THESIS FOR THE MASTER'S DEGREE IN CHEMISTRY

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TWO DIMENSIONAL LIQUID CHROMATOGRAPHIC SEPARATIONS OF PLANTS EXTRACTS

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Contents

Preface

The work presented in this master thesis was carried out at the Department of Chemistry, University of Oslo from August 2008 to May 2010. Prof. Elsa Lundanes, Prof. Tyge Greibrokk and Post Doc. Steven Wilson served as my supervisors.
Early in this study, preliminary experiments were performed to gain knowledge about the principle of liquid chromatography and column packing. Some of this work can be found in appendix.

I would like to thank my supervisors for giving me the opportunity to take a masters degree in organic analytical chemistry, and for giving me an interesting and challenging project. Thank you for the guidance, support, trust and motivation.
Special thanks to Steven Wilson for providing help at any time.
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Many thanks to my parents and my family especially Mr. & Mrs Kalim, ALI & Roza for their support, love, and giving me so many prayers. I am also very thankful to my friends specially Naeimah for her countless prayers and support.
Finally, I would like to thank my wife Sadia for giving me a new happy lovely life.

Oslo, Norway, September 2010

Abstract

In this study, the potential of using off-line or online two dimensional liquid chromatography (2D LC) separations of plant extracts was examined.

A new approach to high speed, online isocratic elution comprehensive 2D LC based on the use of hydrophilic interaction liquid chromatography (HILIC) chromatography in the first dimension, and a fast second dimension utilizing high temperature and Hypercarb porous carbon stationary phase is described. Entirely conventional isocratic elution instrumentation and columns were assembled in a system which could separate the complex mixture of compounds extracted from rare Serbian plants. The second dimension column was used at high flow rate in isocratic mode and a high column temperature of 110°C. Fractions eluted from the first dimension HILIC column were subjected to the second dimension either online (using an interface) or off-line. The online HILIC-Hypercarb combination demonstrated large peak capacities as compared to other modes.

Abbreviations

AcN	acetonitrile
FA	formic Acid
GC	gas chromatography
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
i.d.	inner diameter
IPA	isopropanol
IC	ion chromatography
LC	liquid chromatography
LOD	limit of detection
MS	mass spectrometry
m/z	mass to charge ratio
MeOH	methanol
MP	mobile phase
NP	normal phase
PEEK	polyether ether ketone
PS-DVB	poly (styrene-divinylbenzene)
PGC	porous graphite carbon
RP	reversed phase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPE	solid phase extraction
TFA	trifluoroacetic acid
t_R	retention time
UV	ultraviolet
ZIC	zwitterionic
#	number
1D	one dimensional
2D	two dimensional
3D	three dimensional

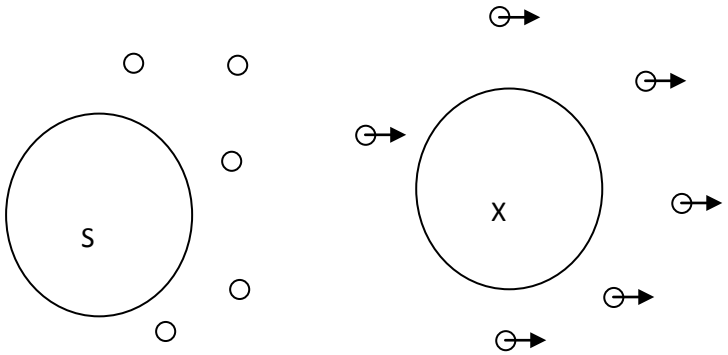
1-Introduction

1.1 Background

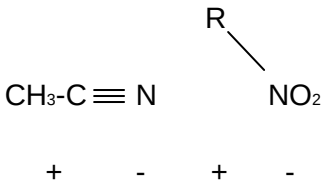
In this study different chromatography principles were used to separate complex mixture of compound in plant extracts. Chromatography is the combined term of techniques for the separation of mixtures. It can be further explained in simple word as, separation of compounds in a mixture based on differential partitioning between the mobile and stationary phases [1]. The combination of HILIC and reversed phase chromatography, including Hypercarb chromatography were used in this project.

There are two basic purposes of chromatography, preparative and analytical separation. Preparative chromatography is used for isolation of different components from of a mixture, while analytical chromatography (used in this thesis) utilizes relatively small sample amounts and used for both qualitative and quantitative purposes. In chromatography, intermolecular interactions play an important role for analyte separations. Intermolecular interactions include different kinds of intermolecular forces. These forces include dispersion, dipole-induced dipole, dipole–dipole, hydrogen bonding and ionic (Columbic) interactions. Different chromatographic separation principles like reversed phase, normal phase, ion-exchange and size exclusion were developed and categorized on the basis of their intermolecular interactions[2]. A Dispersion interaction as shown in Figure 1A is generated by an unsymmetrical electrons arrangement around S (solvent atom) which at any instant of time will cause electrons in adjacent atom X (solute) to move (Columbic repulsion). Thus instantaneous dipole moment between S and X creates electrostatic attraction. Dipole-dipole interaction is described in Figure 1B, and is produced by permanent dipole moment between analyte (R-NO_2) and solvent (CH_3-CN) or stationary phase[2]. Hydrogen bonding interactions of acidic and basic solute and solvent and vice versa are shown in Figure 1C[2]. When a charged analyte interacts with the opposite charged surrounding ionic interaction [2] occur as shown in Fig. 1D.

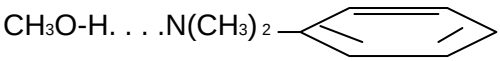
A: Dispersion interaction



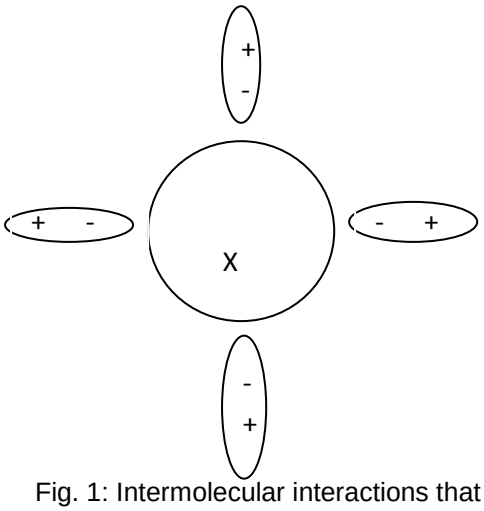
B: Permanent dipole moment



C: Hydrogen bonding interactions



D: Ionic interaction



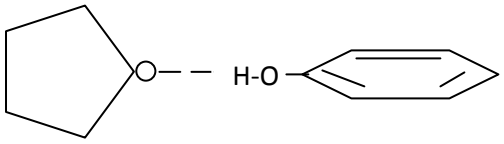


Fig. 1: Intermolecular interactions that can contribute to retention and selectivity [2].

On the basis of intermolecular interaction forces, different chromatographic principles and techniques have been developed. The reversed phase (RP) mode is the most popular mode of LC and it is useful in most cases like in biological studies and for the separation of pharmaceuticals. Normal phase (NP) is perhaps the second most used mode of chromatography and involves polar interactions such as dipole–dipole

and hydrogen bonding, while molecular size has minimal impact on retention in NP [3]. Hydrophilic interaction liquid chromatography (HILIC) is a form of NP chromatography, but is different from both NP and RP chromatography in that it is dominated by a fairly polar stationary phase as does NP, but uses eluents based on mixtures of water with organic solvents as does RP[4]. The intermolecular studies show that by using one type of chromatographic system, one or two types of intermolecular forces are involve but by using a combination of two chromatographic system more intermolecular forces are involved and thus increase the separation power[4].

1.2 Hydrophilic interaction liquid chromatography (HILIC)

HILIC is a kind of NP chromatography because hydrophilic compounds are retained more than hydrophobic compounds. Elution order in HILIC is different compared to RP chromatography, which makes an orthogonal relationship between these systems. Although it utilizes a hydrophilic stationary phase and a more hydrophobic aqueous organic mobile phase, it can be distinguished from NP because of water miscible solvents [5].

HILIC can be used with a vast variety of stationary phases that provide different retention characteristics and separation selectivity with the freedom of buffer selection [5-6]. Underivatized silica with functional groups such as siloxanes, silanols and metals [5, 7], derivatized silica like cation exchanger polysulfoethyl A [5, 7c, 8], weak cation exchanger Polycat A[5, 9], weak anion exchanger PolyWAX[5, 10], TSKgel amide 80[5, 11] and zwitterionic ZIC-HILIC[5, 12] are the most popular stationary phases for HILIC chromatography (Fig. 2).

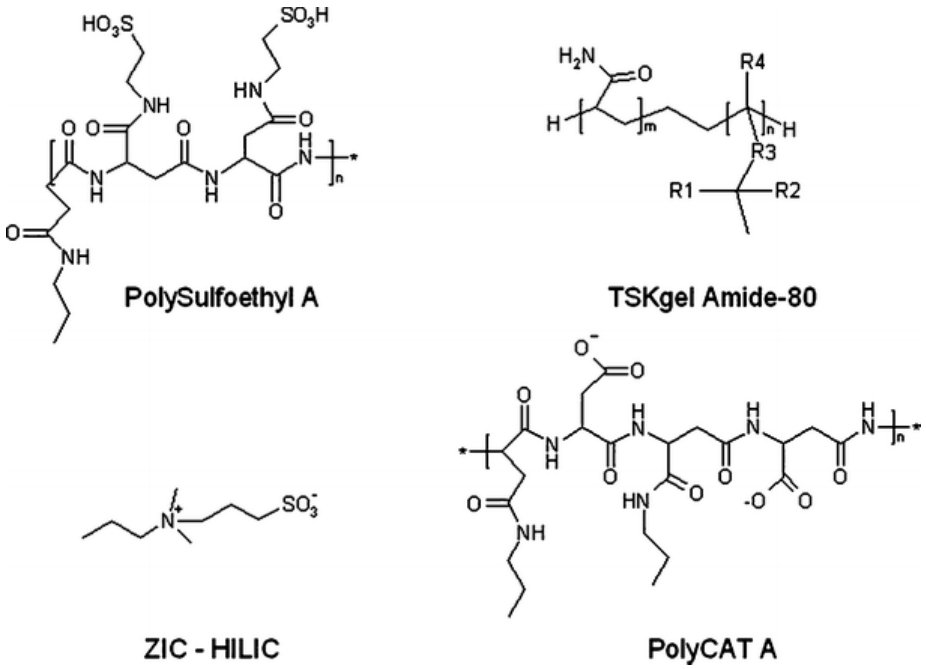


Fig. 2: Functional group structures of different HILIC stationary phases [5, 12].

The durability and popularity of HILIC have increased because of some advantageous features like providing good peak shapes for basic solutes and good sensitivity for mass spectrometry [5, 12]. In HILIC, a sample can be dissolved in pure organic solvent for direct injection while this is difficult in RP. Furthermore, lower mobile phase viscosity generates lower column back pressure high flow rate can be used [13]. HILIC stationary phases generally give stronger retention of compounds which have little or no retention on RP columns, for instance due to wettability problems which is explained in Fig. 3 which shows two phases treated with water and water/methanol mixture. The phase which has been subjected to100 % water for some time loses its efficiency by collapsing[14].

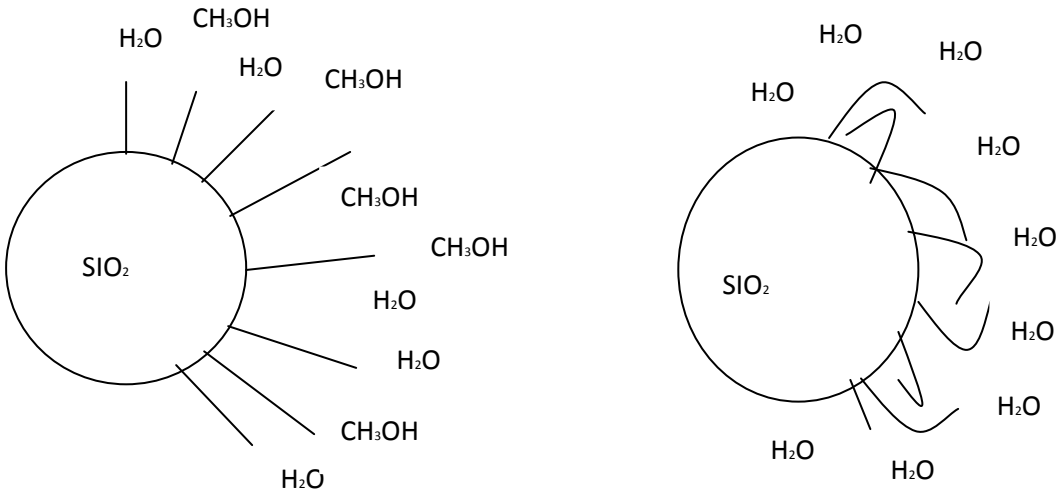


Fig. 3: Explanation of wettability in RP chromatography. Configurations of long-chain bonded alkyl phases (a) in water–methanol solution and (b) in 100 % water [14].

