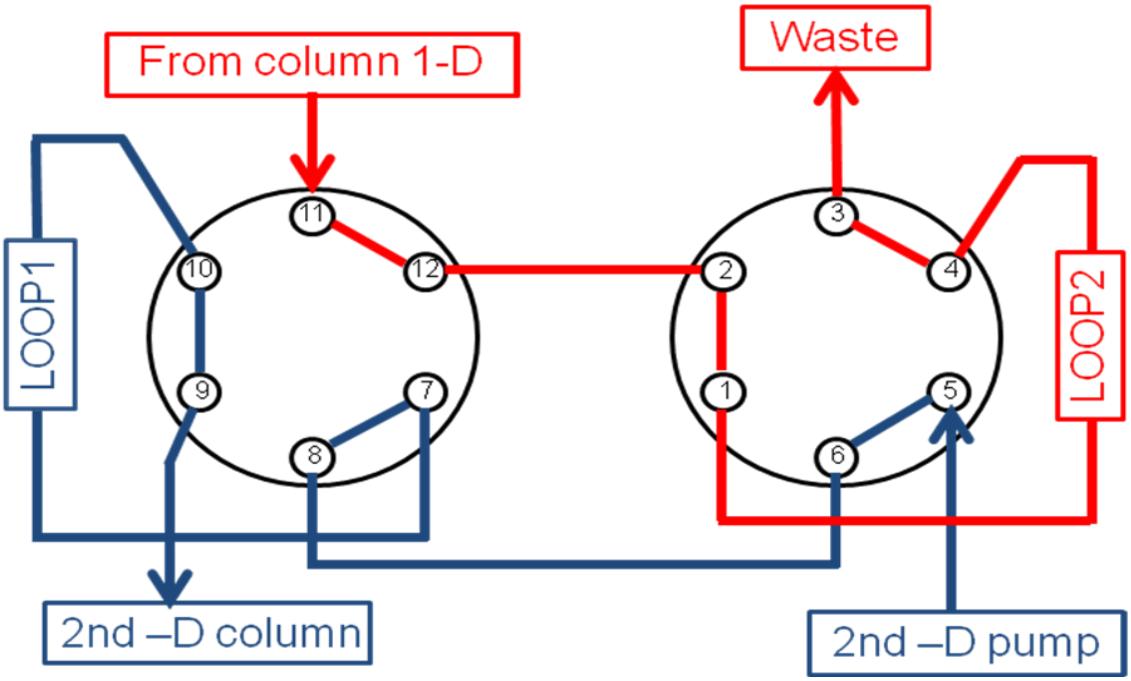


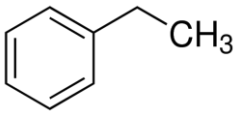
Figure 13: Scheme of the 2D LC×LC sample transfer interface using two six–port switching valves with two sampling loops. Position A and Position B

In the Figure 13 is the schematic presentation of the 2x6-port valve system which was developed and used in this study. In the position A, a fraction of the eluent is loaded from the first dimension to the sampling loop 1 and back-flushed the previous fraction from the sampling loop 2 to the second dimension. In the following step, the valves switch and their functions are changed. In the position B the eluent fraction is back-flushed from the sampling loop 1 to the second dimension and the next fraction of the effluent from the first dimension loaded to the sampling loop 2. The switching between the valves takes place at regular intervals throughout the whole time of the analysis.

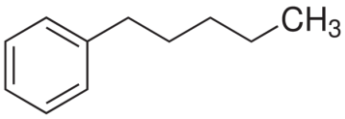
5. Proof of 2D-HPLC principles with alkyl-benzene derivatives

5.1. Sample preparation

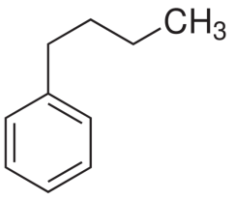
There were used 5 benzene derivatives standards: ethylbenzene, pentylbenzene, butylbenzene, propylbenzene, and nitrobenzene. Standards were dissolved with acetonitrile concentration 2mg/ml. [129].



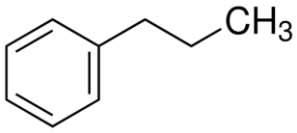
Ethylbenzene



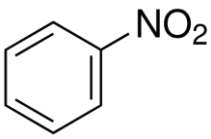
Pentylbenzene



butylbenzene,



propylbenzene



nitrobenzene

These benzene derivatives where used to control the condition of the 2D-HPLC.

5.2. Parameters

The parameters which have been used in these methods were

Mobile phase: water: Acetonitrile (A: B)

Column: Kinetex 2.6 µm C18, 100 A 30 x 4.60 mm

Column temperature 40 ^{o}C

Table 2: Parameters for the separation of alkyl benzene derivatives with kinetex 2.6 µm C18, 100 A 30 x 4.60 mm

	setup i	setup ii
time (min)	2.55	1.50
B%	60.00	70.00
flow ml/min	2.00	3.00

The separation of the derivatives has been check by using different percentages of acetonitrile. The runs were isocratic. The LC-chromatographs were extracted at 213 nm.

5.2.1.1. Results

setup i

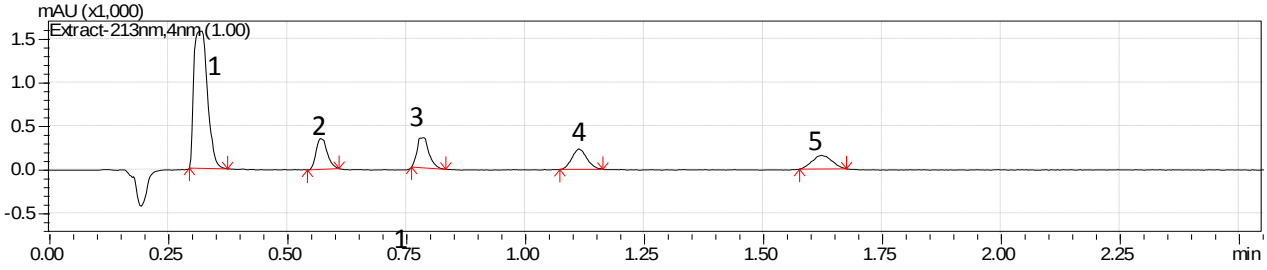


Figure 14: LC-chromatogram extracted at 213 nm for the separation of alkyl-benzene derivatives mixture 1. nitrobenzene, 2. ethylbenzene, 3. propylbenzene, 4. butylbenzene and 5. Pentylbenzene, with kinetex 2.6 µm C18, 100 A 30 x 4.60 mm 60% ACN and flow rate: 2ml/ml (setup I).

The last compound has been eluted before 1.75 min so that the running time has been reduced to 1,90min and also the flow rate of the mobile phase has been increased to 3 ml/min setup ii.

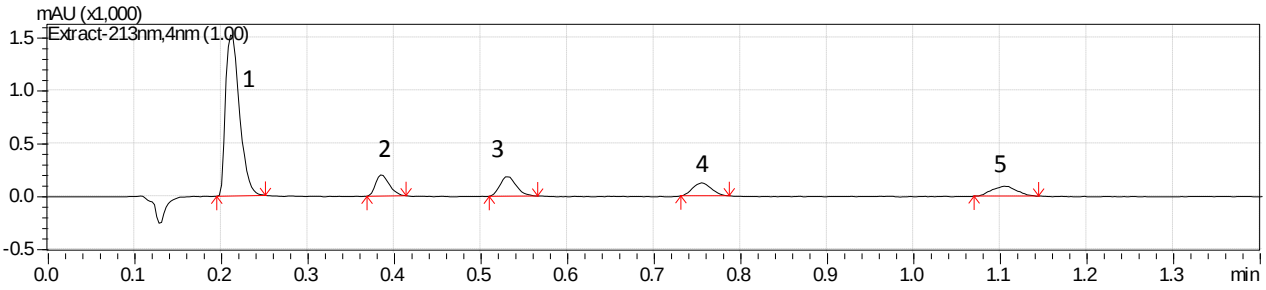


Figure 15: LC-chromatogram extracted at 213 nm for the separation of alkyl-benzene derivatives mixture 1. nitrobenzene, 2. ethylbenzene, 3. propylbenzene, 4. butylbenzene and 5. Pentylbenzene, with kinetex 2.6 µm C18, 100 A 30 x 4.60 mm 70% ACN and flow rate: 3ml/ml (setup II).

5.3. LCxLC separation of alkyl-benzene derivatives

5.4. Sample preparation

There were used 5 alkyl-benzene derivatives standards: ethylbenzene, pentylbenzene, butylbenzene, propylbenzene, and nitrobenzene. Standards were dissolved with acetonitrile concentration 2mg/ml.

5.5. Parameters:

In the two dimensional HPLC were used the following parameters:

Mobile phase: isocratic separations with water (A) and acetonitrile (B)

Column temperature 40 ^{o}C

The first-dimensional analysis was carried out on a pre-column security guard cartridge C8 4x 3.0 mm (Phenomenex). As mobile phases, water (A) and acetonitrile (B), both neutral, were used in isocratic conditions at 60% B;

The second-dimensional analysis was carried out on a Kinetex 2.6 µl C18 100 A 30x 4.60 mm. (Phenomenex). As mobile phases, water (A) and acetonitrile (B), both neutral, were used in isocratic conditions at 60%B;

In order to obtain the 1st dimension chromatogram in a reasonable time a C8 pre column was used. It is operated under small flow rate so a column with small internal diameter was required.

An HPLC oven was used to maintain the column temperature at 40 ^{O}C, flow rate was 0.2 mL/min and the 5µL injections were made by means of an autosampler. The photodiode array detector was operated at 12.5 Hz in the 190–400 nm range.

Table 3: Parameters for the separation of 2 mg/ml alkyl benzene derivatives 1D- analysis with pre-column security guard cartridge C8 4x 3.0 mm and for 2D analysis with Kinetex 2.6 µl C18 100 A 30x 4.60 mm (setup iii)

	setup iii
time (min)	6.00
B%	60.00
flow ml/min	0.1000
D%	60.00
flow ml/min	3.000
injection volume µl	1
Switching of the two six port valves	Every 1,00 min

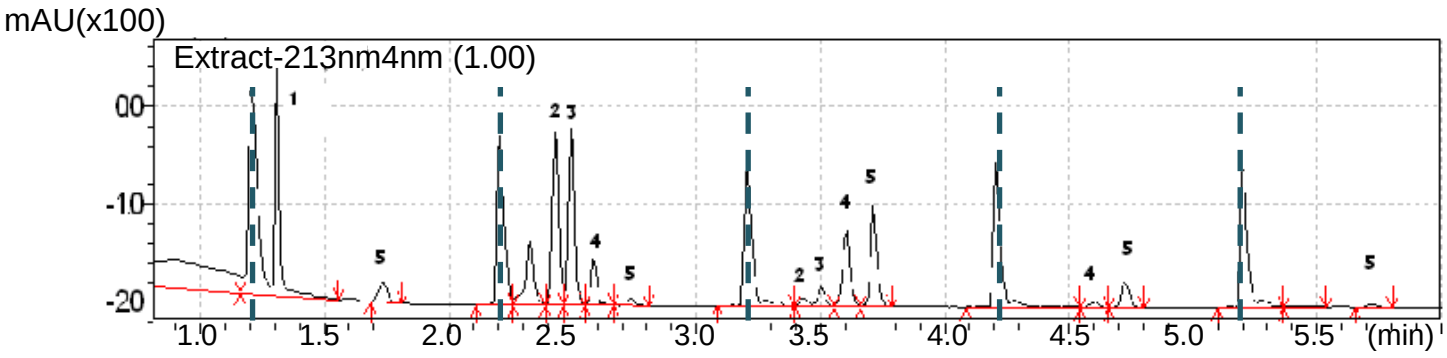


Figure 16: extracted liquid chromatogram at 213 nm of alkyl benzene derivative mixture, 1. nitrobenzene, 2. ethylbenzene, 3. Propylbenzene, 4. Butylbenzene and 5. Pentylbenzene with setup iii. The blue dote-lines indicated the peaks witch caused from the switching of the valves.

5.6. Zone Visualization

In order to gain a better understanding of the results of the two dimensional chromatograms, we cannot solely rely on the classical representation of the chromatogram but also examine the two dimensional contour plots should be studied. In the optimized method of set-up iii the analyzed mixture was successfully separated (Figure 17). The elution order of the alkyl benzene derivatives is 1. nitrobenzene, 2. ethylbenzene, 3. Propylbenzene, 4. Butylbenzene and 5. Pentylbenzene

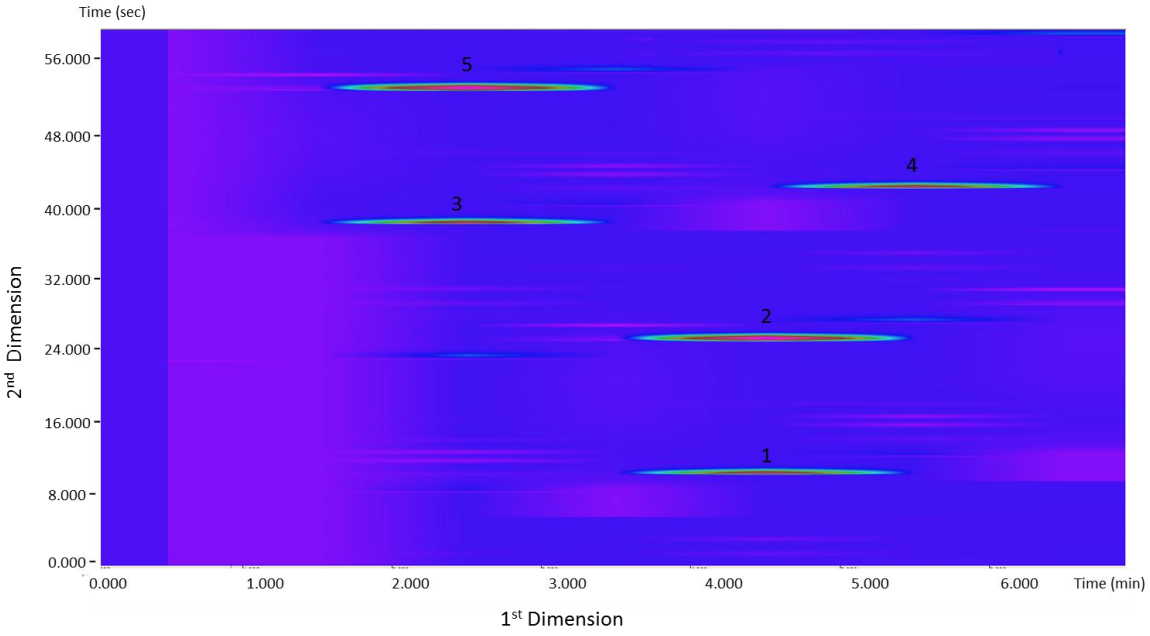


Figure 17: UV Contour plot of alkyl benzene derivative mixture, 1. nitrobenzene, 2. ethylbenzene, 3. Propylbenzene, 4. Butylbenzene and 5. Pentylbenzene with setup iii.

6. Phenolic & Flavonoid compounds

After a series of experiments it was found at the all the standards were soluble in water: acetonitrile 1:1 (Table 4)

Table 4: standard of phenolic & flavonoid compounds which were used in this study and their solubility

Name	Formula	Molecular mass (g/mol)	Predicted m/z [M+H]+	Predicted m/z [M-H]-	Soluble
2-(p-Hydroxyphenyl) ethanol	$C_8H_{10}O_2$	138.1638	-	-	water: methanol 9:1 water: acetonitrile 1:1
Ethyl gallate	$C_9H_{10}O_5$	198.1727	-	-	water: methanol 9:1 water: acetonitrile 1:1
Gallic acid [130]	$C_7H_6O_5$	170.1195	-	169.0142	water: methanol 9:1 water: acetonitrile 1:1
(-) epicatechin [131]	$C_{15}H_{14}O_6$	290.2700	-	289.0718	methanol: water 9:1 water: acetonitrile 1:1
Caffeic acid [132], [133]	$C_9H_8O_4$	180.1574	181.0495	-	water: acetonitrile 1:1
Rutin trihydrate [134]	$C_{27}H_{36}O_{19}.3H_2O$	664.5633	-	-	methanol: water: acetonitrile 9:1:1

6.1. Mobile phase used in this study

During method development different mobile phases were used. These were: pure-water, acetonitrile, water with 0.1% formic acid, acetonitrile with 0.1% formic acid and methanol. Actual conditions are indicated where relevant.

6.2. One dimensional HPLC-MS of phenolic and flavonoid compounds and wine samples

6.2.1. Sample preparation

8 samples of wine from Greece and France were prepared. They were dissolved in water: acetonitrile 1:1 and then diluted with acetonitrile until the concentration of 1mg/ml then filtrated with cellulose filters and transferred to HPLC vials. To control the system reproducibility there were also used 5 standards: 2-(p-hydroxyphenyl) ethanol, ethyl gallate, gallic acid, (-) - epicatechin, and caffeic acid. Standards were dissolved with water: acetonitrile 1:1 and further dilution with water.

6.2.2. Parameters

The parameters for these methods were:

Column: Kinetex 2.6 μm C18 100 A 30*4.60 mm

Pre-column: Security Guard cartridges C8 4x 3.0 mm

Flow rate: setups 4 & 5 0.200 ml/min, setups 6, 7, 8 & 9 0.500 ml/min

Mobile phase: Water: Acetonitrile (A: B)

Column temperature 40 ^{o}C (check)

Ionization: Electrospray

MS: Positive – Negative Ionization

Heat block Temperature 200 ^{o}C.

Table 5: Parameters for the separation of phenolic and flavonoid compounds with Kinetex 2.6 µm C18 100 A 30*4.60 mm and Security Guard cartridges C8 4x 3.0 mm

setup a			setup b			setup c			setup d		
time (min)	%A	%B	Time (min)	%A	%B	time (min)	%A	%B	Time (min)	%A	%B
0	95	5	0	80	20	0	80	20	0	95	5
4.00	95	5	1.00	80	20	1.00	80	20	1.00	95	5
10.00	60	40	10.00	5	95	6.00	30	70	8.00	60	40
11.00	5	95	12.00	5	95	8.00	30	70	10.00	60	40
12.00	5	95	12.01	80	20	10.01	80	20	12.00	95	5
12.01	95	5	16.00	80	20	10.02	80	20	12.02	95	5
17.00	95	5									

6.2.3. Results and discussion

Further research has been done with the 5 standards (2-(p-hydroxyphenyl) ethanol, ethyl gallate, gallic acid, (-) - epicatechin, and caffeic acid) at a concentration level for 10 µg/ml setups a & b µg/ml and 1mg/ml for setups c, & d.

Setup a

The mobile phase was water and acetonitrile, the injection volume 10µl . The initial concentration was 5% B. It was stable for four minutes. At the next six minutes a gradient separation was applied with the mobile phase composition changing to 40% B and in one minute changed to 95% B and remain stable for one minute. After that the composition of the mobile phase went back to 5% of B. The measurement has been done in seventeen minutes. This setup has taken place with standards at the concentration of 5 µg/ml and 20 µg/ml. There was no MS signal.

Setup b

The initial concentration of acetonitrile changed to 20% which was constant for one min. Then a gradient separation occurred and the acetonitrile was increased to 95% in 9 minutes and after two minutes decreased at 20%. The concentration of the standards was 1mg/ml. The injection volume was 5µg/ml. A high amount of the compound went to the detector and the chromatogram at the negative

ionization was saturated. In this setup we had very high ppm difference in the prediction formula. The ethyl gallate had the worst difference 9.64 ppm. And the best difference had caffeic acid with -3.31ppm. An acceptable ppm difference should be in the range 0, 00 to 3, 00 ppm an sometimes at 4, 00 ppm otherwise the results are not acceptable (Table 6).

Table 6: Negative Ionization MS at Ret. Time: 3.290 -> 3.610 min. Ethyl gallate

Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Diff (mDa)	Diff (ppm)	Iso Score
1	44.08	C_{10} H_6 N_4 O	[M-H] -	197.0474	197.0469	0.5	2.54	45.84
2	26.96	C_9 H_{10} O_5	[M-H] -	197.0474	197.0455	1.9	9.64	61.83

Setup c

The setup was similar to setup 5. But now the injection volume was 1µl because we had saturated MS furthermore the gradient went down to 70% B and then went down to 20% until 20min and then went back directly to 95% B. The whole measurement was 10 minutes. Apart from the standards also wine samples were measured.

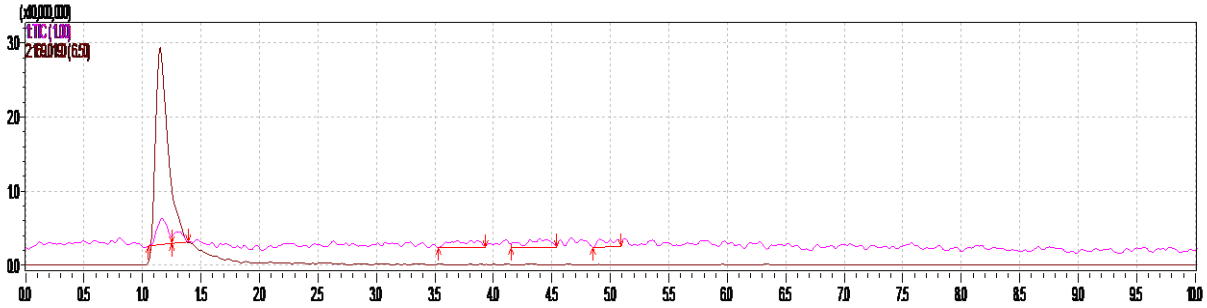


Figure 18: LC-chromatogram of 10 mg/ml gallic acid with Hilic column EC 125/2 Nucleodur 100-3, 95% ACN, flow rate: 1.20 ml/min, run time: 7min

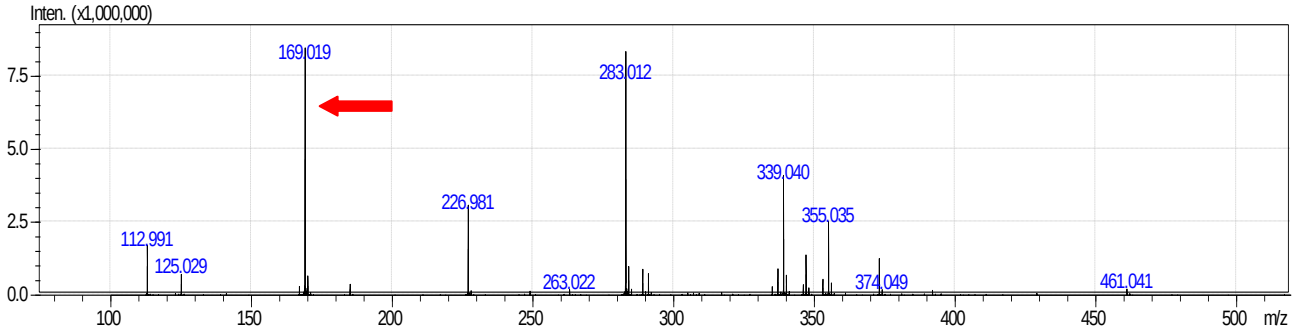


Figure 19: Negative Ionization MS of 10 mg/ml gallic acid (red arrow) with Hilic column EC 125/2 Nucleodur 100-3, 95% ACN, flow rate: 1.20 ml/min, run time: 7min

The samples didn´t have any clear signal in the MS spectrum. The retention times are shown on the Table 7:

Table 7: retention times of 2-(p-hydroxyphenyl) ethanol, ethyl gallate, gallic acid, (-)- epicatechin, and caffeic acid with Hilic column EC 125/2 Nucleodur 100-3, 95% ACN, flow rate: 1.20 ml/min, run time: 7min

Standard compounds	Retention Time (min)
2-(p-hydroxyphenyl) ethanol	2,48
ethyl gallate	2,45
gallic acid	1,06
(-)- epicatechin	1,08
caffeic acid	1,23

For the wine samples the separation of the compounds wasn´t successful since the entire mixture eluted between 0.730 min and 1.780 min.

Setup d

The initial concentration of acetonitrile was kept stable at 5% at the first minute. Then a gradient separation has occurred and we went up to 40% B in seven minutes and after two minutes the concentration of B decreased at 5%. The concentration of the standards was 1mg/ml. The injection volume was 5µg/ml. Only the (-)- epicatechin gave reasonable results both in MS and in UV spectrum. It has high isotope score (88,10%) and low ppm difference (3,11 ppm). However the other standards did´t have the same success. So, there was no sense to run the wine sample as well.

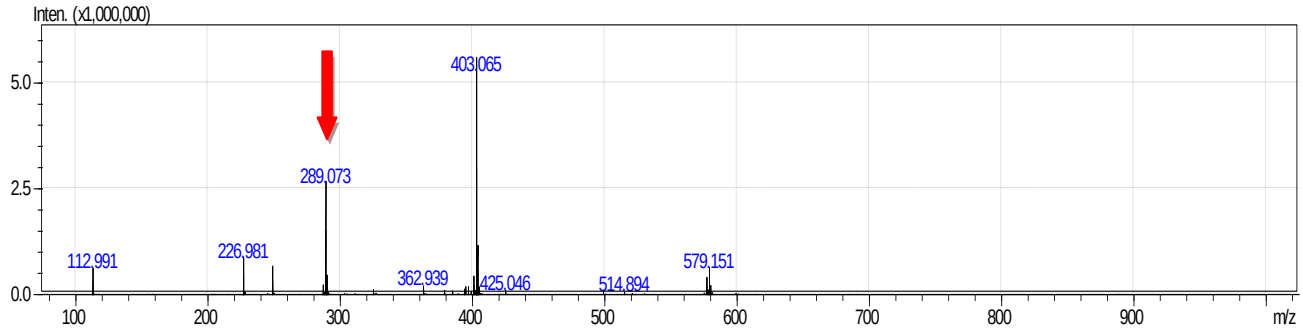


Figure 20: Negative ionization MS at Ret. Time : 5.690 - 5.880 min (-)- epicatechin (red arrow) with setup d.

Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Diff (mad)	Diff (ppm)	Iso Score
1	83.46	C15 H14 O6	[M-H] -	289.0727	289.0718	0.9	3.11	88.10

Figure 21: Negative ionization MS ([M-H]-) at Ret. Time: 5.690 - 5.880 min (-)- epicatechin (red arrow) with setup d

6.3. One dimensional HPLC of phenolic and flavonoids compounds

6.3.1. Sample preparation

We used 5 standards: 2-(p-hydroxyphenyl) ethanol, ethyl gallate, gallic acid, (-)- epicatechin, and caffeic acid. Standards were dissolved with water: acetonitrile 1:1 and further dilution with acetonitrile. The 5 solutions of standards (100 µg/ml): 2-(p-hydroxyphenyl) ethanol, ethyl gallate, gallic acid, (-) - epicatechin, and caffeic acid were diluted with acetonitrile to obtain 10 µg/ml of polyphenols. This concentration has been used for the following setups: 15-17 and 29-33. For the other setups, 1-14, and 18-28 the phenolic and flavonoids concentration was 100 µg/ml.

6.3.2. Parameters

Column: Kinetex 2.6 µg C18 100A 30*4.60mm

Kinetex 2.6u PFP 100A 50*4.60mm

Hilic column EC 125/2 nucleodur 100-3 Hilic

Mobile phase: water: Acetonitrile (A: B)

Column temperature 30 ^{o}C

6.3.3. Method A

Further research and development has been done in order to find the optimum column for the 1st dimension separation of the 5 standards (2-(p-Hydroxyphenyl) ethanol, Ethyl gallate, Gallic acid, (-)- Epicatechin, and Caffeic acid) at concentration of 100 µg/ml. The Kinetex 2.6 µm C18 100A 30*4.60 mm column for setups 1-3,8-9 and 23-28 and the Hilic column EC 125/2 nucleodur 100-3 Hilic for setups 4-7, 10-22 and 29-30. The parameters are discripted on Table 8. The column was always equilibrated with the initial gradient mobile phase.

Table 8: parameters for the separation of 100 µg/ml phenolic and flavonoid compounds with Kinetex 2.6 µg C18 100A 30*4.60mm and Hilic column EC 125/2 nucleodur 100-3 Hilic

	Column	Total flow ml/min	Time (min)	%A	%B	Injection volumn µl
Setup 1	Kinetex 2.6 µm C18 100 A. 30 mm x 4.60 mm	2.00	1.45	80.00	20.00	10
Setup 2		2.00	2.00	88.00	12.00	10
Setup 3		2.00	1.45	90.00	10.00	10
Setup 4	Hilic column EC 125/2 nucleodur 100-3 Hilic	2.00	10.00	5.00	95.00	10
Setup 5		2.00	10.00	15.00	85.00	10
Setup 6		2.00	10.00	25.00	75.00	10
Setup 7		1.20	10.00	15.00	85.00	10
Setup 8	Kinetex 2.6 µm C18 100 A 30*4.60 mm	2.00	1.00	70.00	30.00	10
Setup 9		2.00	1.00	75.00	25.00	10
Setup 10	Hilic column EC 125/2 nucleodur 100-3 Hilic	2.00	5.00	5.00	95.00	10
Setup 11		1.20	7.00	5.00	95.00	10
Setup 12		1.00	10.00	5.00	95.00	10
Setup 13		0.50	10.00	5.00	95.00	10
Setup 14		0.10	30.00	5.00	95.00	10
Setup 18		2.00	3.00	5.00	95.00	10
Setup 19		1.20	4.00	5.00	95.00	10
Setup 20		1.00	5.00	5.00	95.00	10
Setup 21		0.50	15.00	5.00	95.00	10
Setup 22		0.10	40.00	5.00	95.00	10
Setup 23	Kinetex 2.6 µm C18 100 A 30*4.60 mm	1.00	2.50	80.00	20.00	100
Setup 24		1.00	2.50	85.00	15.00	100
Setup 25		1.00	2.00	90.00	10.00	100
Setup 26		0.50	2.50	90.00	10.00	100
Setup 27		0.50	2.00	95.00	5.00	100
Setup 28		0.50	2.00	100.00	0.00	100
Setup 29	Hilic column EC 125/2 nucleodur 100-3 Hilic	0.05	70.00	100.00	0.00	10
Setup 30		0.10	50.00	100.00	0.00	10

6.3.4. Method B

To check witch column could be used as a 2nd dimension column a series of experiments had been done with Kinetex 2.6 µg C18 100 A 30*4.60mm for setups 15-17 and with Kinetex 2.6 µg PFP 100 A 50*4.60 mm for setups 31-33. The parameteres for each column where discripted on **Table 9**. The columns where tested for the separation of the 5 phenolic and flavonoids standards, concentration 10 µg/ml each.