As mentioned above different types of HILIC phases have been used. In principle HILIC stationary phases can be divided into three different groups. Neutral phases which give no electrostatic interactions like diol phases and amide phases, charged phases which give strong electrostatic interaction like plain silica phases and aminopropyl phases, and zwitterionic phases which give weak electrostatic interaction like zwitterionic ZIC-HILIC [6-7, 15]. ZIC-HILIC (shown in Fig. 4) is unique stationary phase which complementary to cation exchange and particularly useful for the separation of peptides and polar compounds. Its weak electrostatic effects improve selectivity and resolution. Some other advantages of ZIC-HILIC phases are a balanced stoichiometry and zero net charge, absence of hydrophobic interaction (only 10 % of carbon) which makes high recoveries [10, 16].

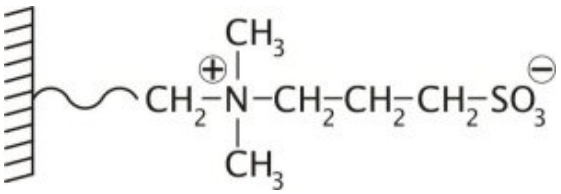


Fig. 4: Schematic illustration of the ZIC®-HILIC stationary phase[15b].

HILIC has been found to be orthogonal to RP regarding separation of proteins[7c], peptides[17], amino acids [7c], oligonucleotides [7c], carbohydrates [18], histones [9] and some natural product extracts [19] [20]. Since the retention mechanism is somewhat

opposite to RP, the combination of HILIC and RP appears to be a very good tool for multidimensional techniques [21].

1.2.1 Column temperature impact on HILIC

Temperature influences the analyte diffusivity, mobile phase viscosity and analyte transferring enthalpy between mobile and stationary phases. Hence the column temperature can significantly improve the performance and selectivity of an HPLC method[22]. With thermally stable column materials higher temperatures can be used and reduce the analysis time remarkably without loss of efficiency [23]. The response of temperature on ZIC-HILIC phases can be opposite. In ZIC-HILC the increased temperature has been seen to increase the retention[24].

1.3 Reversed phase chromatography

RP chromatography involves the use of non-polar stationary phases and polar eluents. The present form of RP chromatography has developed spectacularly since the introduction of chemically bonded stationary phases in 1969 by Halasz and Sebestian [25]. RP LC has a stationary phase that works in such a way that retention time is longer for molecules which are more hydrophobic, while polar molecules elute more readily. Retention time can be increased by adding more water to the mobile phase, thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, retention time can be decreased by adding more organic solvent to the eluent [2, 26]. Compared to other separation principles RP is more stable and reproducible, allows the use of various mobile phases and buffer additives and retention can be easily controlled either isocratically or in gradient mode RP chromatography has surface modifications with many different selectivity available[2, 26]. More hydrophobic compounds spend more time associated with the bonded phase and are eluted last as explained in Fig. 5 [27].

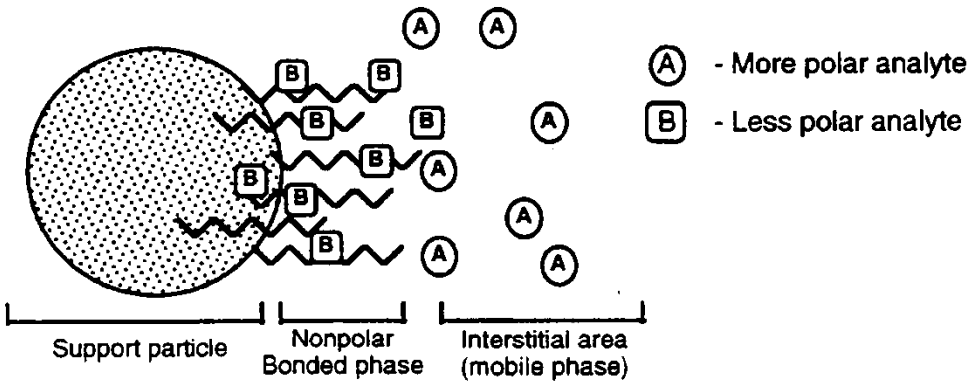


Fig. 5: Explanation of the behaviour of RP stationary phase towards polar and less polar compounds [27].

1.4 Chromatography on Porous Graphite Carbon (PGC) Hypercarb columns

The history of the PGC Hypercarb material is not very long. In 1978 Knox and Gilbert designed a new material for chromatography called “porous glassy carbon”[28] Originally this material had poor chromatographic performance but provided the deliberate beginning for further research. The reason for the poor performance of “porous glassy carbon” material was that was a microporous material [29]. Further research confirmed that by heating porous glassy carbon to 2273-3073 K a new material was formed called porous graphite carbon[29].

Basically the PGC material is fully spherical and porous with porosity around 75 %. As it is mentioned above it does not contain micropores and it is highly reproducible to manufacture [30]. The microscopic study of the PGC material showed that it consists of “flat sheet of hexagonally arranged carbon atoms” [28, 30-31]. The PGC material does not have functional groups on the surface because the aromatic carbon atoms have fully satisfied valances within the graphite sheets [28, 30-31]. The sheet of carbon atoms are held by the dispersion interaction the spacing between these sheets are 2 or 3 dimensional as shown in Fig. 6.

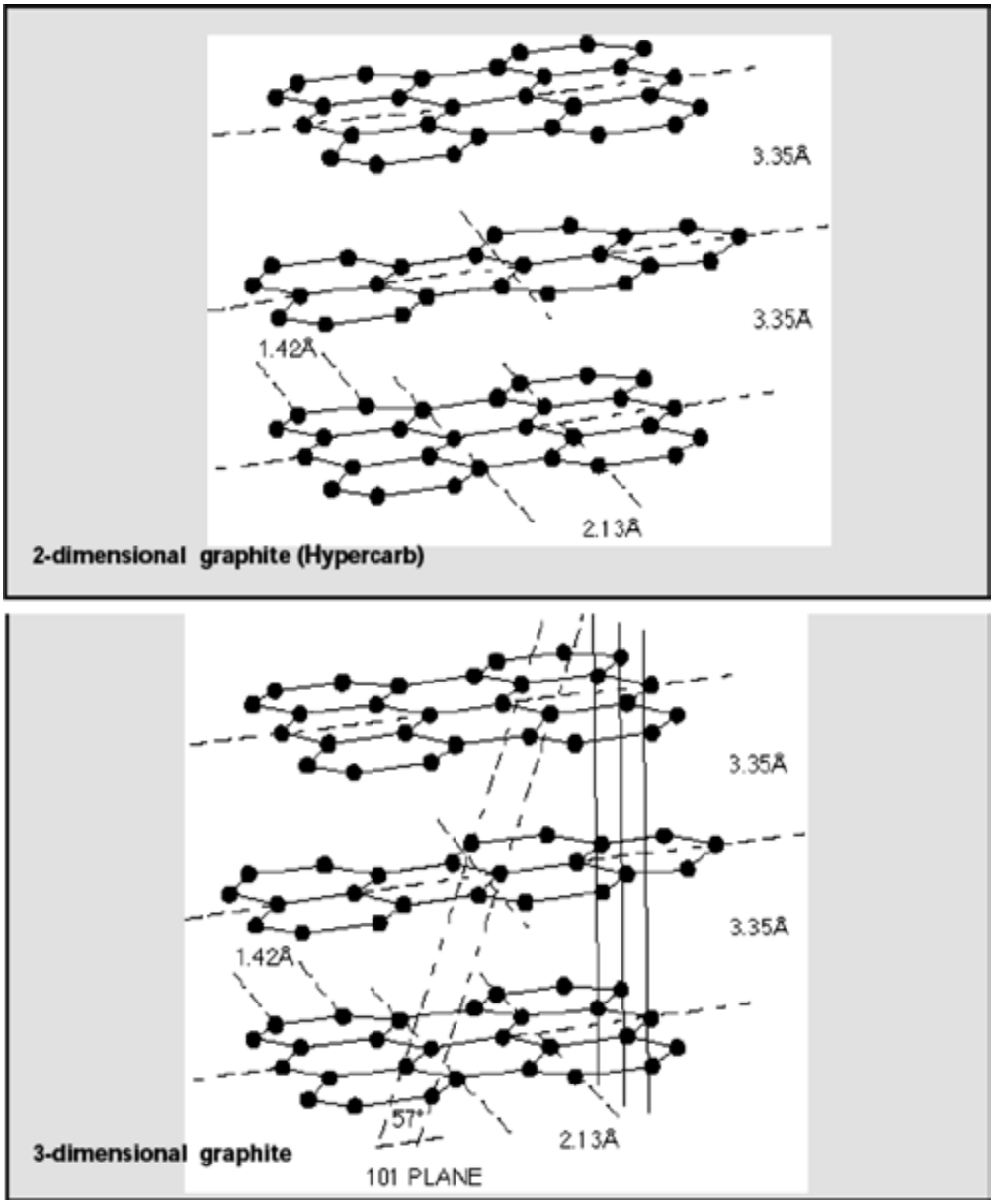


Fig. 6: Crystal structure of graphite: (a) 2-dimensional graphite with no layer registration (b) 3-dimensional graphite with ABAB [28, 30].

Because the PGC Hypercarb material consists of flat sheets of carbon, the retention is based on interaction with the planar analyte[30] (Fig. 7). Similarly charged molecules interact differently with the planar sheets as show in Fig. 8.

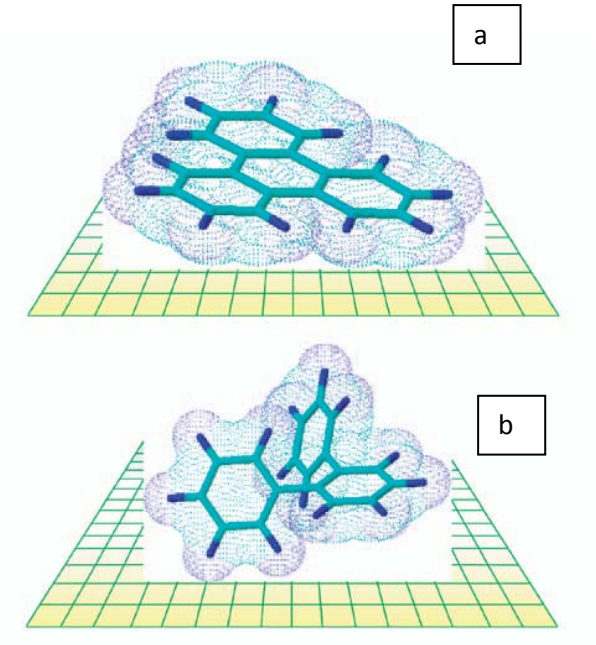


Fig.7: Retention mechanism of PGC material (a) Good alignment of planar molecule to the flat graphite surface; (b) Poor alignment of non-planar molecule to the flat graphite surface [30].