Table 9: parameters for the separation of the 5 phenolic and flavonoid compounds, in the concentration of 10 µg/ml with Kinetex 2.6 µg C18 100 A 30*4.60 mm and Kinetex 2.6 µg PFP 100 A 50*4.60 mm

	Column	Total flow ml/min	Time (min)	%A	%B	Injection volumn µl
Setup 15	Kinetex 2.6 µm C18 100 A 30*4.60 mm	1.00	1.00	100.00	0.00	100
Setup 16		1.00	1.00	95.00	5.00	100
Setup 17		1.00	1.00	90.00	10.00	100
Setup 31	Kinetex 2.6 µm PFP 100A 50*4.60mm	1.00	2.00	100.00	0.00	100
Setup 32		0.50	2.00	100.00	0.00	100
Setup 33		0.30	3.00	100.00	0.00	100

6.3.5. Results and discussion

6.3.5.1. Method A

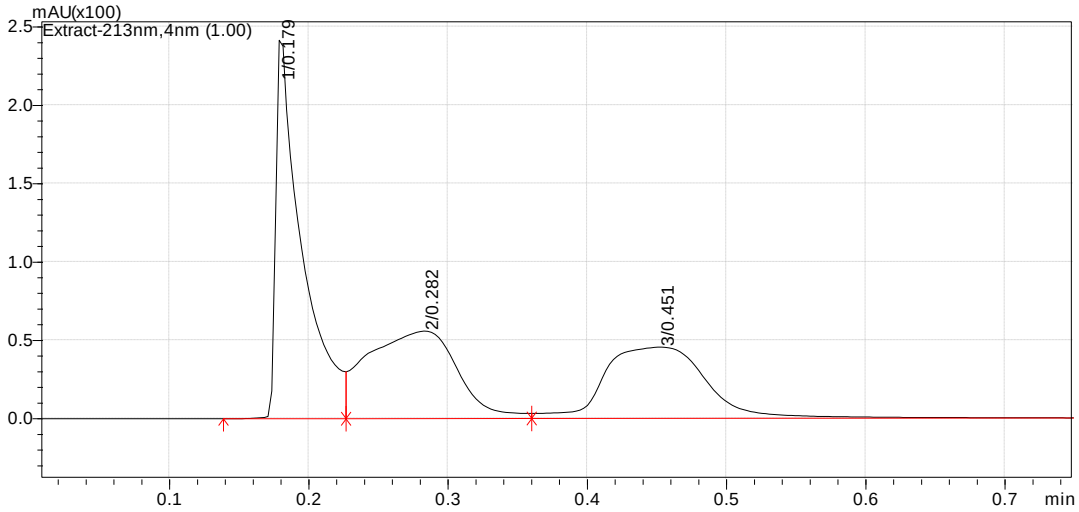


Figure 22: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Kinetex 2.6 µm C18 100 A 30*4.60 mm column. 20% ACN. flow rate: 2.00ml/min

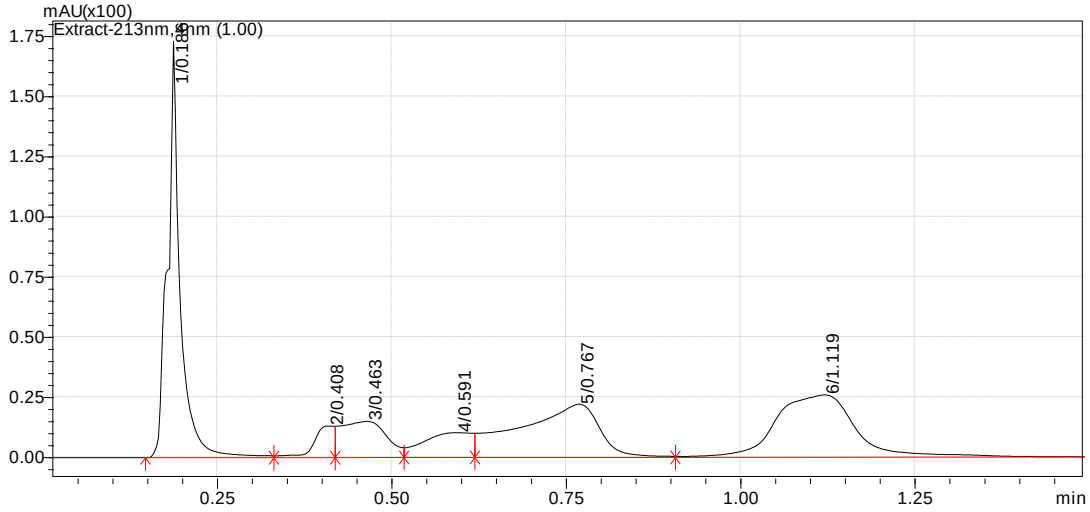


Figure 23: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Kinetex 2.6 µm C18 100 A 30*4.60 mm column. 12% ACN. flow rate: 2.00ml/min

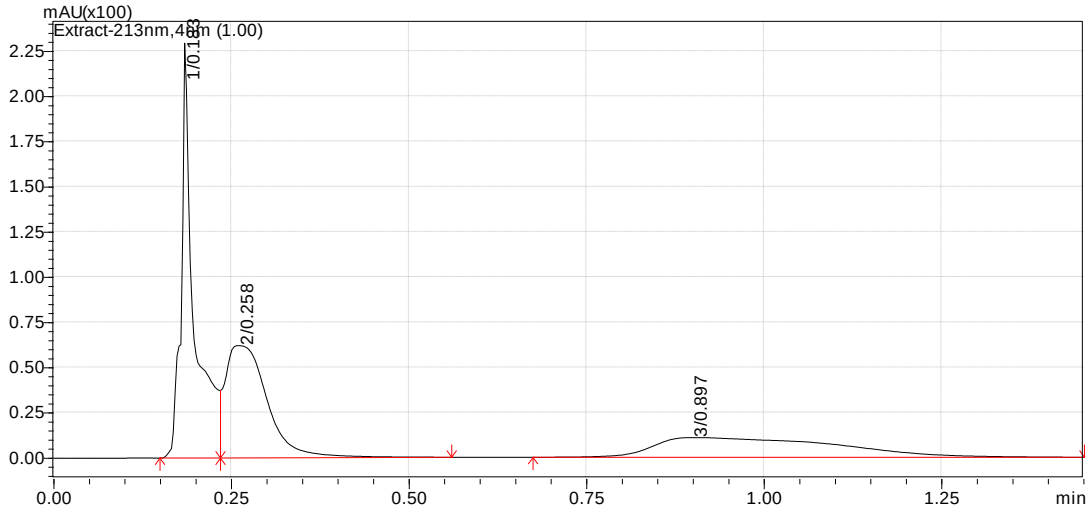


Figure 24: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Kinetex 2.6 µm C18 100 A 30*4.60 mm column. 10% ACN. flow rate: 2.00 ml/min

With 20% ACN as mobile phase the peaks of 2-(p-hydroxyphenyl) ethanol and (-)-epicatechin were broad and co – elute so it was impossible to separate them. Decreasing the ACN concentration to 12% the problem increased. By decreasing more the ACN to 10% the peaks became broader and the resolution of the peaks became deteriorate. A gradient separation should be considered for future research.

The separation with Hilic column was promising seems the mixture of the standards were separated. Next step was to optimize the parameters of this method. So several experiment were done.

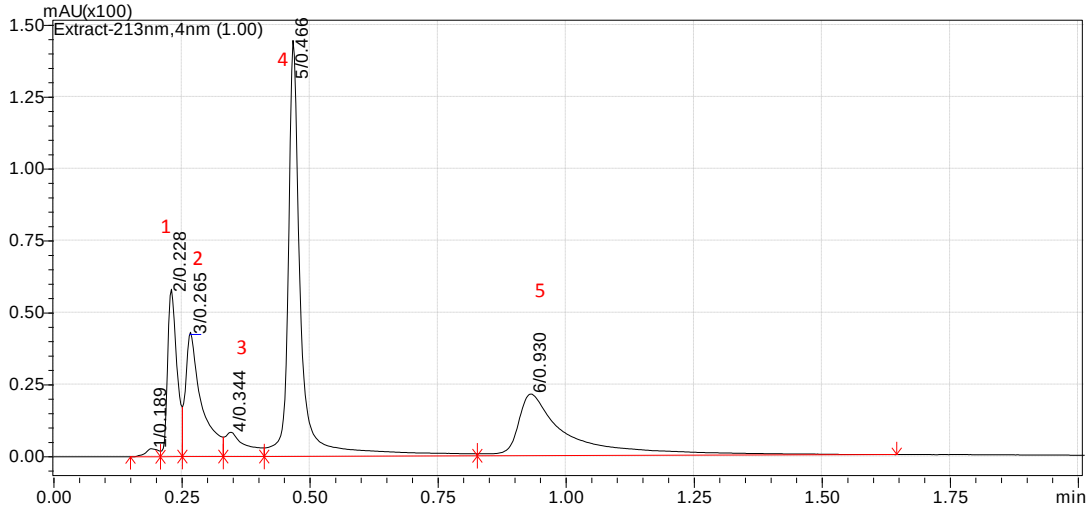


Figure 25: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3 Hilic. 95% ACN. flow rate: 2.00 ml/min. 1) gallic acid 2) (-)- epicatechin 3) caffeic acid 4) Ethyl gallate and 5) 2-(p-hydroxyphenyl) ethanol.

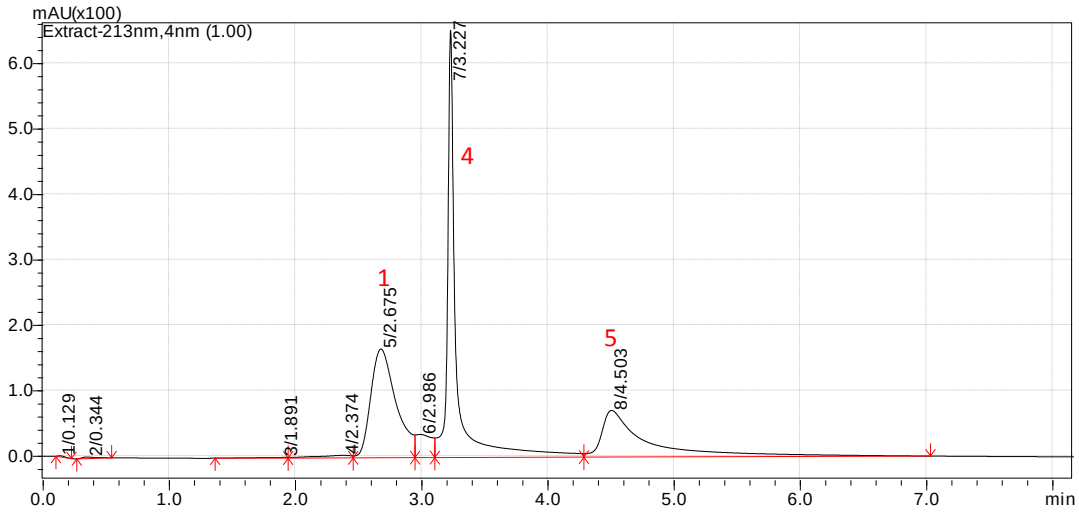


Figure 26: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3 Hilic. 85% ACN. flow rate: 2.00 ml/min. 1) gallic acid 2) (-)- epicatechin 3) caffeic acid 4) Ethyl gallate and 5) 2-(p-hydroxyphenyl) ethanol

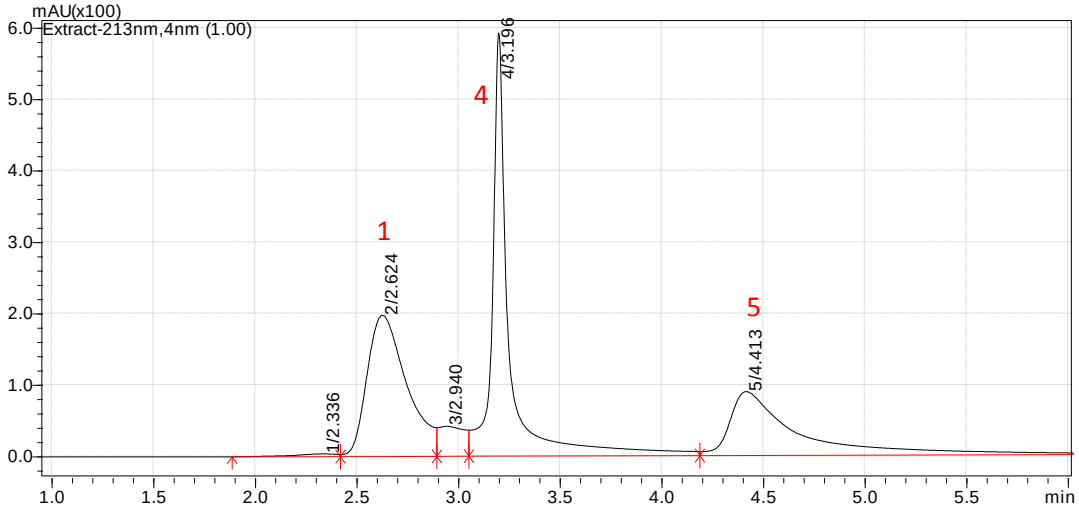


Figure 27: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3 Hilic. 75% ACN. flow rate: 2.00 ml/min. 1) gallic acid 2) (-)- epicatechin 3) caffeic acid 4) Ethyl gallate and 5) 2-(p-hydroxyphenyl) ethanol

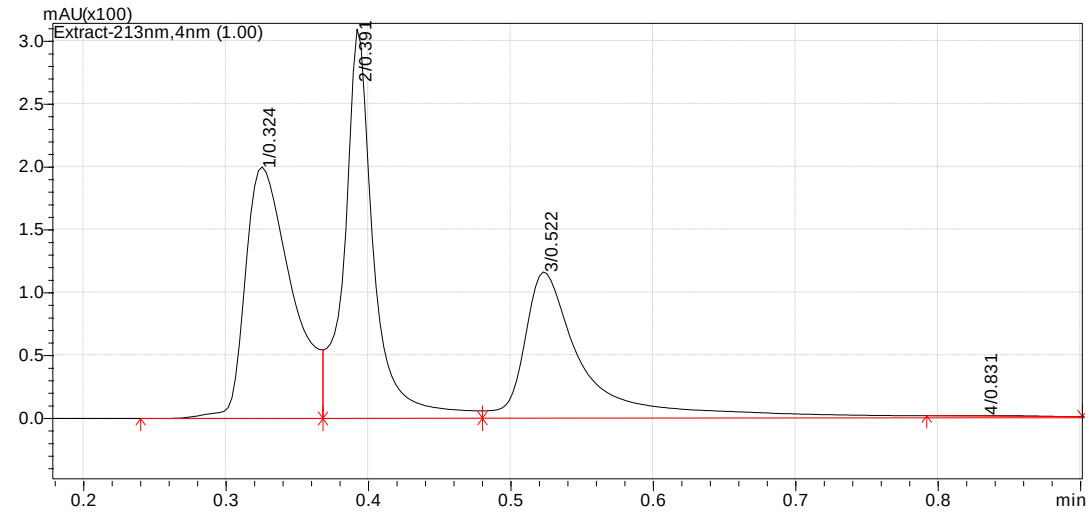


Figure 28: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3 Hilic. 85% ACN. flow rate: 1.20 ml/min. 1) gallic acid 2) (-)- epicatechin 3) caffeic acid 4) Ethyl gallate and 5) 2-(p-hydroxyphenyl) ethanol

With 95% ACN as mobile phase the resolution of the compounds was satisfactory. We try to increase the resolution by decreasing the presentence of ACN in the mobile phase. As a result the elution time has been tripled increased but the resolution between the peaks deteriorated.

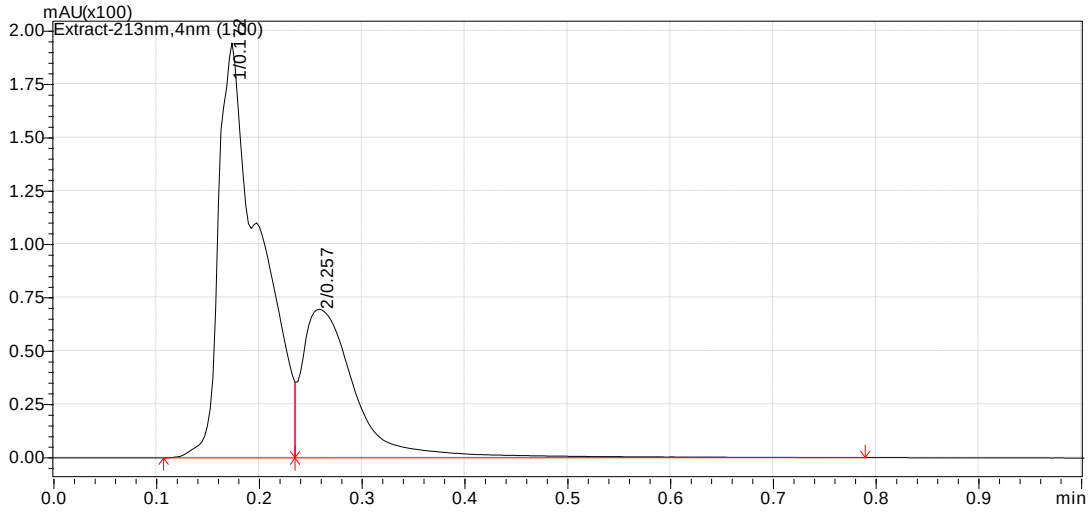


Figure 29: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Kinetex 2.6 µm C18 100 A 30*4.60 mm column. 30% ACN. flow rate: 2.00ml/min

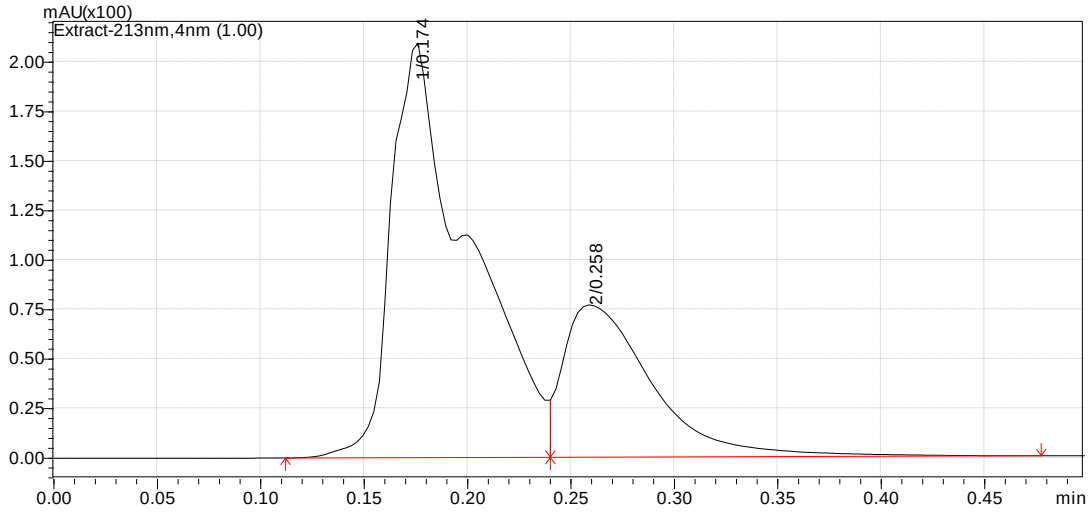


Figure 30: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Kinetex 2.6 µm C18 100 A 30*4.60 mm column. 25% ACN. flow rate: 2.00ml/min

With increasing the concentration of water in the mobile phase increase also the resolution of the polyphenols.

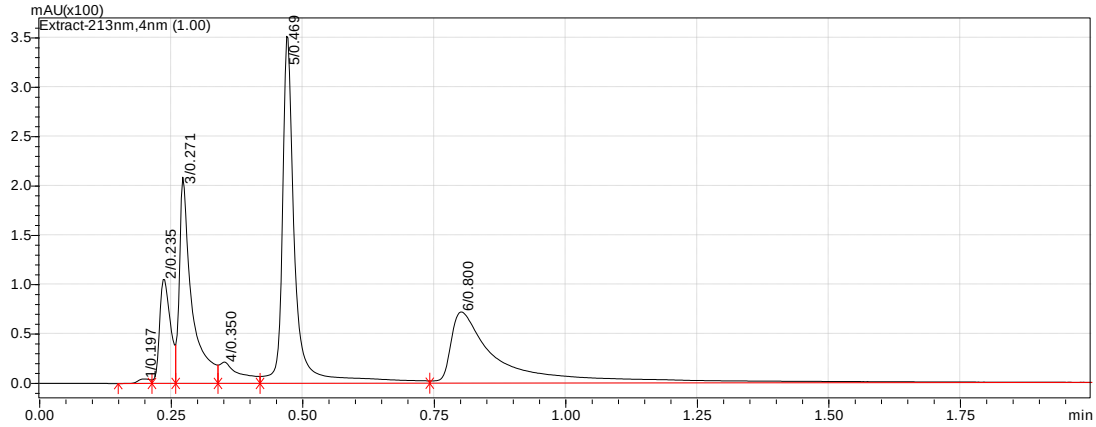


Figure 31: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 95% ACN. flow rate: 2.00ml/min. run time: 5min.

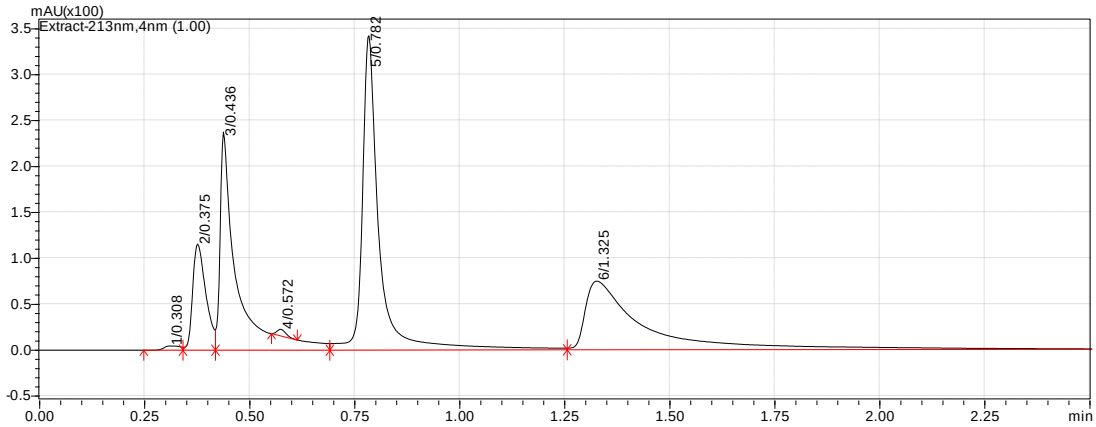


Figure 32: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 95% ACN. flow rate: 1.20 ml/min. run time: 7min.

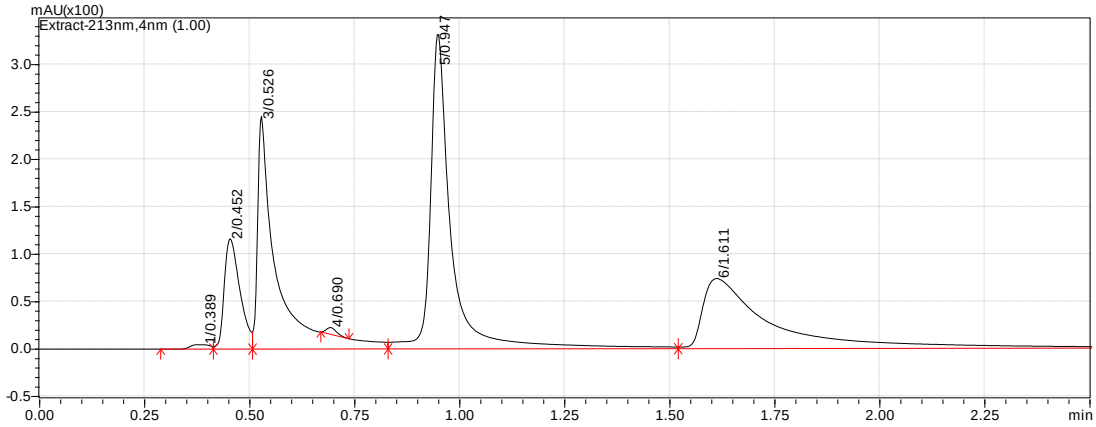


Figure 33: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 95% ACN. flow rate: 1.00 ml/min. run time: 10min

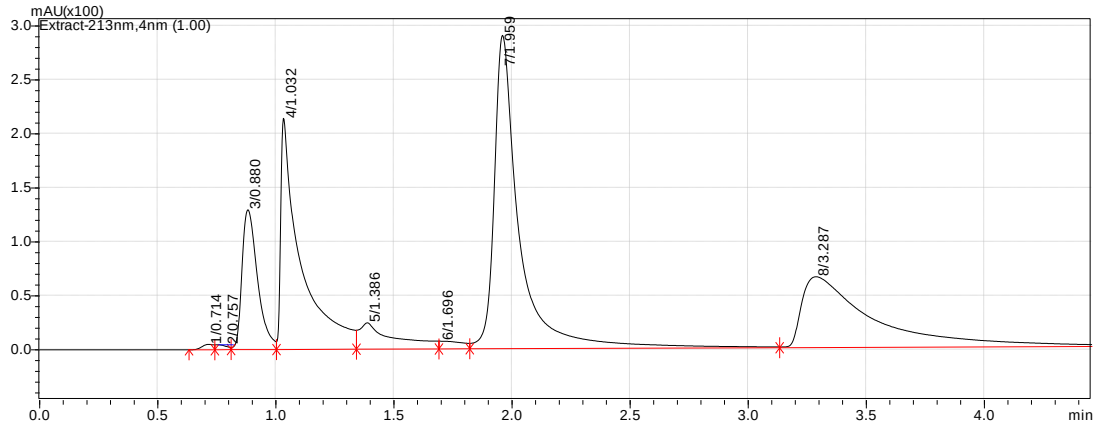


Figure 34: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 95% ACN. flow rate: 0.50 ml/min. run time: 10min.

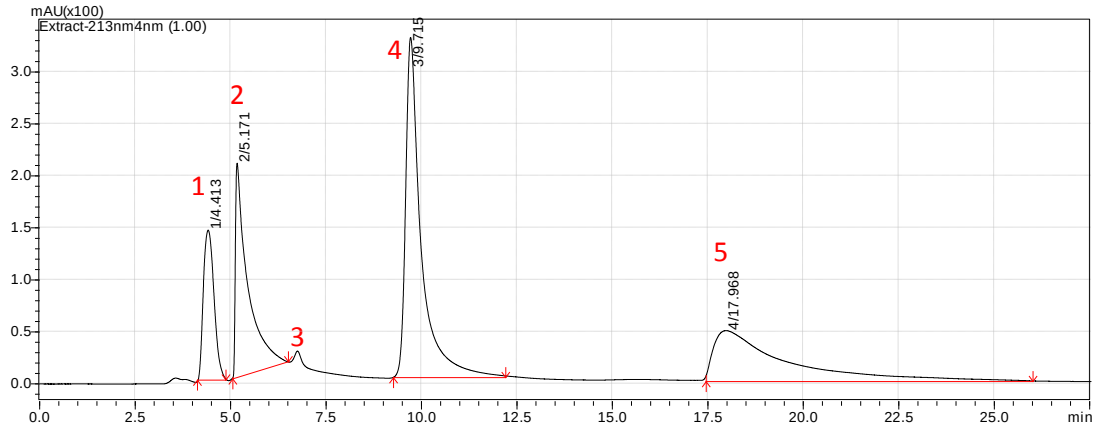


Figure 35: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 95% ACN. flow rate: 0.10 ml/min. run time: 30min. 1) gallic acid 2) (-)-epicatechin 3) caffeic acid 4) Ethyl gallate and 5) 2-(p-hydroxyphenyl) ethanol

Nice separation has occurred for these standards and also through small flow rates there was the desired result. By decreasing the flow rate of the mobile phase increases the time of the analyses. The long Hilic column could be used in comprehensive LCxLC system as the first dimensions column. So the optimum parameters for the 1st dimension column were: Hilic column EC 125/2 nucleodur 100-3, mobile phase 95% ACN- 5% H_2O and flow rate: 0.100 µg/ml

Table 10: Retention times of 2-(p-hydroxyphenyl) ethanol. ethyl gallate. gallic acid. and (-)- epicatechin

Standards	Retention time (min)
2-(P-HYDROXYPHENYL) ETHANOL	0.881
ETHYL GALLATE	1.025
(-)- EPICATECHIN	1.945
GALLIC ACID	3.208

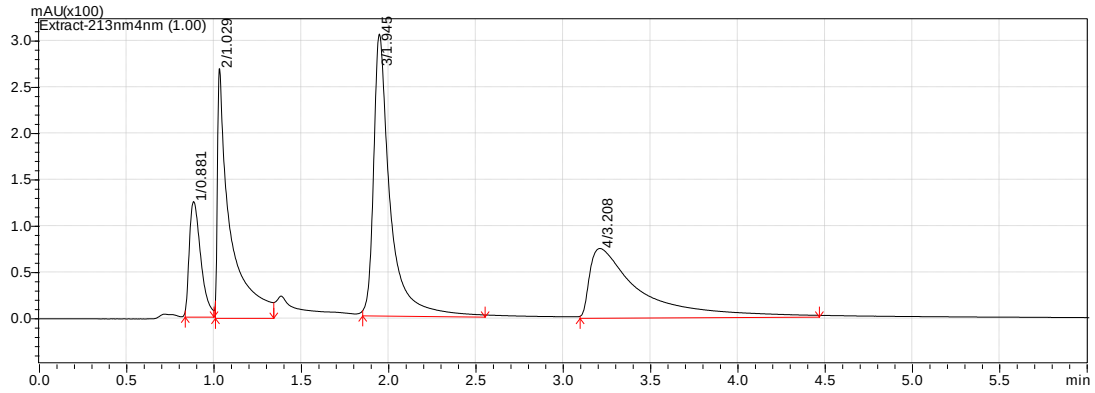


Figure 36: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoids standard with Hilic column EC 125/2 nucleodur 100-3. flow rate: 0.50 ml/min. run time:15.00 min.

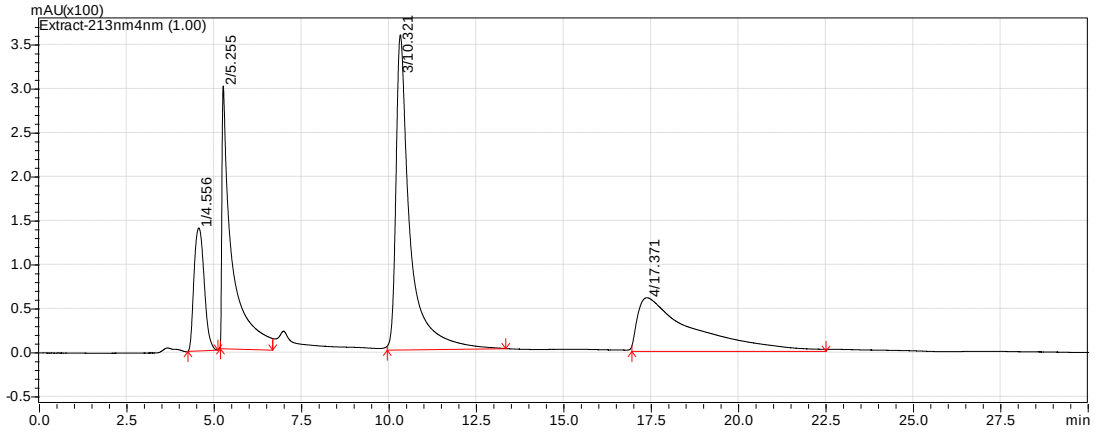


Figure 37: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards Hilic column EC 125/2 nucleodur 100-3. flow rate: 0.10 ml/min. run time: 40.00 min.

The hilic column could be used as 2nd dimension column but then a longer column C18 should be used for the first dimension.

Also the 1D column should be long with small diameter and should be operated at low flow rates 40µl/min-100µl/min. because the loop has very small capacity and it should be filled with eluent coming out of the 1D column. During the time that the chromatographic separation in the 2D column takes place. The 2D column should be short with big diameter and should be operated at higher flow rates 1-3ml/min so that the 2D chromatographic separation analysis will be over within 1-2 minutes. And this is something that can be studied in the feature.

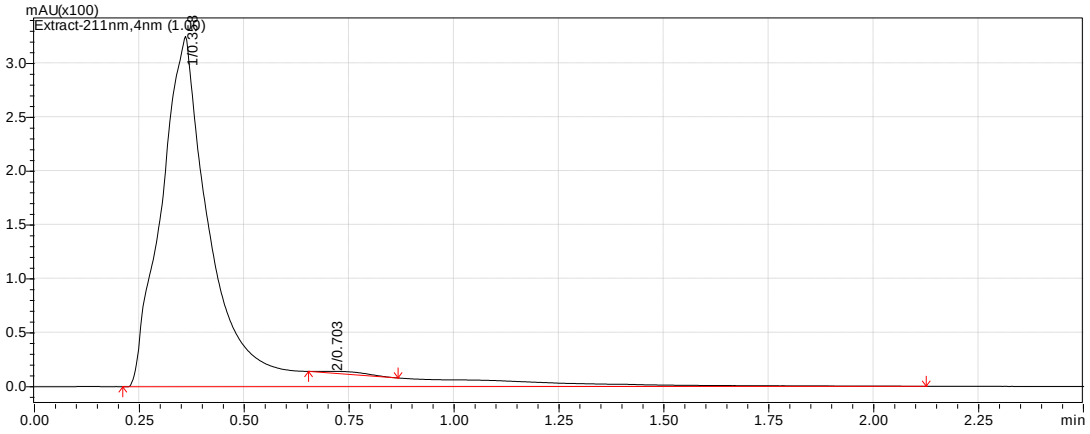


Figure 38: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoids standard with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 20% ACN. flow rate: 1.00 ml/min. run time: 2.50 min.