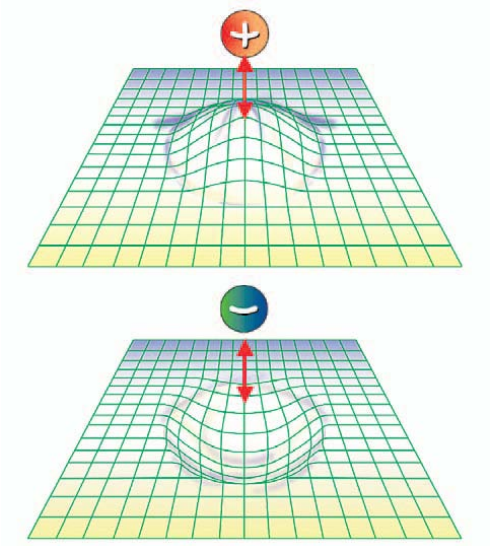


Fig. 8: Schematic representation of polar analyte retention in which (a) positive charge and (b) negative charges approach the graphite surface, resulting in a charge-induced dipole at the graphite surface [30].

The PGC Hypercarb packing is stable across the entire pH range of 0-14. It can be used for both normal and reversed phase separations, and the very robust nature of the material gives exceptional column lifetimes [28, 30]. The Hypercarb also gives good peak shapes for mono functional like benzene, phenol, ethers, acids, and amines. PGC Hypercarb can also separate inorganic anions in ion chromatography and heterocyclic compounds with ordinary mobile phases [28-32]. Some of the above

mentioned advantages can be explained in Fig. 9, which show the difference between silica based reversed phase and Hypercarb.

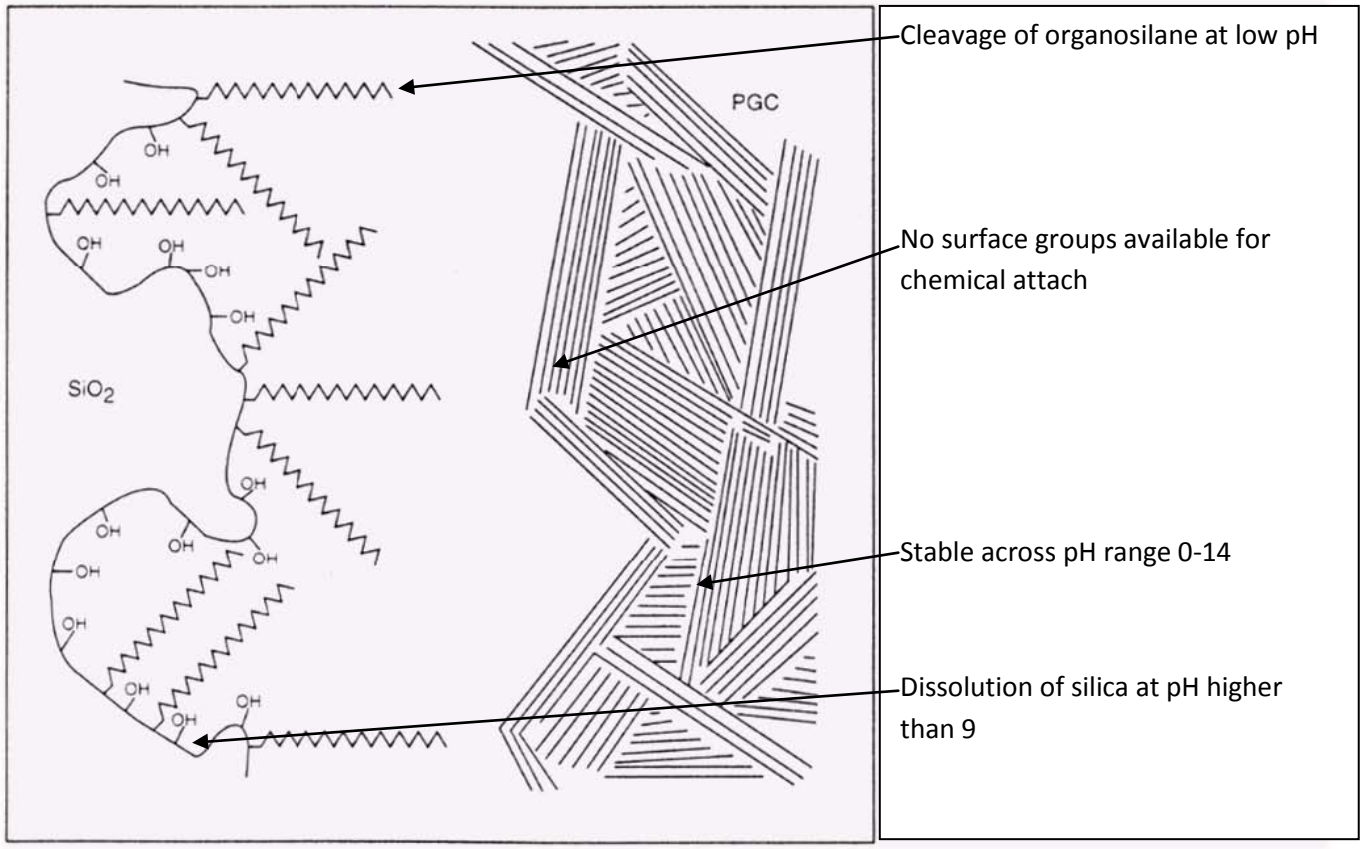


Fig. 9: Surface comparison between C-18 bonded silica and Hypercarb PGC. [32]

1.5 Use of temperature in chromatography

When LC analyses are performed at higher than ambient temperatures, improved peak shapes and faster analysis times are obtained. This phenomenon can be explained as mobile phase flow and diffusion characteristics are totally influenced by the relationship between temperature and viscosity [23]. In general, by increasing the temperature the following chromatographic parameters are influenced, retention is reduced by increasing temperature and the peak shape (efficiency) is also improved. Higher temperature also improves selectivity and reduces the column back pressure due to less viscosity [23, 33]. The temperature has different effects on RP and HILIC separations. This has been investigated in the present work.

1.6 Comprehensive two dimensional chromatography

The meaning of 2D LC is that two independent liquid phase separation systems (such as HILIC and RP, or HILIC and Hypercarb) are used together to separate the compounds in a sample. 2D LC can be performed in two ways. By transferring only one interesting or useful portion of the first dimension system effluent to the second dimension, “heart cutting” chromatography is performed. By transferring the whole of first dimension effluent to the second dimension “comprehensive 2D” chromatography is performed [4].The interest in the 2D LC has increased because of the increasing need to separate complex biological sample in the field of e.g. proteomics and metabolomics. The plants samples investigated in the present study also contain a large number of compounds like fatty acid, essential oil and metabolites. Since plant samples contain many compounds, the peak resolving power of conventional one-dimensional LC is not sufficient. To resolve the compounds in these complex samples a system with a large peak capacity is needed (separate more compounds, see below) [4].

In 2D LC the second dimension column should provide separation of the compounds eluted in fractions from the first dimension. The whole second dimension system can be considered a chemical selective detector which further separates the compounds of the first dimension eluted the flow diagram of this concept is shown in Fig. 10 [4].

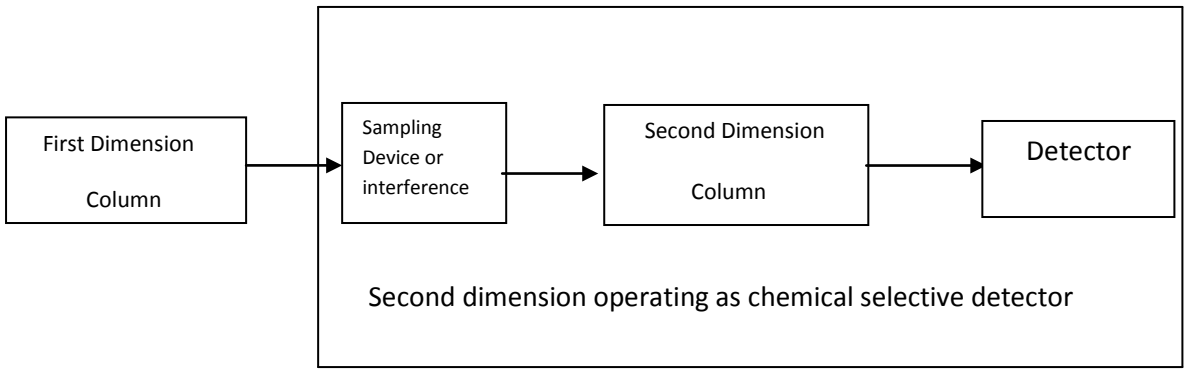


Fig. 10: Block diagram of instrumentation for 2D LC. The box indicates that the second dimension of the system effectively acts as a chemically selective detector for the compound that elute from the first dimension column [4].

1.7 Peak capacity

It is important to understand the idea of peak capacity; under ideal conditions the overall peak capacity of the 2D LC system is the multiple of peak capacity of the first dimension and the second dimension. This concept of peak capacity was first study by Karger in 1973 [34] later Giddings [35] and Guiochon et al. [36] proved this concept by further theoretical and practical research. The overall peak capacity ($n_{c,2D}$) is equal to the product of the individual peak capacities of the first and second dimension separations (^1n_c and ^2n_c, respectively) as shown in Fig. 11 and eq. 1.

$$n_{c,2D} = {}^1n_c \times {}^2n_c \quad (1)$$

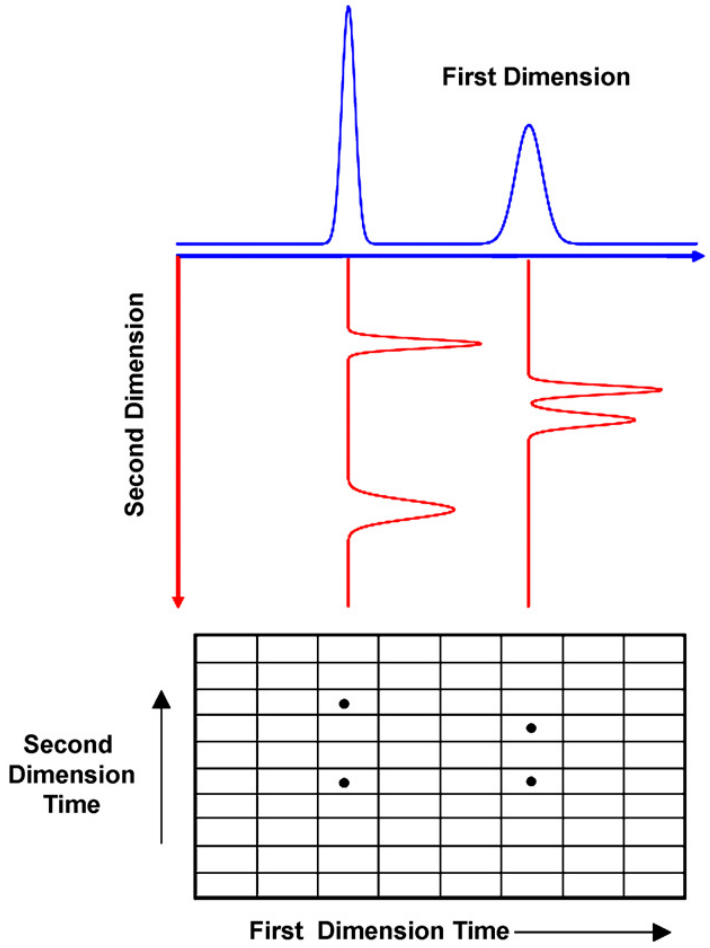


Fig. 11: Illustration of the multiplicative relationship between the peak capacities of the independent first and second dimensions in comprehensive two dimensional separations[4].

1.8 History of 2D LC

The history of multi dimensional liquid chromatography is not very long. The first comprehensive review of 2D LC was presented by Schure [37]. The work of Erni and Frei[38] was very important for the development of online 2D LC system because they provide the initiated work on the coupling of two different chromatographic system in a successful way. In the field of proteomics, a 2D LC system was introduced in 1990 by Jorgenson and Bushey[39]. Wolters[40],Regnier[41] and Moore [42] utilized the same 2D LC technique for the separation of a complex protein sample, but it took 6 h to complete the analysis of single sample. The idea of fast second dimension analysis was introduced by Stoll et al.[43] in 2006. By the introduction of high temperature to obtain fast second dimensional analysis, the capacity of 2D LC to separate complex sample is also improved.

1.9 Representation of 2D LC results

Displaying the result from both dimensions in such way that it covers all the data provided is important. Long complex chromatograms data from first and second dimension chromatograms can be presented in 2D [44]. The eluted first dimension fraction that was unresolved in first dimension column is subjected to the second column, and the data from these two systems are combined in such a way that it can be expressed in 2 or 3 dimensions Fig 11 shows in step 1 the collection and the transfer of first dimension column effluent to the second dimension. In the subsequent separation on the second dimension column, a series of sequential second dimension chromatograms are collected as one string of data. Data from the sequential second dimension chromatograms can be reshaped to produce a variety of different representations of the 2D chromatogram[44].

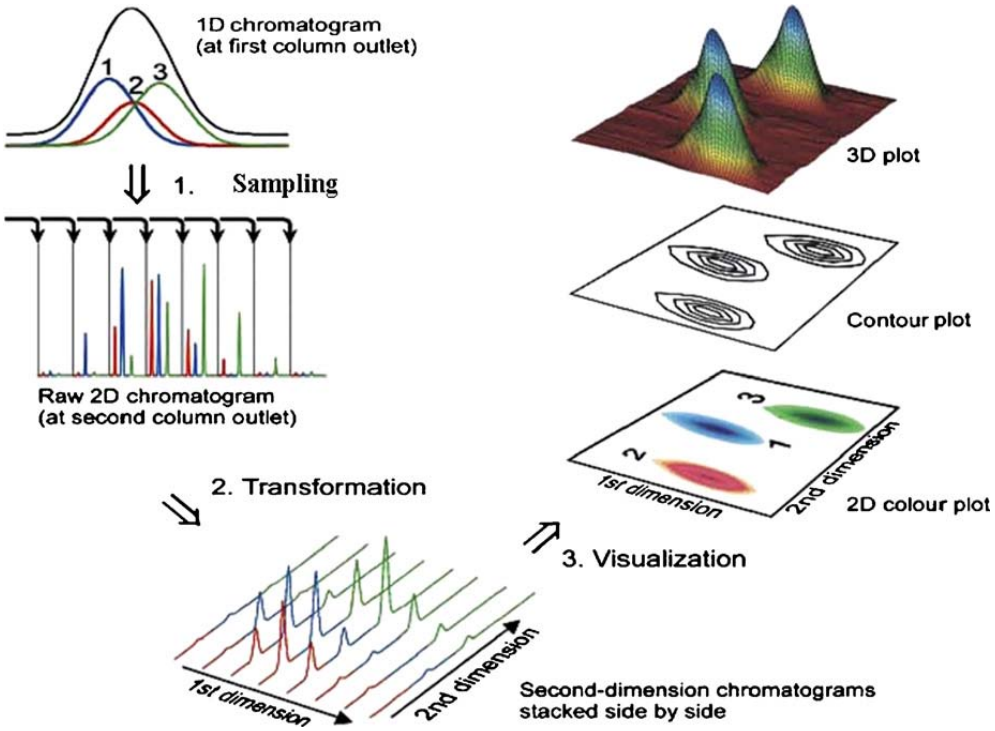


Fig. 11: Illustration of the information as it is collected and analyzed in comprehensive 2D separation experiments [44].

1.10 Off-line 2D LC

Off-line 2D LC has been successfully applied in number of application like the separation of peptides and protein digests [39, 45]. Comprehensive off-line 2D LC has also been successfully used for the separation and identification of procyanidins [46] and polyphenols in red wines [47] [48]. The main disadvantage to used off-line 2DLC is that the analysis time is long and sometime hours are needed to complete one analysis. Off-line 2DLC systems require more operator attention since automation is difficult. On the other hand it has capacity to separate more compound than online.

1.11 Online 2D LC

Online 2D LC has also been successfully applied in the field of proteomics [49], and has showed its resolving power for the determination of fatty acid in fish oil[50]. Successful serial coupling of RP and hydrophilic interaction liquid chromatography has been used for the separation of pharmaceutical complex compounds[51]. By applying two parallel columns in the second dimension the online 2D LC system can provide more orthogonality and peak capacity[52]. Online 2D LC also successfully used for the separation and identification of saponins from *Quillaja saponaria* [53] by coupling anion-exchange and hydrophilic interaction columns [54]. Determination of bradykinin in rat muscle tissue dialysate was obtained with online solid phase extraction (SPE)-HILIC-SPE-RP-MS [55] and recently 2D LC has been used in a number of applications, e.g. for identification of small hydrophilic angiotensin I-inhibiting peptides in milk hydrolysates [56]. In these studies mostly HILIC has been used as the first dimension and RP as second dimension. Some new combinations have been applied for the separation of complex compounds, one of the most developed of these methods are using an online HILIC x RP LC system with two detectors [57], by using fully automatic HILIC/RP column-switching LC chromatographic system for the complementary analysis of complex plant samples [58]. To explore the different combinations of multi dimensional LC system a comprehensive study was conducted and the results of this study are arranged in tabular form to make it brief and short as shown in Table 1. From the literature survey it is clear that very little or may be no work has been done on 2D LC system with HILIC and Hypercarb combinations, as will be discussed in this thesis. The analysis time is generally small compared to off-line 2DLC. Totally automated system can be used and high peak capacity as compared to off-line 2D LC is obtained. The main disadvantage of online 2D LC is that the whole system is automated so any fault at any part of system can destroy the whole analysis.

Table 1: 2D LC methods

Mode of combination	Stationary phases	Mobile phases	Analysis time (min)	Sample	Type of 2D LC	Ref.
RP X RP	CN X monolith C18	Gradient (MeOH and H2O X AcN and H2O)	220	Adinandra nitida, one type of traditional Chinese medicine (TCM)	Online	[59]
RP X RP	HS-F5×carbon clad Zirconia	Gradient (phosphate buffer and AcN X 20 mM perchloric acid in water and AcN)	30	Corn metabolomics	Online	[43]
RP×RP	Monolith C18×monolith C18	Isocratic (gradient) water and tetrahydrofuran (THF) × isocratic (gradient) water and MeOH	60	Aromatic compounds	Online	[60]
RP×RP	C5×C18	Gradient(AcN (TFA) × Formic acid, IPA		Peptide Purification	Online	[61]
RP×RP	X-terra C18×SB-Phenyl	Gradient AcN, TFA in H2O X AcN,H2O	20	Proteomics	Online	[62]
RP×RP	ODS-Q × monolith C18	Gradient (AcN, TFA	40	Mixture of some aromatic amines and non-amines	Online	[63]
RP×RP	C18 × carbon clad zirconia (CCZ)	MeOH × can	250	Complex mixture of Oligostyrenes	Online	[64]
RP×RP	CN×C18	Gradient and isocratic MeOH, H2O × acetic acid buffer and can	200	Pharmaceutical traditional Chinese medicines	Online	[65]
NP×RP	Microbore silica × monolithic C18	Gradient (*n*-hexane and ethyl alcohol × 2-propanol, AcN, water	>160	Orange essential oil and juice carotenoids	Online	[66]
RP×NP (HILIC)	C18 Microbore × aminopropyl Silica	Gradient (AcN × isocratic ethanol-dichloromethane – water	120	Polymers: ethylene oxide–propylene oxide (EO–PO) (co) oligomers	Online	[67]

NP×RP	Microbore silica × monolithic C18	Isocratic n-hexane / ethylacetate × gradient water / can	50	Lemon oil	Online	[68]
RP×IEC (SCX)	C18 × parallel 48-plexed SCX	Gradient (AcN (TFA) × AcN (phosphate, KCl)		Proteomics	Off-line	[69]
IEC (SCX)×RP	SCX micro-trap × Poroshell 300SB-C18	AcN, water, TFA		Proteomics: phosphopeptides enrichment	Online	[70]
IEC (SCX)×RP	PO4-irconia × SB-C18	Gradient(sodium Phosphate × AcN, TFA)	20	Tryptic peptides	Online	[71]
IEC (SCX)×RP	PL-SAX × polystyrenedivinylbenzene (PS-DVB)RP	pH gradient × pH gradient		Proteomics	Online	[72]
IEC (SCX)×RP	Poly LC-SCX × C18 on Chip	Stepped ammonium acetate salt × gradient can	>480	Proteomics	Off-line	[73]
IEC (SCX)×RP	Polymeric beads bonded with diethylaminoethyl and sulfonic acid groups × C18	Gradient KH2PO4 × gradient AcN (TFA)	20	Proteomics	Online	[74]
IEC (SCX)×RP	BioX-SCX×C18	Stepped NaCl salt solution × gradient AcN	>600	Identify tryptic peptides from the immunoprecipitate	Online	[75]
NP (liquid chromatography at critical conditions (LC-CC))×SEC	Alltech Platinum Silica × HSPgel-RT MB-L/M	Chloroform / diethylether × chloroform		Polymer: degradation product of poly(bisphenol A)carbonate (PC)	Online	[76]
NP×SEC	Hypersil “bare” Silica × PLgel	Isocratic 48% ACN in DCM × THF	90	Polymer Characterization: poly(methylmethacrylate) (PMMA)	Off-line	[77]
NP×SEC	Hypersil “bare” Silica ×Mixed-C	Isocratic THF–hexane × THF	240	Polymer: polystyrene (PS)	Online	[78]
SEC×RP	G2000SWXL × BDS-C18	Water (TFA) × gradient AcN (TFA)	320	Peptides	Off-line	[79]
RP×SEC	NovaPak silica × HSPgel	Gradient × THF	300	Analysis of a series of styrene-methylacrylate (SMA) copolymers	Online	[80]

Affinity (titania) × RP	Titania Column × monolith C18	Gradient potassium Phosphate × gradient AcN (TFA)	30	Phosphopeptides	Online	[81]
Affinity (silver)×RP	Silver column×monolith C18	Isocratic 0.7% (v/v) acetonitrile in *n* –hexane × gradient isopropanol and acetonitrile	140	Food analysis: rice oil	Online	[82]

1.12 Detection

A UV detection system was used in this study. A multi-wavelength technology detector was the detector that was available to the analyst at this time. The UV detector has some limitations since compounds that do not possess UV chromophores cannot be detected. However most compounds absorb UV light in the range of 200-350 nm including all substances having two or more double bonds in conjugation (**π** electrons) and all substances that have unshared (non bonded) electrons; *e.g.* all olefins, all aromatics and all substances containing >CO, >CS, -N=O and –N ≡ N groups.

1.13 Samples

Totally 19 samples of dry plant material were available for the project. These plants belong to 4 different genera. All plants except *Ruta graveolens* are aromatic plants, have interesting compositions of essential oils (which means that they contain mono-, sesqui- and probably di- and triterpenes), and their essential oils have certain antibacterial activity[82-83].

1.13.1 Acinos

Acinos (as shown in Fig.12) constitute a group of ten species of annual and short-lived evergreen perennial woody plants. They are small aromatic plant, tufted, bushy or spreading, growing to 10-45 cm tall [84]. The genus Acinos is represented by five species in the Flora of Serbia and Montenegro. *Acinos arvensis* is used for medicinal purposes as an antiseptic, stimulant, tonic and antispasmotic[84]. Selected species are *Acinos hungaricus, Acinos majoranifolius, Acinos suaveolens , Acinos arvensis, Acinos graveolens , Acinos alpinus.* These species contain 17 fatty acid [85] and 33 essential oils [86].

Fig. 12: Acinos Specie (Photo by: Bernd Gliwa, Source: www.biolib.de)

Ref :(http://en.wikipedia.org/wiki/File:Acinos_arvensis_3.jpg)

1.13.2 Calamintha

The Botanical name of Calamintha is *Calaminthao officinalis* (MOENCH) and it belongs to the family: N.O. *Labiatae*[84]. *Calamintha glandulosa, Calamintha vardarensis, Calamintha ashei, Calamintha coccinea, Calamintha dentata, Calamintha grandiflora, Calamintha nepeta, Calamintha officinalis* are the species. One of them is shown in Fig 13.

Fig. 13: Calamintha Specie (Photo by: Kurt Stueber, Source: www.biolib.de)
Ref: (http://en.wikipedia.org/wiki/File:Calamintha_grandiflora2.jpg)

1.13.3 Satureja

Satureja (as shown in Fig. 14) is a genus of aromatic plants of the family *Lamiaceae,* related to rosemary and thyme. *Satureja cuneifolia, Satureja Montana, Satureja horvatii, Satureja hortensis* are the selected species[84]. These plants contained 19 Essential oils[83b]. *Satureja* species are used for making traditional tea-drinks with its refreshing and pleasant taste. It has also positive effects in case of digestion problems, pain in the stomach, and loss of appetite [83b, 86-87].