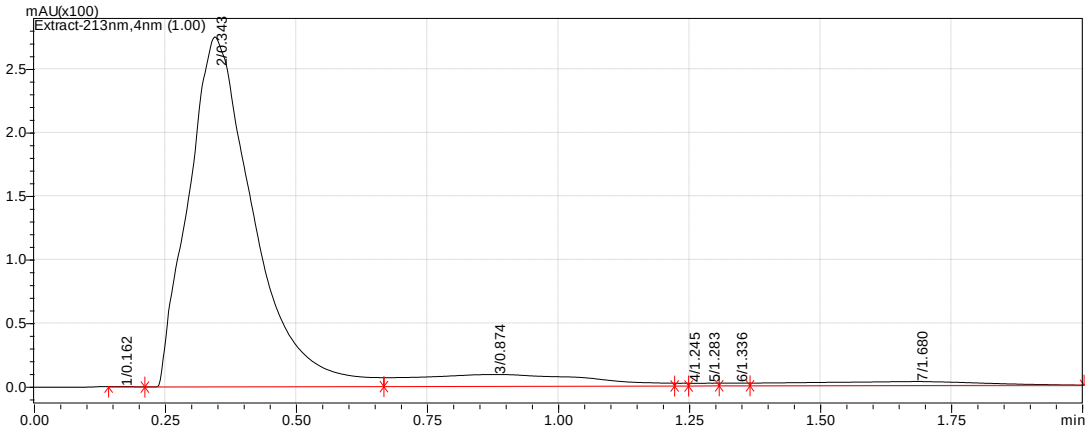


Figure 39: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 15% ACN. flow rate: 1.00 ml/min. run time: 2.50 min.

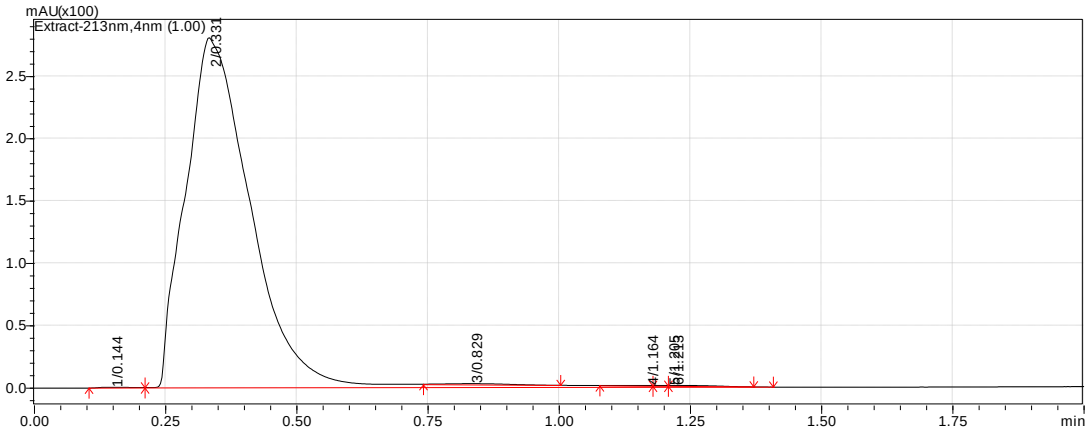


Figure 40: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 10% ACN. flow rate: 1.00 ml/min. run time: 2.00 min.

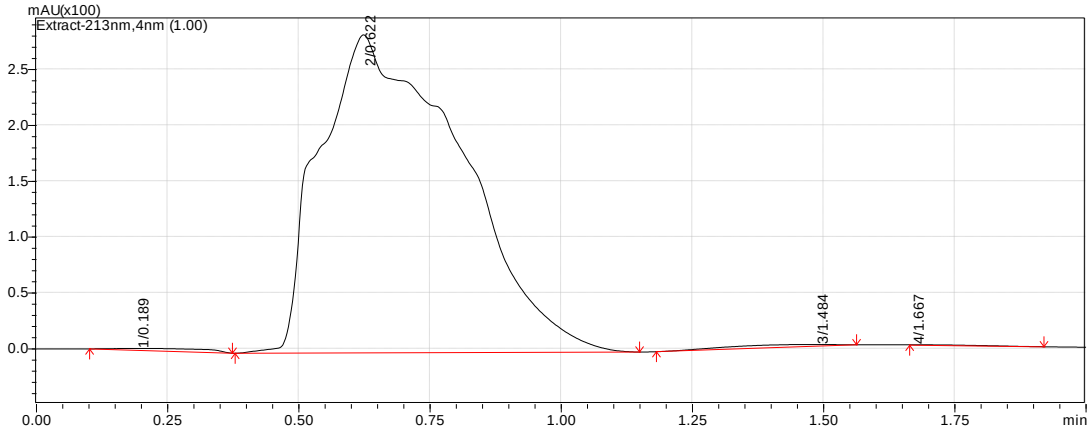


Figure 41: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 10% ACN. flow rate: 0.50 ml/min. run time: 2.50 min.

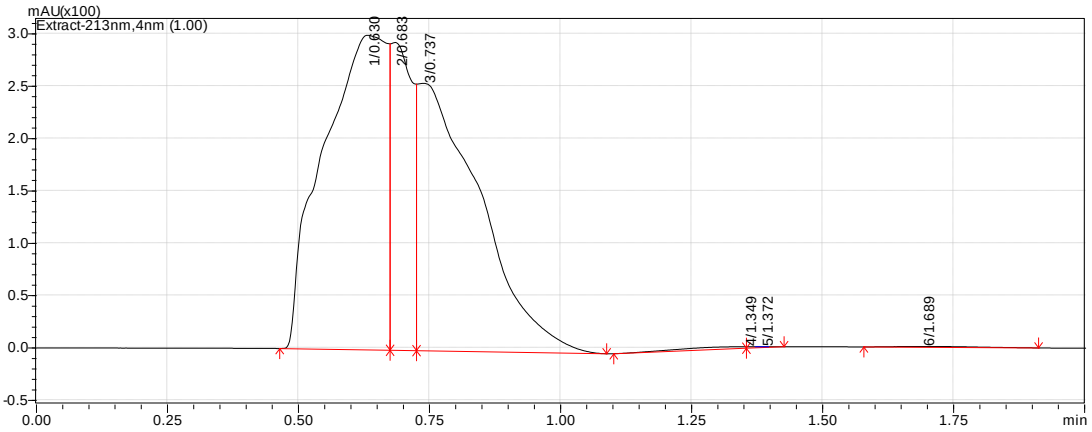


Figure 42: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 5% ACN . flow rate: 0.50 ml/min. run time: 2.00 min.

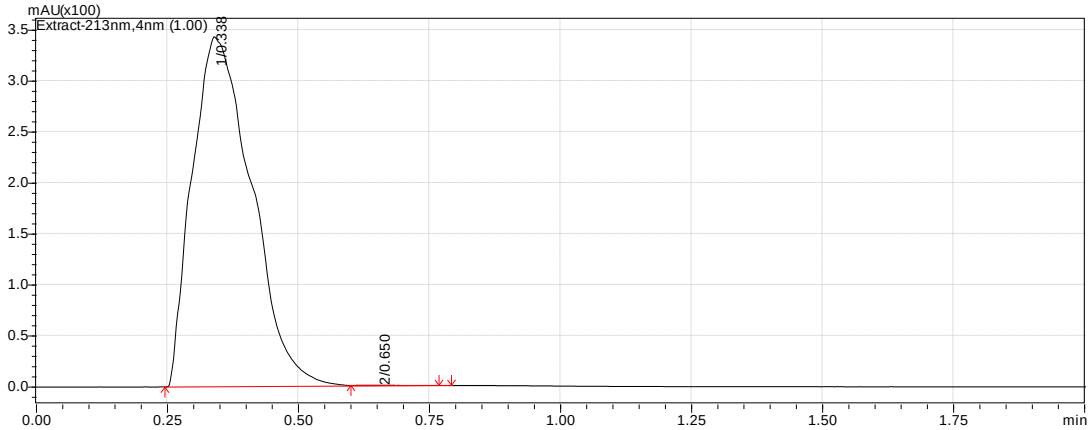


Figure 43: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 0% ACN. flow rate: 0.50 ml/min. run time: 2.00 min.

6.3.5.2. Method B

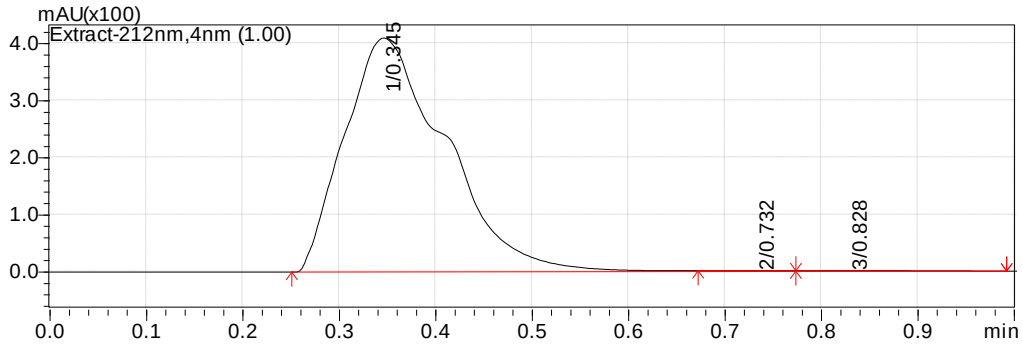


Figure 44: LC-chromatogram for the separation of 10 µg/ml phenolic and flavonoids standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 0% ACN.

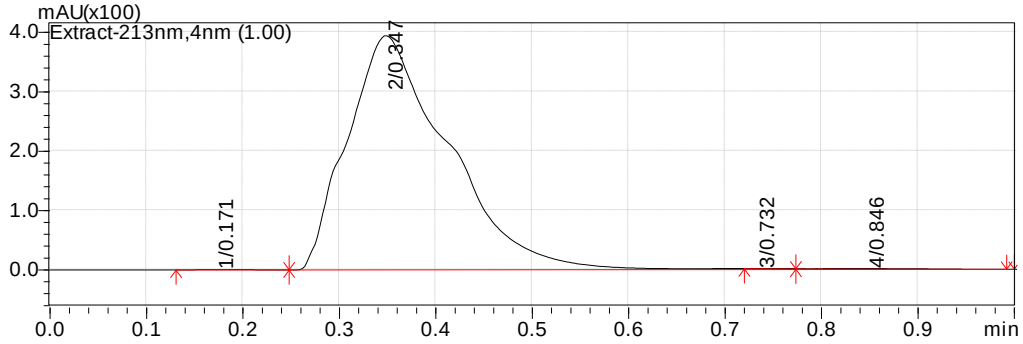


Figure 45: LC-chromatogram for the separation of 10 µg/ml phenolic and flavonoids standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 5% ACN.

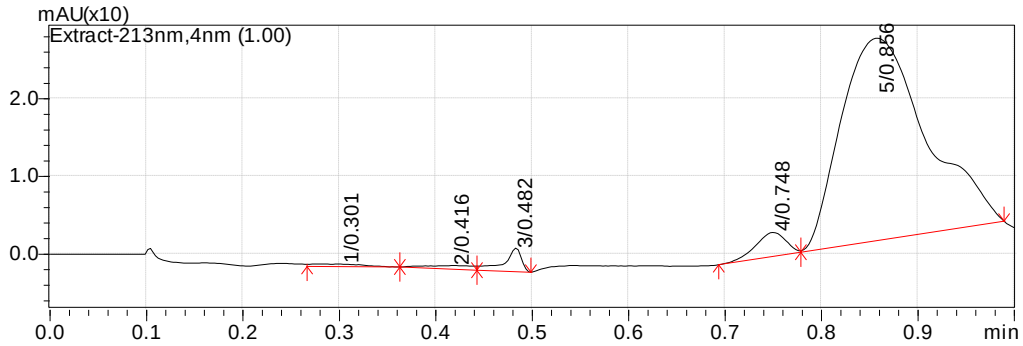


Figure 46: LC-chromatogram for the separation of 10 µg/ml phenolic and flavonoids standards with kinetex 2.6 µm C18 100 A 30*4.60 mm column. 10% ACN.

The LC- chromatograms were measured at 213 nm. With increasing the concentration of ACN in the mobile phase the whole mixture of phenolic and flavonoids standards was shifted but there was hardly any difference in the resolution of the compounds.

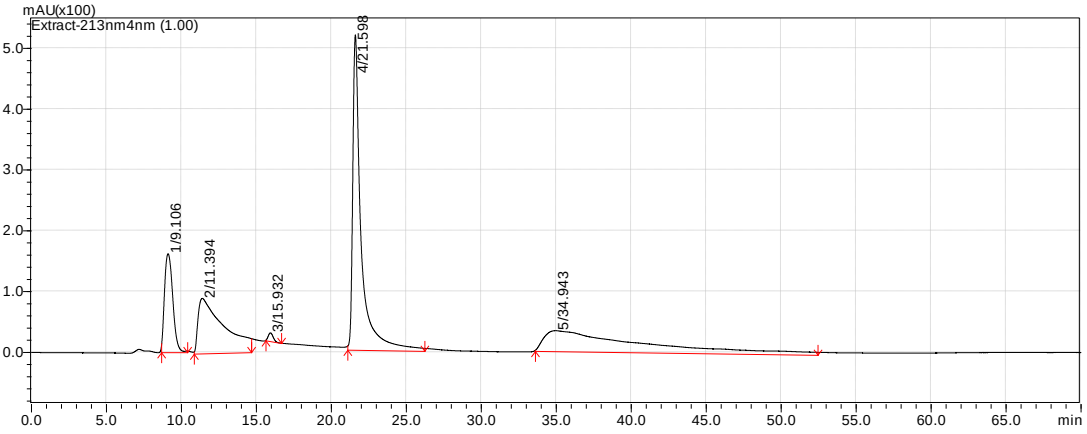


Figure 47: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 0% ACN. flow rate: 0.050 ml/min. run time: 70.00 min.

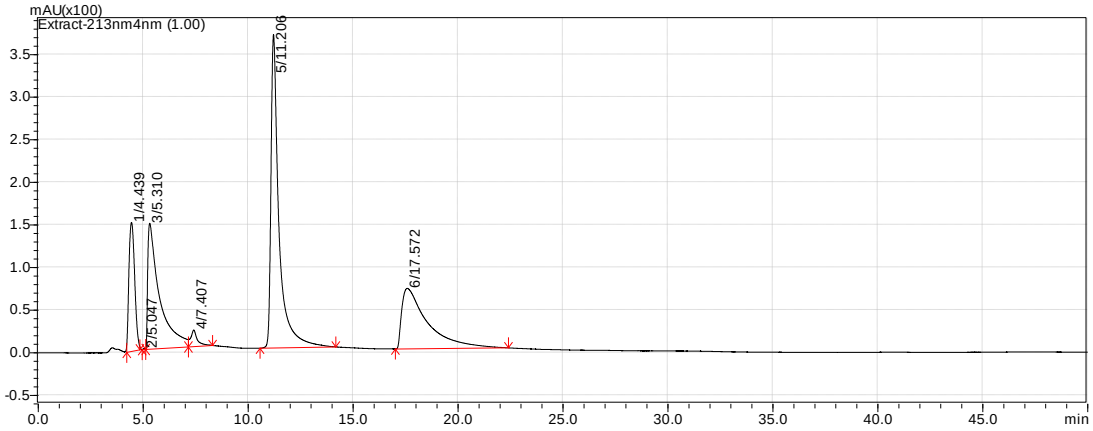


Figure 48: LC-chromatogram for the separation of 100 µg/ml phenolic and flavonoid standards with Hilic column EC 125/2 nucleodur 100-3. 0% ACN. flow rate: 0.10 ml/min. run time: 50.00 min

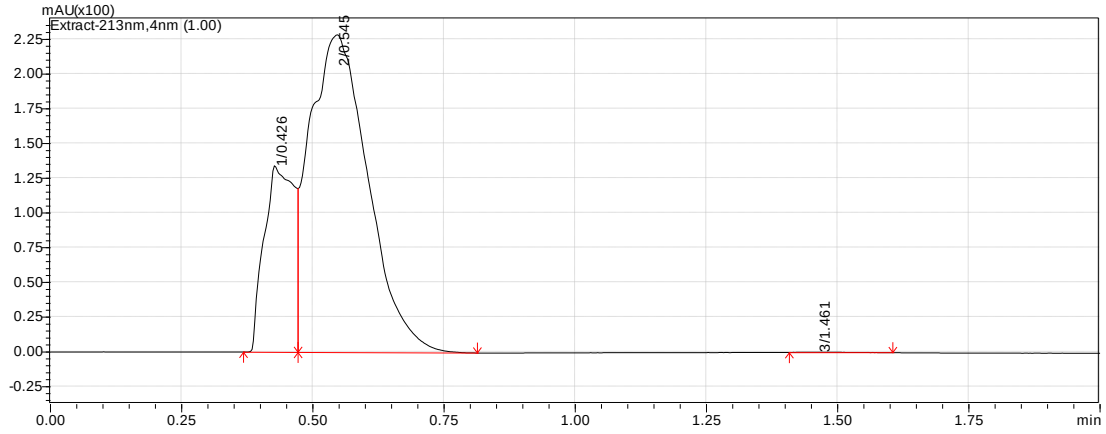


Figure 49: LC-chromatogram for the separation of 10 µg/ml phenolic and flavonoids standards with kinetex 2.6u PFP 100A 50*4.60mm column. 0% ACN. flow rate: 1.00 ml/min. run time: 2.00 min.