Fig. 14: Satureja specie (Photo by: Kurt Stueber, Source: www.biolib.de)
(http://en.wikipedia.org/wiki/File:Satureja_montana0.jpg)

1.13.4 Ruta

Ruta (as shown in Fig. 15) is a genus of strongly scented evergreen, 20-60 cm tall, in the family *Rutaceae*. Between 8 and 40 species have been found in the genus. The most well-known species is the Common Rue[84]. *Ruta graveolens and Ruta graveolens* wild. *Ruta graveolens* is known for its abortive effect in women, and it was used in traditional medicine for interrupting undesired or problematic pregnancy. How effective it is and which compounds are responsible for such an activity are unknown [83b, 86-87].

Fig. 15: Satureja specie (Photo by: Kurt Stueber, Source: www.biolib.de) (http://en.wikipedia.org/wiki/File:Ruta_chalepensis11.jpg)

1.14 Aim of study

The purpose of this study was to develop and examine an online 2D LC system for separating plant extract. These extracts are very complex and cannot be separated by 1D LC alone. An online 2D LC system with two detectors and columns (HILIC X Hypercarb) was used. The effect of temperature and the column-to-column and run-to-run retention time repeatability were examined in order to obtain a fast and reliable method.

2. Experimental

2.1 Materials and reagents

HPLC grade acetonitrile (AcN) was obtained from Rathburn Chemicals Ltd (Walker burn, UK) and VWR international. Methanol was obtained from VWR. Trifluoroacetic acid (TFA) (99%) and formic acid (FA) (50%) were purchased from Fluka (Sigma Aldrich). Analytical grade ammonium acetate from Merck (Darmstadt, Germany) was used as mobile phase additive. Fused silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA), and couplings were made with graphite ferrules (Valco International, Houston, TX, USA) and steel nuts (Valco). Tetrahydrofuran (THF) (>99.7%) and 1-propanol (>99.7%) were purchased from Merck. Toluene (glass distilled grade) was obtained from Rathburn. Ethanol was purchased from Arcus (Oslo, Norway). Nitrogen (99.99%) was obtained from AGA (Oslo, Norway). Type 1 water was produced with a Milli-Q Ultrapure water system (Millipore, Bedford, MA).

2.2 Sample and standards

Green tea bags (Twinings co. limited London) were used as test samples. Satureja Montana I, Acinos Hungariais, Satureja Cunrifolia, Calamintha Glandulosa, Ruta Graveolens, Satureja Montana II, Satureja Montana III were the Serbian plant samples obtained from Serbia. Uracil, benzoic acid, toluene, 3-bromophenols, citric acid, and oxalic acid sodium benzoate were purchased from Merck Sigma Aldrich.

2.3 Preliminary off-line 2D LC experiments

A preliminary off-line system was assembled to develop the method for separation of compounds from Serbian plants extracts.
A commercial green tea plant extract sample was used as a test material in the preliminary experiments due to limited amount of the Serbian plants.

2.3.1 Sample preparation

The samples were extracted in three different solvents. One method involved methanol extraction and one AcN/water (1/1, v/v) extraction. A dried green tea bag was dipped in 100 ml of methanol and the solution was stirred for 5 min before treated ultrasonically for 15 min. The tea bag was then removed. The same procedure was used when extraction was performed with (50/50, v/v) AcN/water. The Serbian plant samples were also extracted with these extractions solvents. In each experiment about 250 mg of plant material were extracted with 100ml of solvent.

2.3.2 First Dimension separation

2.3.2.1 Instrumentation and chromatographic conditions

The conditions used for the first dimension separation of the compounds from the tea sample were obtained by performing some preliminary experiments. For the first dimension separation an Agilent 1100 series (Agilent, Palo, Alto, CA, USA) pump with online vacuum degasser was used for mobile phase delivery. UV detection was performed at 254 nm with a Waters (Waters Milford, MA**,** USA) 486 tunable absorbance detector. Instrument control was carried out by Totalchrome version 6.2.1. All connections were made with tubing (Valco). A Selerity Technology Inc. Polaratherm series 9000 column heating compartment was used to increase the temperature of the column. The instrumentation of the first dimension separation is shown in Fig 14.

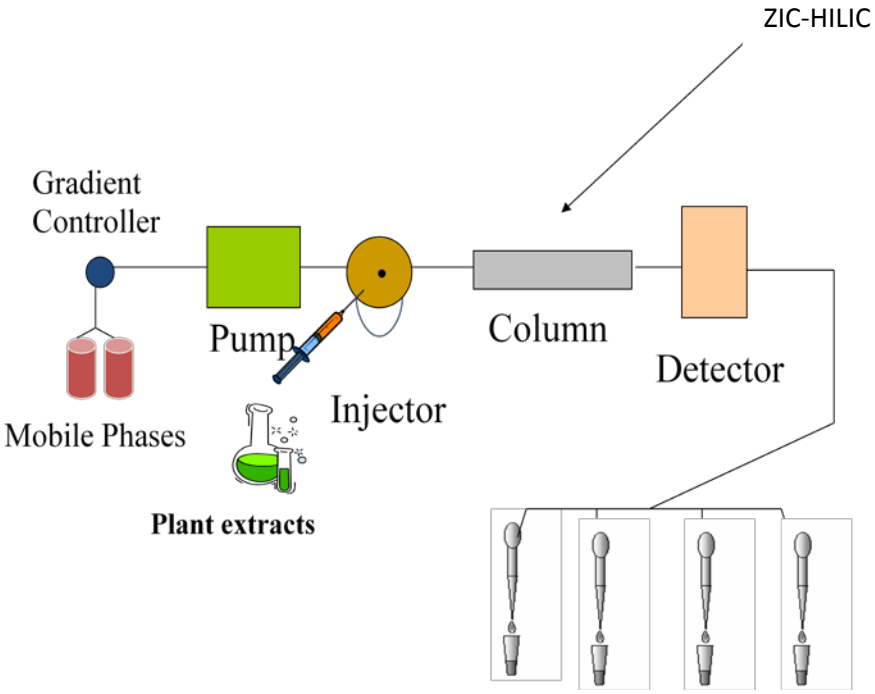


Fig 14: The HILIC first dimension system. The fractions collected after the detector was used for the second dimension separation.

Separations of the green tea sample (both methanol and AcN/ water extracts) were performed on a ZIC-HILIC (150 mm× 4.6 mm i.d. 3.5 µm particle size, 200 Å pore size) column from Merck SeQuant, Umeå, Sweden. The mobile phase consisted of (A) 10 mM ammonium acetate pH 7.9 and (B) acetonitrile. Separations were investigated by an isocratic elution at 85 % B and 15 % A. The flow rate was 0.50 ml/min and UV detection was performed at 254 nm. Separations were performed at 45 °C and 50 µl were injected. For one-dimensional separations, the column outlet was connected directly to the UV detector by using PEEK tubing (O.D. x i.d; 1/16 inc X 0.02 inc).The eluent after the detector was collected in small poly propylene vials every 2 min. Totally 20 vials of samples were collected during 40 min. These vials were stored for further use in the second dimension.

2.3.3 Second dimension separation

Fractions from first dimension were treated with nitrogen gas to evaporate the excess solvent. The dried fractions were subsequently dissolved in the mobile phase solvent. The binary mobile phase consisted of 0.1% formic acid in water (A)/ AcN (B) (30/70 % v/v). UV was recorded at 254 nm with a Shimadzu SPD 10 A (Shimadzu Albert-Hahn, USA) UV detector. The gradient methods were developed for fast RP LC. The separation was performed using a Hitachi L 7100 HPLC Pump (Hitachi Tokyo, Japan) on an ACE RP C18 column (50 mm×4.6 mm i.d., 1.8 µm). A linear gradient was performed from: 5 % B to 100 % B in 20 min. The flow rate was 1.0 ml/min and 50 µL were injected. Instrumentation used for the second dimension separation is shown in Fig 15.

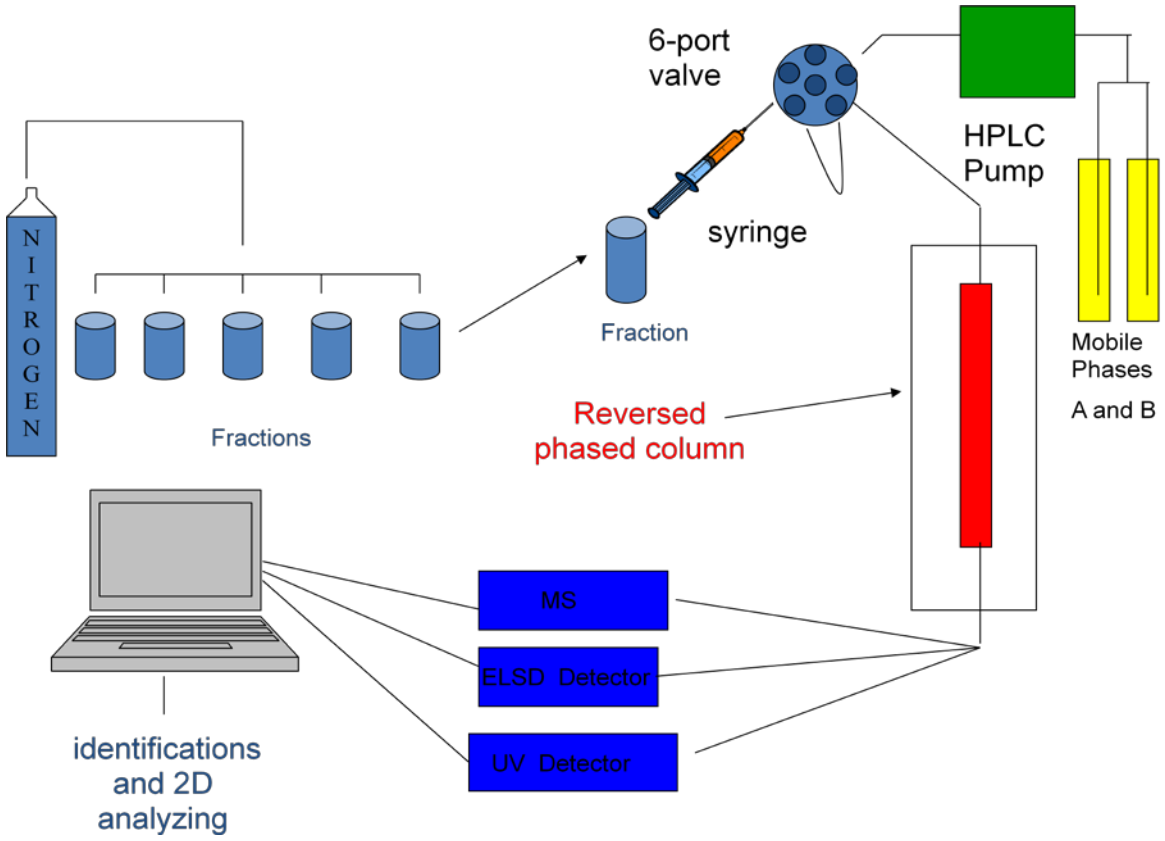


Fig 15: Second dimension separation system.

2.4 Online 2D LC system

The basic model of the online 2D LC system used in this work is shown in Fig 16.

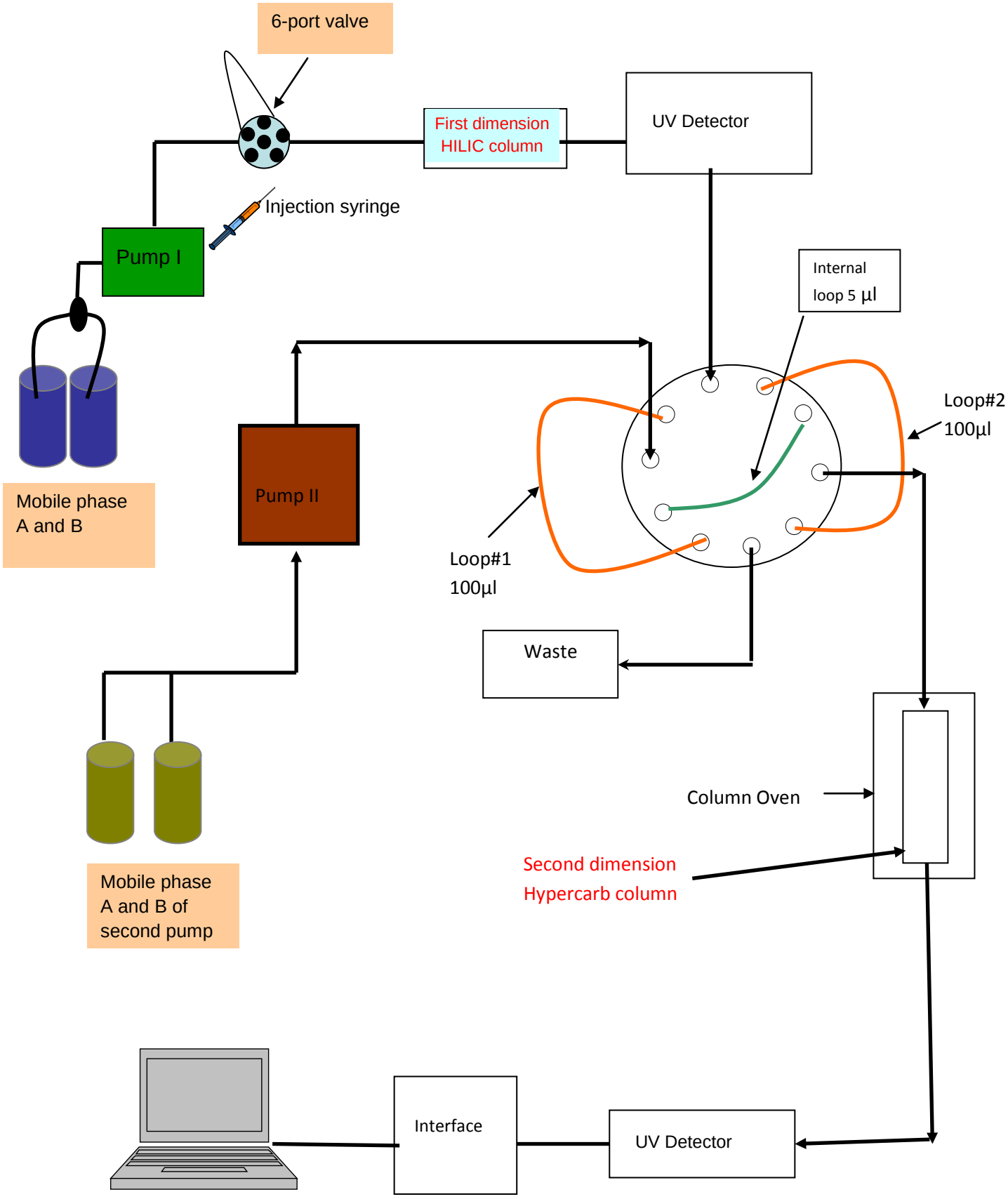


Fig 16: Representation of the online 2D LC system

The first dimension of the 2D LC instrument was comprised of the same components as described above for the off-line 2D separation, including the use of the HILIC column as the first dimension column. The outlet of the first dimension column was connected to the inlet of the 10-port valve (Valco Instruments,Houston, TX) shown in the diagram of the complete 2DLC instrument shown in Figure 16. Switching of 10-port valves was automatically controlled by the Agilent pump (pump1). The two sample loops (loop 1 and loop 2 in Fig. 17) were used to alternately capture fractions from the first dimension separation and deliver them to the second dimension Hypercarb column (50 X 4.6 i.d (mm), particle size 3 µm, Thermo Scientific Germany through PEEK tubing. The Polaratherm series 9000 column heating oven (Selerity Technology Inc Germany) was used to preheat the column at 100 °C The Agilent 1100 series and a Hitachi L 7100 pumps (pumps I and II in Fig. 16) were used in the first and second dimension of the 2D LC system, respectively. The mobile phase used in the first and second dimension was a combination of B (AcN) and 10 mM ammonium acetate pH 7.9 (A). A picture of instrument is shown in Fig. 19.

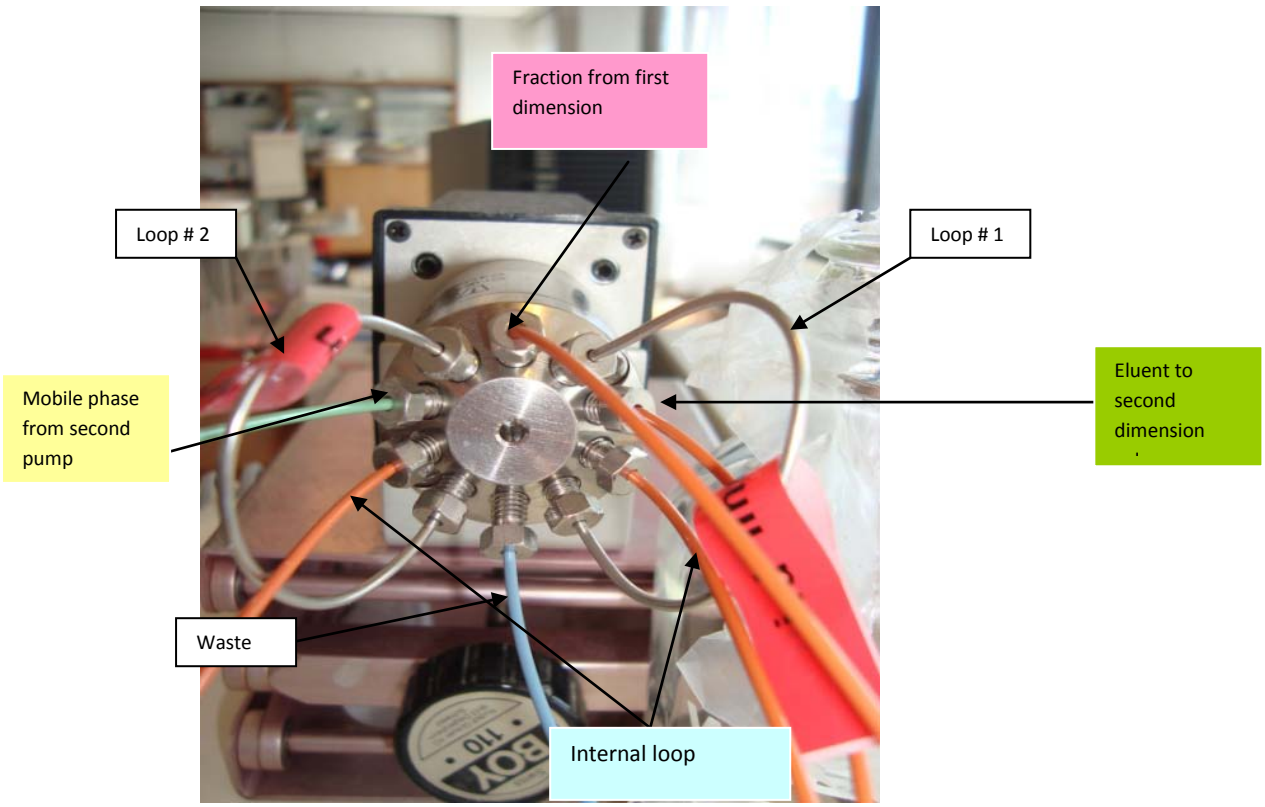


Fig. 17: 10 port switching system for online 2D LC system.

Both the first and the second dimension separation were carried out in isocratic mode. In the first dimension the flow rate was 0.05ml/min with mobile phase composition 85 % B and 15 % A. In the second dimension the flow rate was 2 ml/min and mobile the composition were 70 % B and 30 % A (same mobile phases as in the first dimension). The interface (10 port valve) used to combine first and second dimension is shown in Fig. 18. UV detection of the first dimension separation was performed at 254 nm with a Shimadzu UV detector and the data were collected by Total chrome 6.2.1 software. The 10 port valve was automatically controlled and the positions A and B were kept for 2 min each. When the 10 port valve was in position A, the eluent from first dimension filled the loop #1 (100 µl) in 2 min. When the10 port valve was turned to position B, the eluent from first dimension filled loop # 2, and at the same time the mobile phase from the second dimension pump II transferred the sample from loop # 1, with the help of the inter loop. The first dimension fraction was separated on the second dimension column in 2 min and detected by Waters **486** tunable absorbance detector. The chromatograms of both detectors were recorded simultaneously by the Totalchrom software. Some of the data were sent to Svein Mjøs (Department of Chemistry at the University of Bergen) who has made the contour 2D plot that were presented in this study.

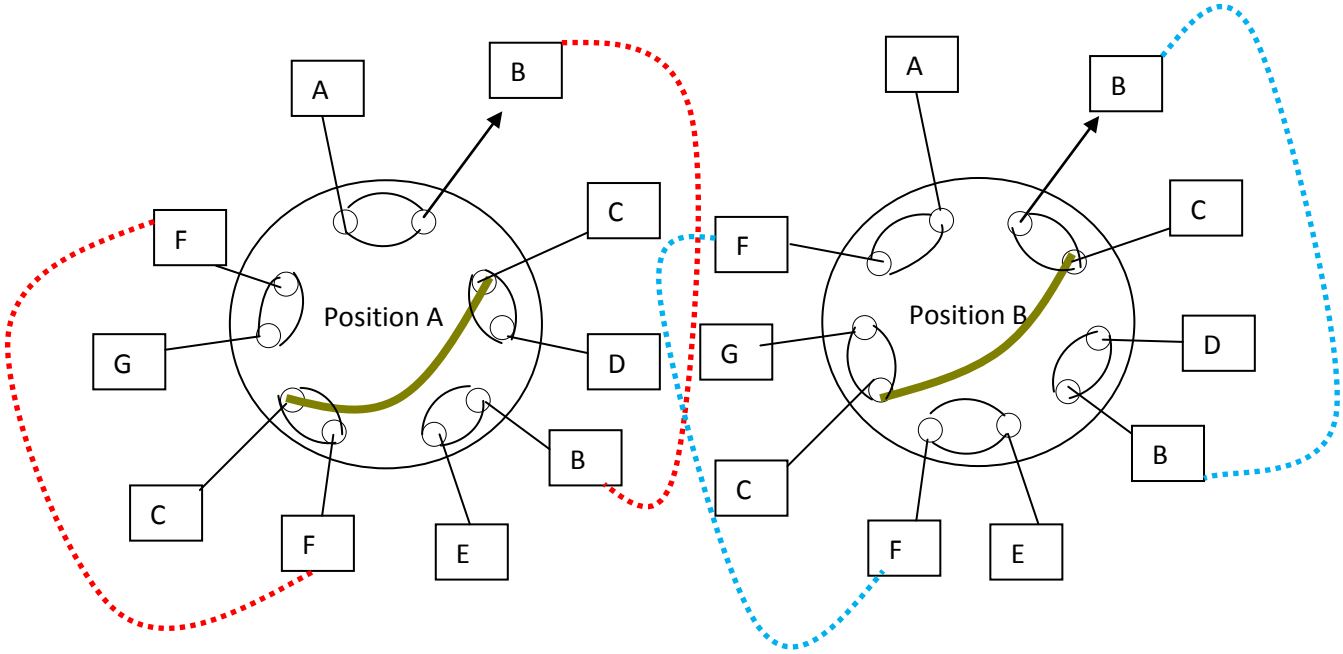


Fig 18: Switching system of online 2D LC system with two different positions. Left: sampling of fraction in loop #1, and analyzing fraction in loop #2. Right (opposite)

Fraction from first dimension	A	Waste	E
Loop # 1	B	Loop # 2	F
Internal loop	C	Mobile phase from second pump	G
Eluent to second dimension column	D		