During the next step the compounds were separated with two dimension liquid chromatography. For the first dimension a long column was needed and for the second dimension short column was needed (setups I-III). For better separation the two columns should have different separation selectivity. After these experiments the system turned to two dimension system (see setups I-III)

6.4. Two dimensional HPLC of phenolic and flavonoids compounds

6.4.1. Sample preparation

There were used the 5 phenolic and flavonoids standards: 2-(p-Hydroxyphenyl) ethanol. Ethyl gallate. Gallic acid. (-) - Epicatechin. and Caffeic acid. Standards were dissolved with water: acetonitrile (1:9) to obtain 10 µg/ml of polyphenols.

6.4.2. Parameters

Columns:

1-D: Hilic column EC 125/2 nucleodur 100-3 Hilic (No 1)

2-D: Kinetex 2.6 µm C18 100 A 30*4.60 mm (No 2) or

Kinetex 2.6 µm C18 100 A 50*4.60 mm (No 3)

Mobile phase: water: acetonitrile (A: B)

Column temperature: 30 ^{o}C

Considerations before establishing the 2-D system:

Several considerations have to be made before establishing the two dimensional system. Since the loop volume has been selected. (In this study was 100µl). The flow rates and modulation time should be matched. The compound eluted from the first dimension should be sampled at least 3 or 4 times in order to maintain the highest overall 2-D separation resolution. This is achieved by employing low flow rates in the first dimension (broader peaks provide more sampling fractions for the second dimension) and fast flow rates (short modulation times) in the second [135]. The individual one dimensional chromatograms of each column should be examined.

6.4.3. Method I

All chromatographic separations were carried out at 30 ◦C. In the comprehensive LC×LC mode the whole effluent from the first dimension column (Hilic column EC 125/2 nucleodur 100-3 Hilic (No 1)) was transferred on-line to the second-dimension column (Kinetex 2.6 µm C18 100 A 30*4.60 mm (No 2)) in subsequent fractions via the 2x6-port switching valves equipped with 100µl sampling loops using the LC-20AB pump (Shimadzu). The 2x6-port valves were switched every 1 min. Three experimental comprehensive LC×LC setups with different composition mobile phase were compared. Before the running of each 2-D setup were equilibrated with the initial gradient mobile phase for 5 min with blank injection.

6.4.3.1. Setup I

In the first dimension was used the Hilic column (No. 1) with isocratic elution (0 min: 85% ACN; 100 min: 80% ACN; F = 0.1 mL/min). In the second dimension was employed a short Kinetex column (No. 2) with isocratic elution (0 min: 0% ACN; 100 min: 0% ACN; F = 2.0 mL/min).

6.4.3.2. Setup II

In the first dimension was used the Hilic column (No. 1) was used with isocratic elution (0 min: 85% ACN; 100 min: 85% ACN; F = 0.1 mL/min). In the second dimension was employed a short Kinetex column (No. 2) with isocratic elution (0 min: 0% ACN; 100 min: 0% ACN; F = 2.0 mL/min).

6.4.3.3. Setup III

In the first dimension was used the Hilic column (No. 1) was used with isocratic elution (0 min: 90% ACN; 100 min: 90% ACN; F = 0.100 mL/min). In the second dimension was employed a short Kinetex column (No. 2) with isocratic elution (0 min: 0% ACN; 100 min: 0% ACN; F = 2.000 mL/min).

6.4.4. Method II

All chromatographic separations were carried out at 30 °C. In the comprehensive LC×LC mode. the whole effluent from the first dimension column. (Hilic column EC 125/2 nucleodur 100-3 Hilic (No 1)) was transferred on-line to the second-dimension column (Kinetex 2.6 µm C18 100 A 50*4.60 mm (No 3)) in subsequent fractions via the 2x6-port switching valves equipped with 100µl sampling loops using the LC-20AB pump (Shimadzu). The 2x6-port valves were switched every 1 min. Two experimental comprehensive LC×LC setups with mobile phase of different composition were compared.

6.4.4.1. Setup IV

For the first dimension a Hilic column (No. 1) was used with isocratic elution (0 min: 95% ACN; 28 min: 95% ACN; F = 0.1 mL/min). In the second dimension. a short Kinetex column (No. 3) was employed with isocratic elution (0 min: 0% ACN; 28 min: 0% ACN; F = 1 mL/min). The injection volume was 10µl.

6.4.4.2. Setup V

For the first dimension a Hilic column (No. 1) was used with isocratic elution (0 min: 95% ACN; 52 min: 95% ACN; F = 0.05 mL/min). In the second dimension. a short Kinetex column (No. 3) was employed with isocratic elution (0 min: 0% ACN; 52 min: 0% ACN; F = 2 mL/min). The injection volume was 10µl.

6.4.5. Results and discussion

6.4.5.1. Method I

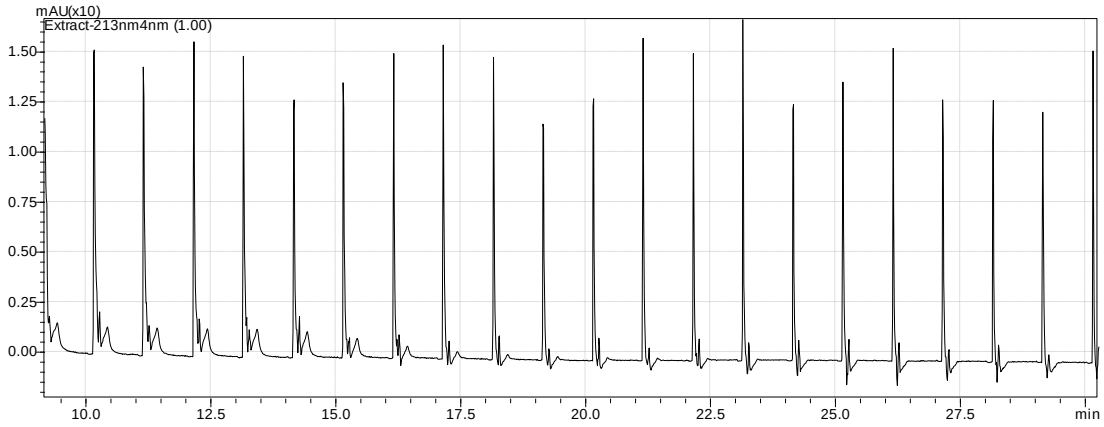


Figure 50: LC chromatogram of separation of 10 mg/ml phenolic and flavonoid standards with setup III with columns Hilic column EC 125/2 nucleodur 100-3 Hilic and Kinetex 2.6 µm C18 100 A 30*4.60 mm. F_1= 0.100ml/min and F_2= 2. 00 ml/min port valves switched every 1 min

The samples were eluted during the injections peak. So there was no separation. This could be because of the high concentration of ACN after the first column separation. The ACN decrease the polarity of the sampling injection and in the 2nd dimension the column has non polar stationary phase and as a result the retention time of the compounds decrease as well.

6.4.5.2. Method II

The samples were eluted after the injections peak (coloured arrows on Figure 51). It was increased the resolution strength but this wasn't enough for the separation of the mixture.

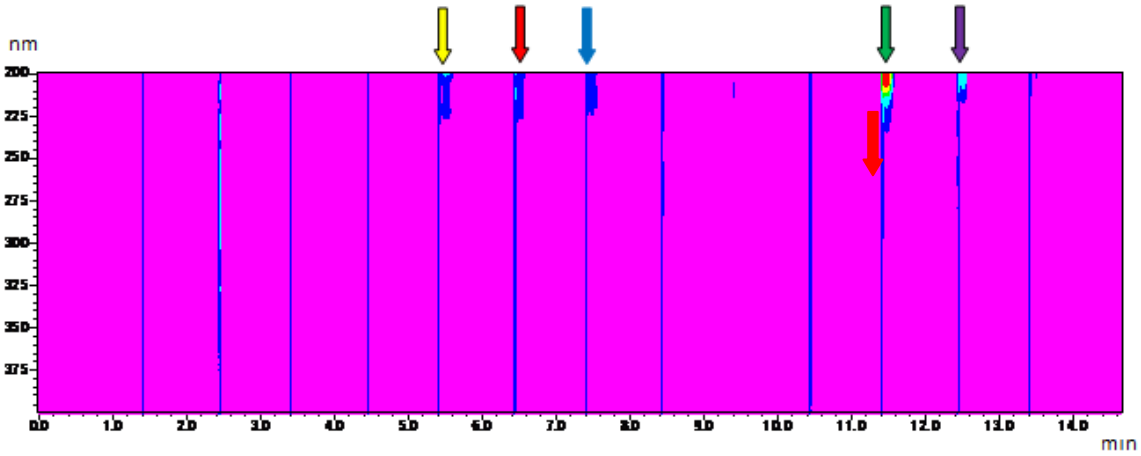


Figure 51: wavelength/time contour plot for the separation of 10 mg/ml phenolic and flavonoid standards with columns Hilic column EC 125/2 nucleodur 100-3 Hilic and Kinetex 2.6 µm C18 100 A 50*4.60 mm. F_1= 0.100ml/min and F_2= 2.000 ml/min port valves switched every 2 min (setup IV)

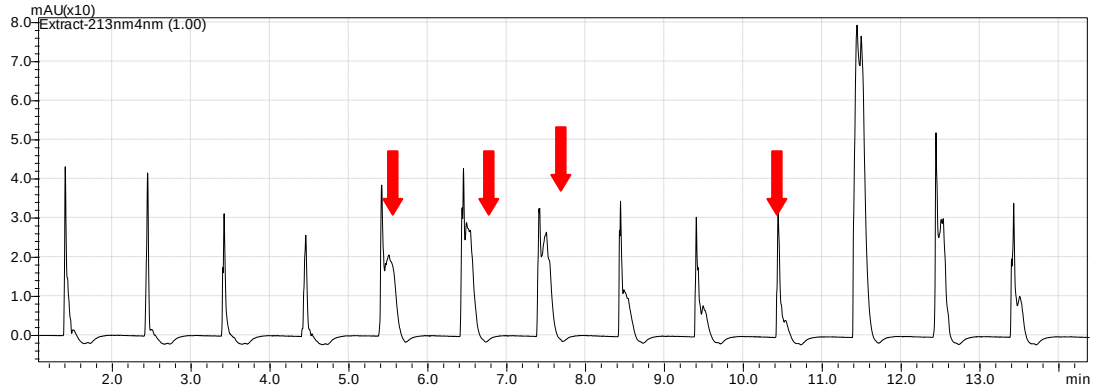


Figure 52: LC chromatogram of separation of 10 mg/ml phenolic and flavonoid standards with columns Hilic column EC 125/2 nucleodur 100-3 Hilic and Kinetex 2.6 µm C18 100 A 50*4.60 mm. F_1= 0.100ml/min and F_2= 2.000 ml/min port valves switched every 2 min (setup IV). The arrows indicated the elution of sample direct after the injection peaks

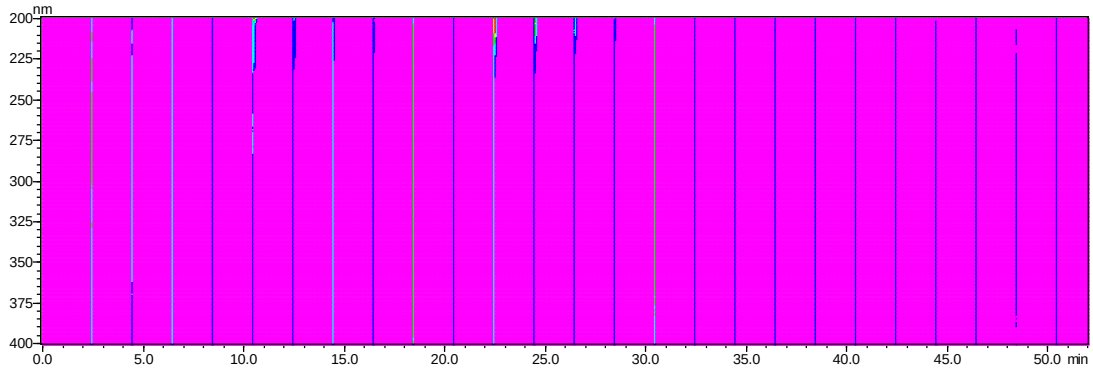


Figure 53: wavelength/time contour plot for setup V with columns No1 and No3. F_1=0.050ml/min and F_2=1.00 ml/min port valves switched every 1 min

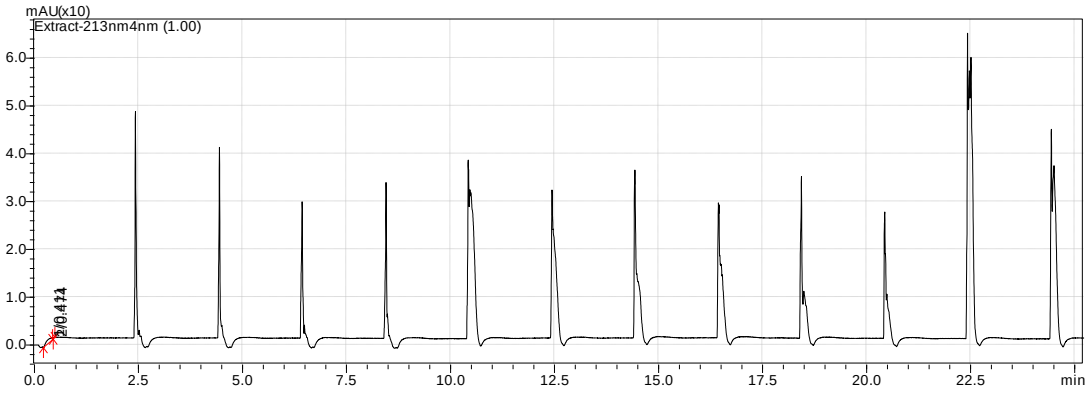


Figure 54: Liquid Chromatogram for setup V with columns No1 and No3. F_1=0.050ml/min and F_2=1.00 ml/min port valves switched every 2 min

In the setup IV there was a marginal separation of the standards compounds: There are some peaks direct after the injection peaks (Figure 51) but the peaks were hardly retained. In setup V the flow rate has been reduce in both dimension to increase the resolutions of the compounds. But the analyzed peaks got lost again in the injection peaks (Figure 53)

The problem could be that after the 1st dimension column the sample is highly concentrated in ACN so the mobile phase has similar polarity to the stationary phase of the kinetex C18 column and as result the resolution has been decreased.

6.4.5.3. Analysis of red wine Samples

The setup IV of the two dimensional system was applied to the analysis of red wine samples. The samples were diluted (as already been described in section 6.2.1) in acetonitrile filtrated and injected. None of the experiments provided an efficient separation of the phenolic compounds. In all cases there was co-elution of the compounds with the injection peaks.