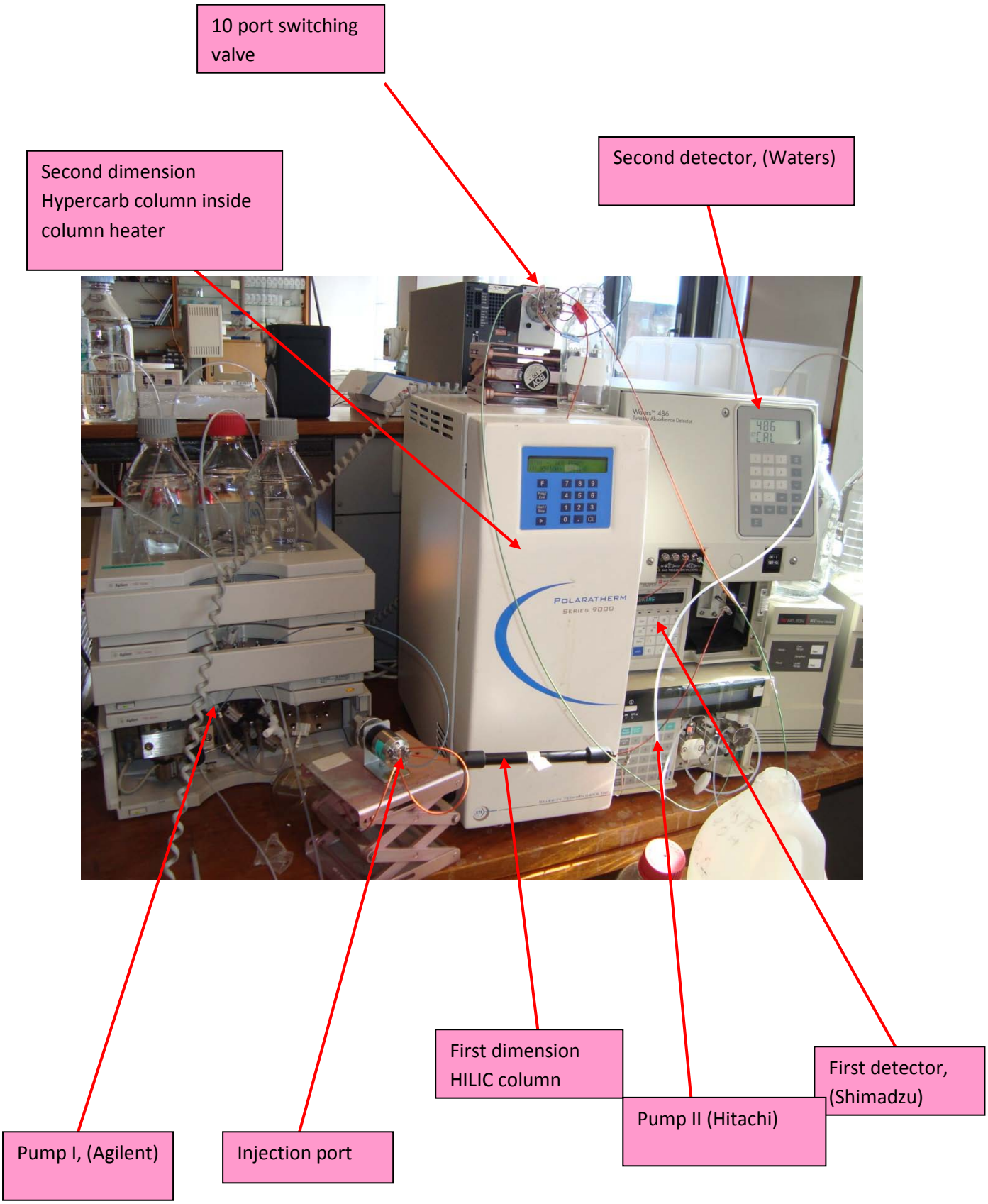


Fig 19: Online 2D LC instrumentation.

3 Results and discussion

It is difficult to appreciate preliminary 2D LC results (presented throughout this section) without first seeing a combined 2D plot (the finished result). An example is presented with description to make it easy for the reader to understand the 2D plot (Fig. 21). A 2D plot displays the result from both dimensions in such way that it covers all the data provided. The compounds that were unresolved in first dimension column (a chromatogram with poor resolution is shown in Fig. 20A) is subjected to the second column by the two loops 10 port interface in online or as fractions in off-line mode. In the second dimension the fractions are analyzed separately. In our online 2D LC system (explained in experimental part) the flow rate of the first dimension was 50 µl/min and one loop of the (10 port switch) interface could store 100 µl in 2 min. The second dimension system has thus 2 min for analying the fraction, so fast (high flow rate) second dimension is needed. During 80 min of first dimension the second dimension separately analyze 40 fractions (as shown in Fig. 20B). The data from these two systems are combined in such a way that it can be expressed in 2D with resolved compounds as shown in Fig. 21. The system can be explained as the collection and the transfer of first dimension column effluent to the second dimension. Separation in the second dimension column produces a series of sequential chromatograms collected as one string of data. Data from the sequential second dimension chromatograms can be reshaped to produce a variety of different representations of the 2D chromatogram as shown in Fig. 11.

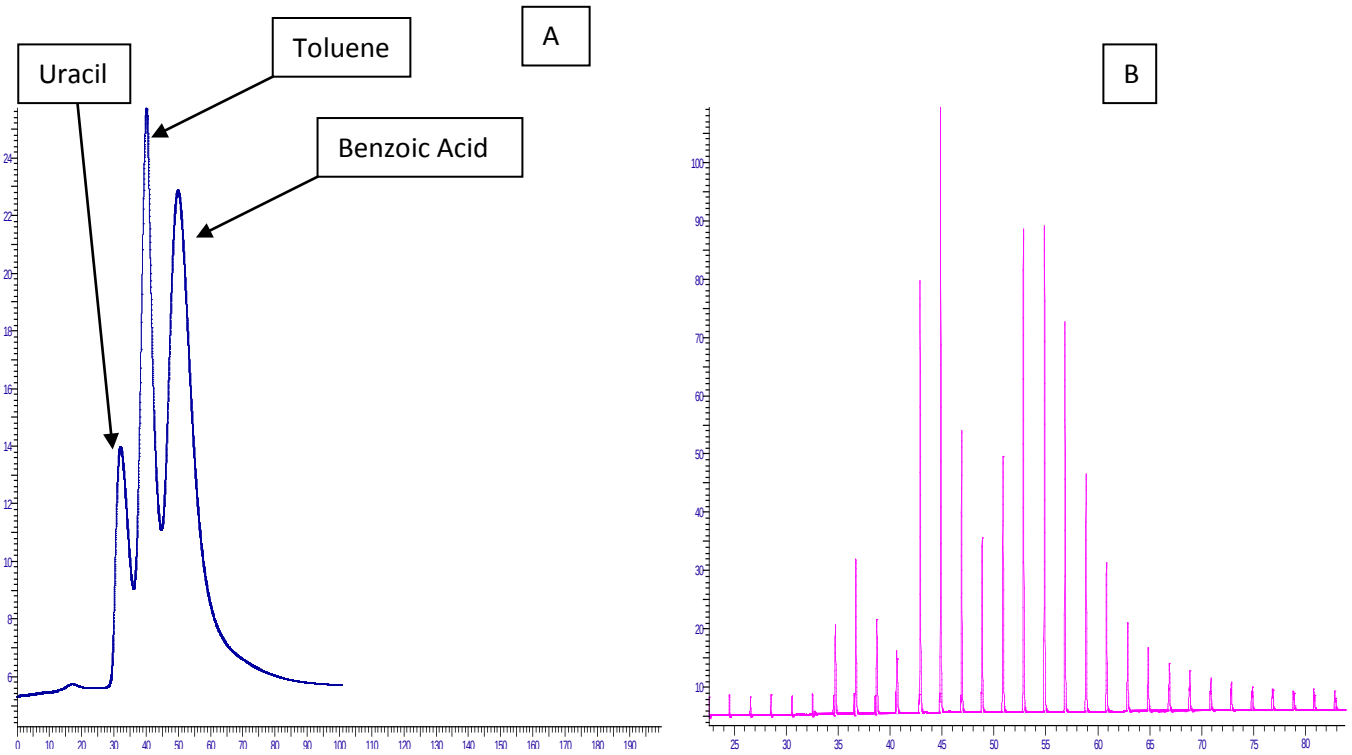


Fig. 20: Chromatograms of three standards, uracil, toluene, benzoic acid on a HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10 mM ammonium acetate (A)/AcN (B) with isocratic elution 85/15 % v/v min with a flow rate of 0.05 ml/min. UV detection of first detector was performed at 254 nm. Second dimension separation was performed on a Hypercarb column (50 mm× 4.6 mm i.d) with isocratic elution with 10 mM ammonium acetate (A)/AcN (B) 30/70 % v/v with a flow rate of 2 ml/min at 110 °C. Detection of second detector was also performed at 254 nm. Injection volumes of 50 µl were used. (A) first dimension and (B) second dimension chromatogram.

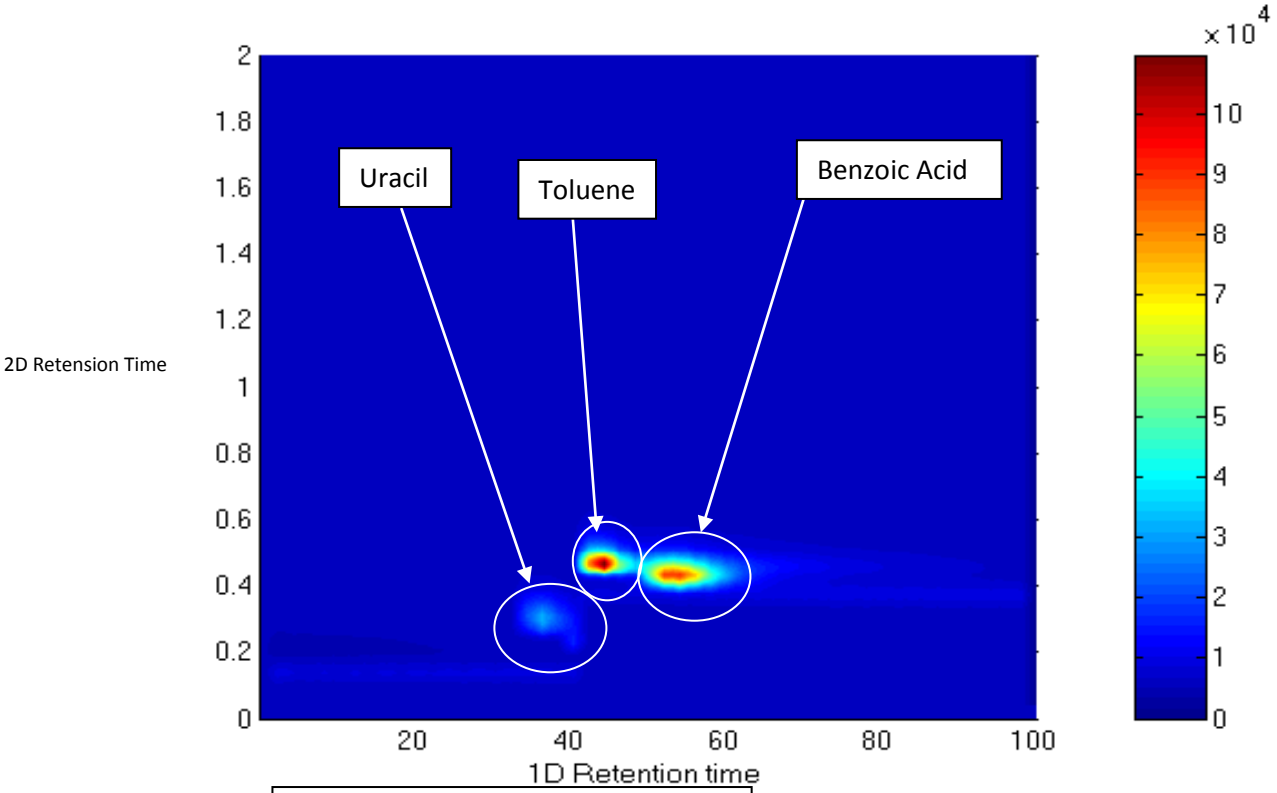


Fig.21: contour 2D plot of Fig. 20 A and B

High resolving power is the main advantage of 2D LC. We will here discuss the optimalization of the 2D LC combination of HILIC and Hypercarb chromatography.