7. Conclusion

Following a long series of experiments it has been proven that the phenolic- flavonoids standards could already be separated with the Hilic column EC 125/2 nucleodur 100-3 Hilic (No. 1) in one dimension with acceptable resolution using an isocratic mobile phase of 95% ACN. Despite the already successful separation in one dimension, a two dimensional HPLC separation was to be developed due to the fact that in the real samples there are high amounts of phenolic- flavonoid compounds, which may lead to co-elution. The 2D-HPLC as has already been mentioned in the theory increases the peak capacity of the chromatogram. To achieve this uncorrelated two dimensional system should be employed. In this study after a series of experiments the Kinetex column 2.6 µm C18 100A 50*4.60mm (No. 3) was chosen as the 2nd dimension column. The main problem concerning this method was that the after the 1st dimension column due to the high concentration of the mobile phase with ACN the sample got highly concentrated with ACN. As a result the retention time of the phenolic and flavonoid compounds was low on the Kinetex column. This problem was partially solved by using pure high polar solvent in the mobile phase (100% water) during the 2nd dimension analysis. The setup IV was partially successful. The mixture sample hardly retained in the column and eluted direct after the injection peaks. However this leads to the conclusion that this method looks promising and further research should be done along this line.

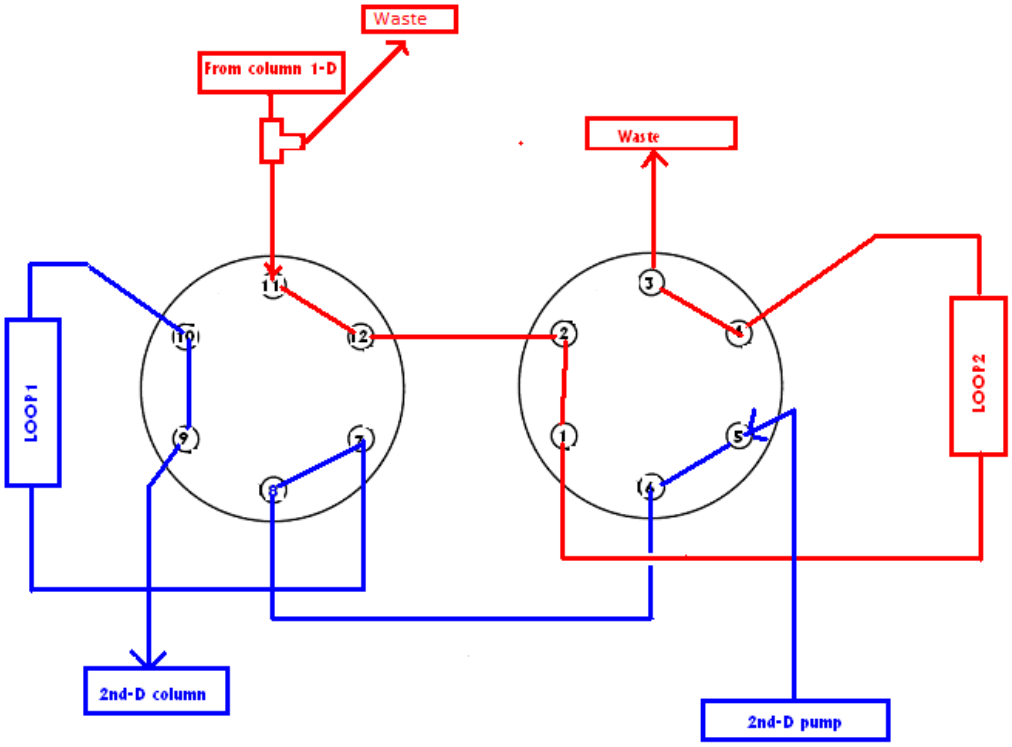
7.1. Impact

Phenolic and flavonoid compounds are natural compounds in several red wines and non-alcoholic beverages and the identification and then the isolation from the beverages could lead on the investment of drugs for different diseases for example coronary heart disease (CHD)

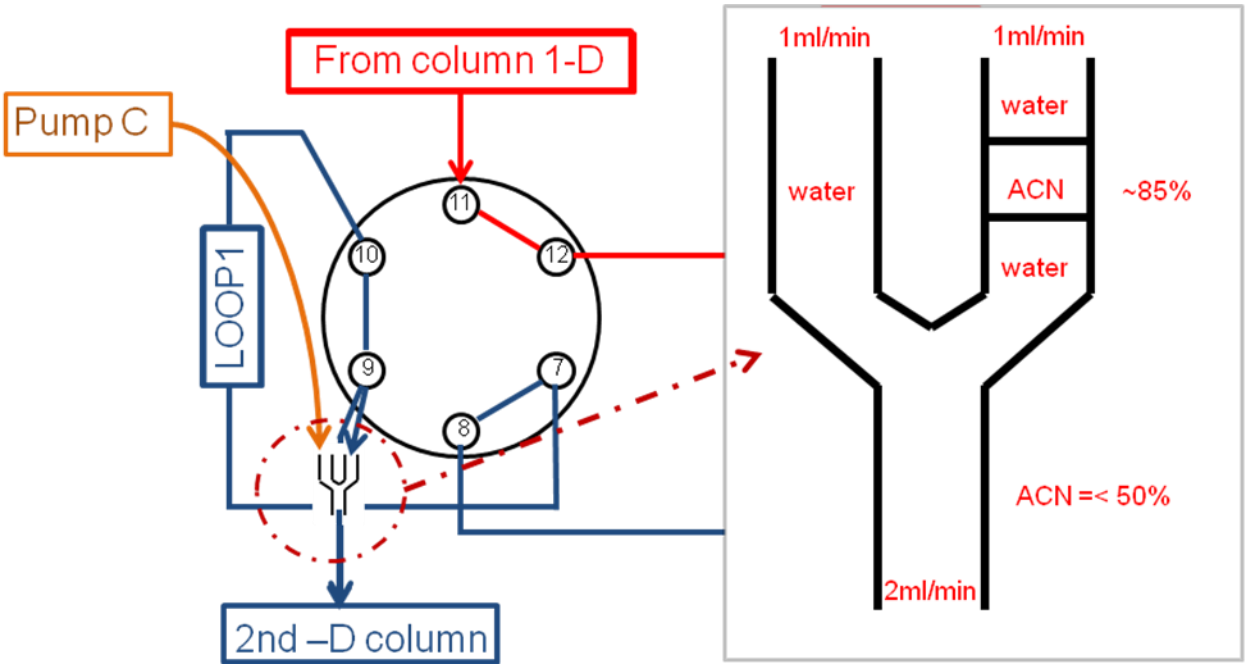
Most importantly, however, this study helps to establish the ground for further investigation of methods for the analysis of phenolic and flavonoid compounds from red wines and non-alcoholic beverages with two-dimensional high performance liquid chromatography. It also demonstrates the value of studying naturally derived molecules for beneficial clinical effects.

7.2. Suggestion for feature researches

For future researches to reduce this problem one solution could be the splitting flow after the 1st dimension column with the consequence of losing some sensitivity depending on the split ratio.



Another solution could be an additional pump after the 1st dimension column so that we can have a mobile phase composition that allows analytes to be retained on the 2nd D column!



Another possible solution could be the exchange of mobile phase from acetonitrile to methanol. Methanol could a more suitable solvent due to two distinct characteristics that it possesses. Firstly, it has higher polarity than acetonitrile; therefore, it can increase the retention time.

And as a final step, it is should be considered to couple the 2D-HPLC system with an MS detector. The MS detector is compatible with flow rates close to 0.5mL, so it is important to consider that the second dimension flow rate is 1.0mL/min or higher, to split the flow before the MS detector.

8. References

1a Dósa, G.; Péter, A. (2002). "Detection of phenoloids in some Hungarian Inula and Centaureaspecies". Acta Botanica Hungarica 44: 129

2 Papp, I.; Apáti, P.; Andrasek, V.; Blázovics, A.; Balázs, A.; Kursinszki, L.; Kite, G. C.; Houghton, P. J. et al (2004). "LC-MS Analysis of Antioxidant Plant Phenoloids". Chromatographia 60.

3 Li, Ning; Tan, Ning-Hua; Zhou, Jun (2004). "New Chlorine-Containing Phenoloid FromCurculigo capitulata". Journal of Asian Natural Products Research 6 (1): 7–10

4 Hättenschwiler, Stephan; Vitousek, Peter M. (2000). "The role of polyphenols in terrestrial ecosystem nutrient cycling". Trends in Ecology & Evolution 15 (6): 238

5 http://www.merriam-webster.com/dictionary/polyphenol

6 Macheix, J. J., Fleuriet, A., Billot, J., Fruit Phenolics, CRC Press, Boca Raton FL 1990

7 P. Dugo, F. Cacciola, M. Herrero, P. Donato, L. Mondello. 2008. Use of partially porous column as second dimension in comprehensive two-dimensional system for analysis of polyphenolic antioxidants J. of separation science 31 (19), 3297-3308

8 Mobh, Shiro (1938). "Research for Vitamin P". The Journal of Biochemistry.

9 Flavonoids (isoflavonoids and neoflavonoids)., IUPAC Compendium of Chemical Terminology

10 http://en.wikipedia.org/wiki/Flavonoid

11 J.B. Harborne, The Flavonoids: Advances in Research Since 1986, Chapman and Hall, London, 1994, p. 64.

12 A. Moure, J.M. Cruz, D. Franco, J.M. Dominguez, J. Sineiro, Natural antioxidants from residual sources,(2001) Food Chemistry 72, 2, p. 145–171.

13 A.J.J. Dugas, J. Castaneda-Acosta, G.C. Bonin, K.L. Price, N.H. Fischer, G.W. Winston, Evaluation of the total peroxyl radical-scavenging capacity of flavonoids: Structure-activity relationships**,** J. Natl. Prod. 63 (2000) 327.

14 T.B. Ng, F. Liu, Z.T. Wang, Antioxidative activity of natural products from plants Life Sci. 66 (2000) 709.

15 M.E. Crespo, J. Galvez, T. Cruz, M.A. Ocete, A. Zarzuelo, Anti-inflammatory activity of diosmin and hesperidin in rat colitis induced by TNBS**,** Planta Med. 65 (1999) 651.

16 V. Butterweck, G. Jurgenliemk, A. Nahrstedt, H. Winterhoff, Planta Med. 66 (2000) 3.

17 L.L. Marchand, Biomed. Pharmacother. Cancer preventive effects of flavonoids - A review**,** 56 (2002) 296-301.

18 J.A. Milner. Reducing the risk of cancer. In Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals; Goldberg, I., Ed.; Chapman & Hall: New York, NY, USA, 1994; pp. 39-70.

19 G.G. Duthie, K.M. Brown, Reducing the risk of cardiovascular disease. In Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals; Goldberg, I., Ed.; Chapman & Hall: New York, NY, USA, 1994; pp. 19-38

20 H. Wang, G. Cao, R.L. Prior, Total antioxidant capacity of fruits. J. Agric. Food Chem. 1996, 44, 701-705.

21 C.A. Rice-Evans, N.J. Miller, G. Paganga. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 1996, 20, 933-956.

22 P.G. Pietta, Flavonoids as antioxidants. J. Nat. Prod. 2000, 63, 1035-1042.

23 B. Zhou, L.-M. Wu, L. Yang, Z.-L. Liu, Evidence for alpha-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. Free Radic. Biol. Med. 2005, 38, 78-84.

24 S. Renaud, M. de Lorgeril Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet. 1992;339:1523–1526.

25 E. Fragopoulou, C. A. Demopoulos, S. Antonopoulou, Lipid minor constituents in wines. A biochemical approach in the French paradox I. J. of Wine Research 2009:1, 131–143

26 C.D. Stalikas, Extraction, separation, and detection methods for phenolic acids and flavonoids. J. Sep. Sci. 2007, 30, 3268-3295.

27 R. Tsao, Deng, Z. Separation Procedures for naturally occurring antioxidant phytochemicals. J. Chromatogr. B 2004, 812, 85-99.

28 C.D. Stalikas, Phenolic acids and flavonoids: occurrence and analytical methods. Methods Mol. Biol. 2010, 610, 65-90.

29 R. Tsao, Chemistry and Biochemistry of Dietary Polyphenols, Nutrients, vol. 2, Dec. 2010, pp. 1231-1246.

30 C.D. Stalikas, Extraction, Separation, and Detection Methods for Phenolic Acids and Flavonoids, Journal of Separation Science, vol. 30, no. 18, Dec. 2007, pp. 3268-3295.

31 F. Shahidi, and M. Naczk, Phenolics in Food and Neutraceuticals, 1st ed., CRC Press, Boca Raton, 2004, pp. 525, 517.

32 M. Azar, E. Verette, and S. Brun, Identification of Some Phenolic Compounds in Bilberry Juice Vaccinium Myrtillus, Journal of Food Science, vol. 52, no. 5, Sep. 1987, pp. 1255-1257.

33 Y. Peng, Q. Chu, F. Liu, and J. Ye, Determination of Phenolic Constituents of Biological Interest in Red Wine by Capillary Electrophoresis with Electrochemical Detection, Journal of Agricultural and Food Chemistry, vol. 52, no. 2, Jan. 2004, pp. 153-156.
34 C.A. Hall, A. Zhu, and M.G Zeece, Comparison Between Capillary Electrophoresis and High-Performance Liquid Chromatography Separation of Food Grade Antioxidants, Journal of Agricultural and Food Chemistry, vol. 42, no. 4, Apr. 1994, pp. 919-921.
35 Y. Zuo, C. Wang, and J. Zhan, Separation, Characterization, and Quantitation of Benzoic and Phenolic Antioxidants in American Cranberry Fruit by GC-MS, Journal of Agricultural and Food Chemistry, vol. 50, no. 13, Jun. 2002, pp. 3789-3794.
36 G.J. Soleas, J. Yan, and D.M. Goldberg, Ultrasensitive Assay for Three Polyphenols (Catechin, Quercetin and Resveratrol) and Their Conjugates in Biological Fluids Utilizing Gas Chromatography with Mass Selective Detection, Journal of Chromatography B, vol. 757, no. 1, Jun. 2001, pp. 161-172.
37 H.T. Lu, Y. Jiang, and F. Chen, Application of Preparative High-Speed Counter-Current Chromatography for Separation of Chlorogenic Acids from Flos Lonicerae, Journal of Chromatography A, vol. 1026, no. 1, Feb. 2004, pp. 185-190.
38 X. Vitrac, C. Castagnino, P. Waffo-Teguo, J.C. Deleunay, J. Vercauteren, J.P. Monti, G. Deffieux, G., and J.M. Merillon, Polyphenols Newly Extracted in red Wine from Southwestern France by Centrifugal Partition Chromatography, Journal of Agricultural and Food Chemistry, vol. 49, no. 12, Dec. 2001, pp. 5934-5938.
39 W. Pongsuwan, T. Bamba, K. Harada, T. Yonetani, A. Kobayashi, and E. Fukusaki, High-Throughput Technique for Comprehensive Analysis of Japanese Green Tea Quality Assessment Using Ultra-Performance Liquid Chromatography with Time-of-Flight Mass Spectrometry (UPLC/TOF MS), Journal of Agricultural and Food Chemistry, vol. 56, no. 22, Nov. 2008, pp. 10705-10708.
40 http://www.sigmaaldrich.com/catalog/product/aldrich/188255?lang=de&region=AT
41H.H Weetall, Enzymic synthesis of gallic acid esters. Eur. Pat. 137601 (1985)
42 http://www.sigmaaldrich.com/catalog/product/sigma/g7384?lang=de&region=AT
43 http://www.sigmaaldrich.com/catalog/product/sigma/e4018?lang=en&region=GB
44 W. Boerjan; J. Ralph; M. Baucher, (2003). "Ligninbiosynthesis". *Annual Review of Plant Biology* **54**: 519–46
45 Purification and identification of active antibacterial components in Carpobrotusedulis L. Elmarie van der Watt and J. C Pretorius, Journal of Ethnopharmacology, Volume 76, Issue 1, June 2001, pp. 87–91
46 J. Cazes, R.P.W. Scott, Chromatography theory, Marcel Dekker, Inc., New York, 2002.
47 V.R. Meyer, Practical high-performance liquid chromatography, 4th ed., John Wiley & Sons, Chichester, 2004.
48 http://en.wikipedia.org/wiki/Chromatography
49 http://old.iupac.org/goldbook/IT06941.pdf
50 D.C. Harris, Quantitative Chemical Analysis, W.H. Freeman and Co., New York, 1999.
51 A.J. Alpert J.Chromatography, A 499 (1990) 177-196
52
http://www.cienytech.com/tablas/HILIC%20%20What%20is%20Hydrophilic%20Interaction%20Chromatography%20II.pdf
53 D.V. McCalley, U.D. Neue, Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography, J. Chromatogr. A 1192 (2008) 225.
54 J. Pesek, M.T. Matyska, A comparison of two separation modes: HILIC and aqueous normal phase chromatography, LC-GC N. Am. 25 (2007) 480.
55 U.D. Neue, HPLC Columns: Theory, technology and practice, Wiley-VCH, Inc., New York, 1997.
56 P. Hemström, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29 (2006) 1784.
57 P. Dallet, L. Labat, E. Kummer, J.P. Dubost, Determination of urea, allantoin and lysine pyroglutamate in cosmetic samples by hydrophilic interaction chromatography, J. Chromatogr. B 742 (2000) 447.
58 C. McClintic, D. Remick, J. Peterson, D. Risley, Novel Method for the Determination of Piperazine in Pharmaceutical Drug Substances Using Hydrophilic Interaction Chromatography and Evaporative Light Scattering Detection,J. Liq. Chromatogr. Relat. Technol. 26 (2003) 3093.
59 X. Wang, W. Li, H.T. Rasmussen, Orthogonal method development using hydrophilic interaction chromatography and reversed-phase high-performance liquid chromatography for the determination of pharmaceuticals and impurities, J. Chromatogr. A 1083 (2005) 58
60 A. Yanagida, H. Murao, M. Ohnishi-Kameyama, Y. Yamakawa, A. Shoji, M. Tagashira, T. Kanda, H. Shindo, Y. Shibusawa, Retention behavior of oligomeric proanthocyanidins in hydrophilic interaction chromatography**,** J. Chromatogr. A 1143 (2007) 153.
61 H. Zhang, Z. Guo, F. Zhang, Q. Xu, X. Liang,
HILIC for separation of co-eluted flavonoids under RP-HPLC mode, J. Sep. Sci. 31 (2008) 1623.

62 Nic, M.; Jirat, J.; Kosata, B., eds. (2006–). "reversed-phase chromatography". IUPAC Compendium of Chemical Terminology (Online ed.). doi:10.1351/goldbook.R05376. ISBN 0-9678550-9-8. http://goldbook.iupac.org/R05376.html.
63 I. Molnar and C. Horvarth, Reverse phase chromatography of polar biological substances: separation of catechol compounds by high performance liquid chromatography**,** Clin.Chem., 22 (1976) 1497.
64 http://en.wikipedia.org/wiki/Reversed-phase_chromatography
65 http://www.standardbase.com/tech/HPLC.pdf
66 http://www.ssi.shimadzu.com/products/literature/HPLC/SSI_Prominence_UFLC.pdf
67 http://ssihplc.com/hplc-pumps/
68 http://en.wikipedia.org/wiki/Csaba_Horv%C3%A1th_%28chemical_engineer%29
69 L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC method development, 2nd ed., John Wiley & Sons, Inc., New York, 1997.
70 B.A. Bidlingmeyer, Practical HPLC methodology and applications, John Wiley & Sons, Inc., New York, 1992.
71 L.R. Snyder, J.J. Kirkland, Introduction to modern liquid chromatography, John Wiley & Sons, Inc., New York, 1974.
72 http://en.wikipedia.org/wiki/High-performance_liquid_chromatography
73 van Deemter JJ, Zuiderweg FJ and Klinkenberg A (1956). Longitudinal diffusion and resistance to mass transfer as causes of non ideality in chromatography, Chem. Eng. Sc. 5: 271–289
74 http://www.labhut.com/products/hplc-columns.html
75 http://www.silicycle.com/products/siliachrom-hplc-columns?gclid=CJa-7ZC4y68CFYnO3wod13-iZQ
76 W.M.A. Niessen, Liquid chromatography-mass spectrometry, 3rd ed., CRC Press, New York, 2006.
77 B.B. Wheals, in C.F. Simpson (Editor), Techniques in liquid chromatography, Wiley Heyden Ltd., West Sussex, 1982, p. 121.
78 D.A. Skoog, F.J. Holler, T.A. Nieman, Principles of Instrumental Analysis, 5th ed., Saunders College Publishing Philadelphia, 1998.
79 G.D. Christian, Analytical Chemistry, 6th ed., John Wiley & Sons, Inc., MA, 2004.
80 http://www.hplc.cz/Teorie/UV_VIS_detector.html
81 http://www.hwe.oita-u.ac.jp/kiki/ronnbunn/paper_choi.pdf
82 http://en.wikipedia.org/wiki/Liquid_chromatography%E2%80%93mass_spectrometry
83 Kumaguai H., Application of Liquid Chromatography/Mass Spectrometry to the Analysis of Sugars and Sugar-Alcohol., DOI: 5988-4236EN.
84 R.A. Shellie, P.R. Haddad, Comprehensive two-dimensional liquid chromatography, Anal. Bioanal. Chem. 386 (2006) 405.
85 S.P. Dixon, I.D. Pitfield, D. Perrett, Comprehensive multi-dimensional liquid chromatographic separation in biomedical and pharmaceutical analysis: A review, Biomed. Chromatogr. 20 (2006) 508.
86 U.D. Neue, Peak capacity in unidimensional chromatography, J. Chromatogr. A 1184 (2008) 107.
87 S.A. Cohen, M.R. Schure: MD-LC theory and applications in industrial chemistry and the life sciences 1
88 J.C. Giddings, in H.J. Cortes (Editor), (1990) Use of Multiple Dimensions of in Analytical Separations, in Multidimensional Chromatography: Techniques and Applications, Marcel Dekker, Inc., New York, p. 1.
89 J.C. Giddings, in H.J. Cortes (Editor).(1967) Maximum number of componets resolvable by gel filtration and other elution chromatographic methods. Anal. Chem. 39, 1027-1028, p. 1.
90 J.C. Giddings, (1991) Unified Separation Science. John Wiley & Sons, Inc. New York.
91 E. Grushka (1970) Chromatographic peak capacity and the factors influencing it. Anal. Chem. 42, 1142-1147.
92 C.G. Horvath, S.R. Lipsky (1967). Peak capacity in chromatography. Anal. Chem. 39, 1893
93 J.C. Giddings, Anal. Chem. 56 (1984) 1270A
94 J.C. Giddings, J. High Resolut. Chromatogr. Commun. 10 (1987) 319.
95 P. Dugo, F. Cacciola, T. Kumm, G. Dugo, L. Mondello, Comprehensive multidimensional liquid chromatography: Theory and applications, J. Chromatogr. A 1184 (2008) 353.
96 Z. Liu, D.G. Patterson, M.L. Lee, Geometric approach to factor analysis for the estimation of orthogonality and practical peak capacity in comprehensive two-dimensional separations, Anal. Chem. 67 (1995) 3840.
97 X. Li, D.R. Stoll, P.W. Carr,Equation for peak capacity estimation in two-dimensional liquid chromatography, Anal. Chem. 81 (2009) 845.
98 M. Gray, G.R. Dennis, P. Wormell, R. Andrew Shalliker, P. Slonecker, Two dimensional reversed-phase-reversed-phase separations: Isomeric separations incorporating C18 and carbon clad zirconia stationary phases, J. Chromatogr. A 975 (2002) 285-297.
99 K. Horie, H. Kimura, T. Ikegami, A. Iwatsuka, N. Saad, O. Fiehn, N. Tanaka, Calculating optimal modulation periods to maximize the peak capacity in two-dimensional HPLC, Anal. Chem. 79 (2007) 3764.
100 J.V. Seeley, Theoretical study of incomplete sampling of the first dimension in comprehensive two-dimensional chromatography, J. Chromatogr. A 962 (2002) 21.

101 F. Cacciola, P. Jandera, Z. Hajdu´, P C˘ esla, L. Mondello, (2007) Comprehensive two-dimensional liquid chromatography with parallel gradients for separation of phenolic and flavone antioxidants Journal of Chromatography A, 1149 (2007) 73–87
102 C.J. Venkatramani, Y. Zelechonok, Two-dimensional liquid chromatography with mixed mode stationary phases. J. Chromatogr. A vol. 1066 pp 45-53 (2005)
103 A.R. Shalliker, M.J. Gray, in E. Grushka, N. Grinberg (Editors), Advances in Chromatography, CRC Press, New York, 2006, p. 177.
104 P.J. Schoenmakers, G. Vivo-Truyols, W.M.C. Decrop, A protocol for designing comprehensive two-dimensional liquid chromatography separation systems, J. Chromatogr. A 1120 (2006) 282.
105 I. François, A. de Villiers, B. Tienpont, F. David, P. Sandra, Comprehensive two-dimensional liquid chromatography applying two parallel columns in the second dimension, J. Chromatogr. A 1178 (2008) 33.
106 A. de Villiers, M.K. Kalili, M. Malan, and J. Roodman, Improving HPLC Separation of Polyphenols, LCGC Europe, 1 Oct. 2010.
http://www.chromatographyonline.com/lcgc/article/articleDetail.jsp?id=691153&sk=&date=&pageID=6
107 Z. Liu, D.G. Patterson, M.L. Lee,. Geometric approach to factor analysis for the estimation of orthogonality and practical peak capacity in comprehensive two-dimensional separations Anal. Chem 67 (1995) 3840.
108 M.K. Kalili, and A. de Villiers, Off-Line Comprehensive 2-Dimensional Hydrophilic Interaction × Reversed Phase Liquid Chromatography Analysis of Procyanidins, Journal of Chromatography A, vol. 1216, no. 35, Aug. 2009, pp. 6274-6284.
109 I. François, A. de Villiers, and P. Sandra, Considerations on The Possibilities and Limitations of Comprehensive Normal Phase-Reversed Phase Liquid Chromatography (NPLC×RPLC), Journal of Separation Science, vol. 29, no. 4, Mar. 2006, pp. 492-498.
110 I. François, A. de Villiers, B. Tienpont, F. David, and P. Sandra, Comprehensice Two-Dimensional Liquid Chromatography Applying Two Parallel Columns in The Second Dimension, Journal of Chromatography A, vol. 1178, no. 1, Jan. 2008, pp. 33-42.
111 M. Kivilompolo, and T. Hyötyläinen, Comparison of Separation Power of Ultra Performance Liquid Chromatography and Comprehensive Two-Dimensional Liquid Chromatography in The Separation of Phenolic Compounds in Beverages, Journal of Separation Science, vol. 31, no. 19, Oct. 2008, pp. 3466-3472.
112 M. Kivilompolo, V. Obůrka, and T. Hyötyläinen, Comprehensive Two-Dimensional Liquid Chromatography in The Analysis of Antioxidant Phenolic Compounds in Wines and Juices, Analytical and Bioanalytical Chemistry, vol. 391, no.1, May 2008, 373-380.
113 M. Kivilompolo, and T. Hyötyläinen, Comprehensive Two-Dimensional Liquid Chromatography in Analysis of Lamiaceae Herbs: Characterisation and Quantification of Antioxidant Phenolic Acids, Journal of Chromatography A, vol. 1145, no. 2, Mar. 2007, pp. 155-164.
114 P. Dugo, F. Cacciola, M. Herrero, P. Donato, and L. Mondello, Use of Partially Porous Column as Second Dimension in Comprehensive Two-Dimensional System for Analysis of Polyphenolic Antioxidants, Journal of Separation Science, vol. 31, no. 19, Oct. 2008, pp. 3297-3308.
115 P. Dugo, F. Cacciola, P. Donato, D. Airado-Rodríguez, M. Herrero, and L. Mondello, Comprehensive Two-Dimensional Liquid Chromatography to Quantify Polyphenols in Red Wines, Journal of Chromatography A, vol. 1216, no. 44, Oct. 2009, pp. 7483-7487.
116 F. Cacciola, P. Jandera, Z. Hajdú, P. Česla, and L. Mondello, Comprehensive Two-Dimensional Liquid Chromatography with Parallel Gradients for Separation of Phenolic and Flavone Antioxidants, Journal of Chromatography A, vol. 1149, no. 1, May 2007, pp. 73-87.
117 E. Blahová, P. Jandera, F. Cacciola, and L. Mondello, Two-Dimensional and Serial Column Reversed-Phase Separation of Phenolic Antioxidants on Octadecyl-, Polyethyleneglycol-, and Pentafluorophenylpropyl-Silica Columns, Journal of Separation Science, vol. 29, no. 4, Mar. 2006, pp. 555-566.
118 F. Cacciola, P. Jandera, E. Blahová, and L. Mondello, Development of Different Comprehensive Two Dimensional Systems for The Separation of Phenolic Antioxidants, Journal of Separation Science, vol. 29, no. 16, Nov. 2006, pp. 2500-2513.
119 F. Cacciola, P. Jandera, and L. Mondello, Temperature Effects on Separation on Zirconia Columns: Application to One- and Two-Dimensional LC Separation of Phenolic Antioxidants, Journal of Separation Science, vol. 30, no. 4, Mar. 2007, pp. 462-474.
120 I. François, A. de Villiers, P. Sandra, J. Sep. Sci. 29 (2006) 492.
121 P. Dugo, M. Herrero, D. Giuffrida, T. Kumm, G. Dugo, L. Mondello, Application of comprehensive two-dimensional liquid chromatography to elucidate the native carotenoid composition in red orange essential oil, J. Agric. Food Chem. 56 (2008) 3478.
122 P. Dugo, V. Skerikova, T. Kumm, A. Trozzi, P. Jandera, Elucidation of carotenoid patterns in citrus products by means of comprehensive normal-phase x reversed-phase liquid chromatography, L. Mondello, Anal.Chem. 78 (2006) 7743.

123 P. Jandera, J. Fischer, H. Lahovská, K. Novotná, P. Cesla, L. Kolárová, Two-dimensional liquid chromatography normal-phase and reversed-phase separation of (co)oligomers, J. Chromatogr. A 1119 (2006) 3.
124 F. Cacciola, P. Jandera, E. Blahová, L. Mondello, Development of different comprehensive two dimensional systems for the separation of phenolic antioxidants, J. Sep. Sci. 29 (2006) 2500.
125 F. Cacciola, P. Jandera, Z. Hajdu, P. Cesla, L. Mondello, Comprehensive two-dimensional liquid chromatography with parallel gradients for separation of phenolic and flavone antioxidants, J. Chromatogr. A 1149 (2007) 73.
126 F. Cacciola, P. Jandera, L. Mondello, Temperature effects on separation on zirconia columns: Applications to one- and two-dimensional LC separations of phenolic antioxidants, J. Sep. Sci. 30 (2007) 462.
127 M.J. Gray, G.R. Dennis, P.J. Slonecker, R.A. Shalliker, Comprehensive two-dimensional separations of complex mixtures using reversed-phase reversed-phase liquid chromatography, J. Chromatogr. A 1041 (2004) 101
128 http://www.ssi.shimadzu.com/products/product.cfm?product=LC-MS_it_tof
129 http://en.wikipedia.org/wiki/Benzene#Benzene_derivatives
130 http://webbook.nist.gov/cgi/cbook.cgi?Name=gallic+acid&Units=SI&cMS=on&cUV=on
131 http://en.wikipedia.org/wiki/Catechin
132 http://webbook.nist.gov/cgi/cbook.cgi?ID=C331395&Units=SI&Mask=2000#Gas-Chrom
133 http://webbook.nist.gov/cgi/cbook.cgi?ID=C331395&Units=SI&Mask=200#Mass-Spec
134 http://webbook.nist.gov/cgi/cbook.cgi?Formula=C27H30O16+3H2O&NoIon=on&Units=SI
135 R. Murphy, M.R. Schure, and J.P. Foley, Effect of Sampling Rate on Resolution in Comprehensive Two-Dimensional Liquid Chromatography, Analytical Chemistry, vol. 70, no. 8, Apr. 1998, pp. 1585-1594.