3.1 Preliminary off-line 2DLC experiments

In the beginning of this study, different experiments were performed as off-line 2D LC because this approach was easier to handle regarding the method development of the first dimension separation on HILIC columns and second dimension separation on RP and Hypercarb columns. Another reason to perform off-line 2D LC separation is to compare the peak capacity with the online system and evaluate the best method (comparison is discussed in online section). The experiments involved in both dimensions were performed separately to check the effect of different parameters. Neither first not second dimension gives perfect resolution, but the two dimensions will work together. Therefore, individual chromatograms in both dimensions may look poor, but would look better when plotted in 2D. In online 2D LC, both dimensions are connected via an interface and the eluent of first dimension move directly to the second dimension. The only need of the online 2D LC system is that first dimension should have low flow rate so the second dimension can manage to chromatograph one fraction while another is being (slowly) collected (discussed in the online 2D LC section). Green tea sample was used as test sample in the preliminary experiments. The reason to choose the green tea as test material was that it has some similarities with the plant samples and only small quantities of the Serbian plant samples were that available for the project. These experiments were started with first dimension method development (HILIC).

3.1.1 Method development of first dimension hydrophilic interaction liquid chromatography (HILIC)

First dimension method development for the HILIC stationary phase was divided into three categories. These categories involve comparison between gradient and isocratic elution, use of column temperature and injection volume. A number of experiments were performed in each category.

3.1.1.1 Comparison between gradient and isocratic elution

To optimize the separation and resolution of the HILIC method, gradient and isocratic elution were examined. The main advantage of gradient elution that is it reduces elution time for late eluting components and often improves peak shape, resolution and increase detectability of late eluting components and. However, gradient elution can affect the repeatability. On the other hand, isocratic elution is arguably more repeatable and there is no need to re-equilibrate the column, between analyses which would be useful for online 2D LC. Green tea sample was used as test sample and combinations of 10 mM ammonium acetate (A) and AcN (B) or isopropanol (B) were used as mobile phases. The gradient mobile phase was 85 % B to 45 % B in 40 min and then 85 % B - 60 % B in 40 min. The HILIC phases can only be operated with modest amounts of water (i.e. 95-45 %), so isocratic experiments were performed with different percentages of mobile phase 'B' like 85 %, 70 % and 60 %. The ZIC-HILIC column was used for separation and 50 µl of sample were injected. 9 distinct peaks appeared in the chromatogram (Fig. 20) by using gradient elution, but isocratic elution with 85 % of mobile phase B produced 11 peaks. Isocratic elution with 70 % and 60 % B showed 8 and 6 distinct peaks respectively in the chromatogram. Improved separation of the tea extract was obtained in isocratic mode compared to gradient elution: (see Table 2 for summary of results and conditions).

Table 2: Representation of the data from the experiments of off-line 2D LC

HPLC Mode	(%)mobile phase (A) ammonium acetate 10mM	(%) mobile phase (B) can	No. of peaks appearing in the chromatogram	Peaks distributions
Gradient	15 to 55	85 to 45	9	From 2.5 min to 21 min
Isocratic	15	85	11	From 2.5 min to 11 min
Isocratic	30	70	8	From 2.5 min to 10 min
Isocratic	40	60	6	From 2.0 min to 7.5 min

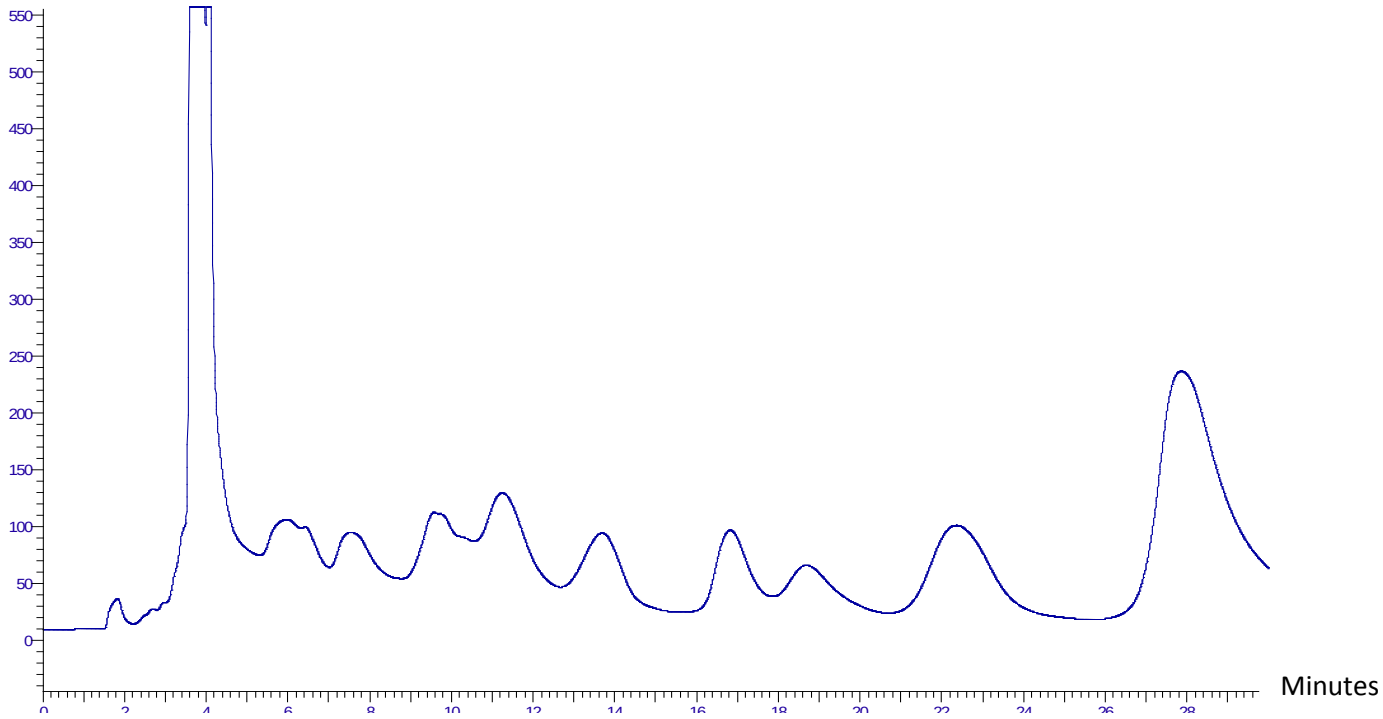


Fig. 20: HILIC separation of Green tea extract on HILIC column (150mm× 4.6mm i.d) with mobile phase consisting of 10mM ammonium acetate (A) and AcN (B) with a linear gradient from 85 to 45 % B in 40 min with a flow rate of 0.5 ml/min at 45 °C.UV detection at 254 nm was performed. (The chromatogram of isocratic elution has bad print quality and it was not possible to regenerate the data).

Different solvents have different selectivity in chromatographic separations. To develop better separation, different solvents with different properties can be examined. Green tea sample was used as test sample and 10 mM ammonium acetate (A) and isopropanol were used as mobile phase. The gradient mobile phase B was from 85 % to 45 % in 40 min and 85 % - 60 % in 40 min. Isocratic experiments were performed with different percentage of mobile phase 'B' like 85 % ,70 % and 60 % . The ZIC-HILIC column was used for separation and 50 µl of sample were injected.

9 peaks appeared in the chromatogram (Fig. 21) by using gradient elution but isocratic elution with 85 % of mobile phase B produced 7 peaks (Fig. 22). Isocratic elution with 70 % and 60 % B resulted in 8 and 6 peaks respectively in the chromatogram. As can be seen in table 3 better separation of the tea extract was obtained in gradient mode compared to isocratic elution by using isopropanol as mobile phase B. If the results of table 2 are compared with the results of table 3, the isocratic elution with 85 % B (AcN) gave the maximum number of eluting peaks, 11, so this composition of isocratic elution was used for the remainder of the study.

Table 3

Representation of the data from the experiments of off-line 2D LC using isopropanol in the mobile phase.

HPLC Mode	(%)mobile phase (A) ammonium acetate 10mM	% mobile phase B *Isopropanol*	No. of peaks appear in the chromatograms	Peaks distributions
Gradient	15 to 55	85 to 45	9	From 3.5 min to 29. 0
Isocratic	15	85	7	From 3.5min to 20.0
Isocratic	30	70	8	From 3.0min to 18.0 min
Isocratic	40	60	6	From 3.0min to 14.0 min

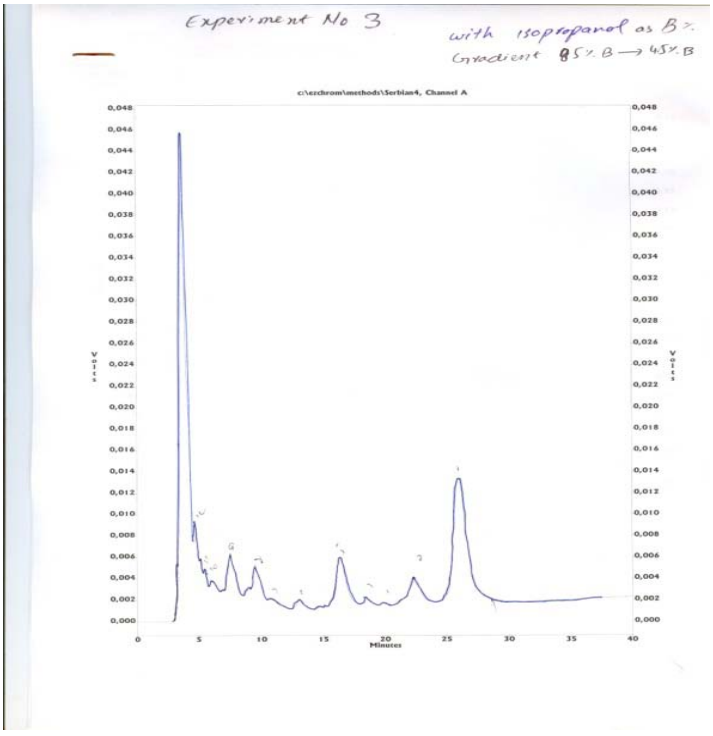


Fig. 21: HILIC separation of Green tea extract on HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10 mM ammonium acetate (A) and isopropanol (B) with a linear gradient from 85 to 45 % B in 40 min with a flow rate of 0.5 ml/min at 45 °C.UV detection at 254 nm was performed. The trace has been made by hand because of bad printer, and not possible to regenerate the data.

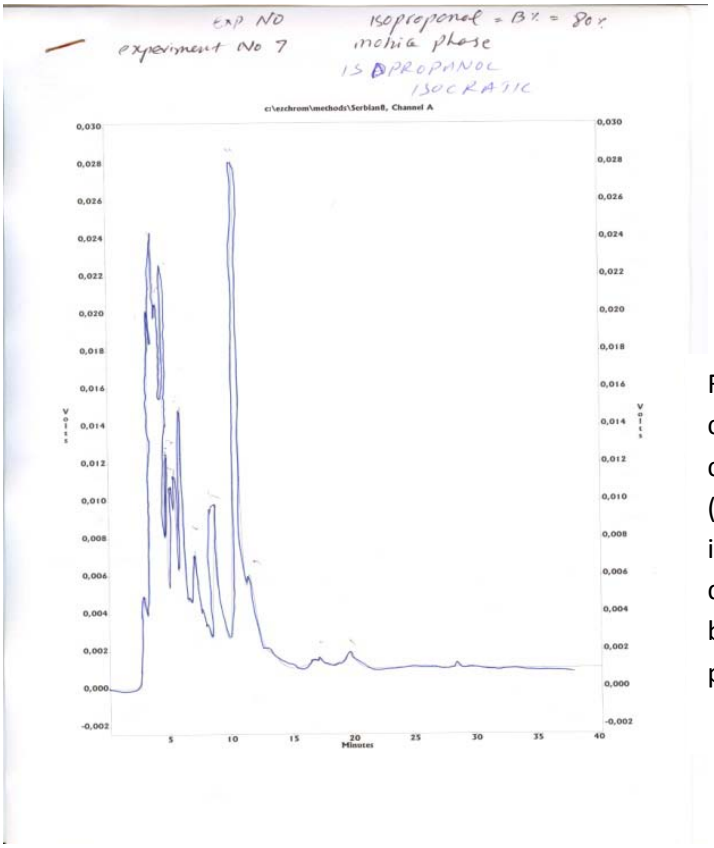


Fig. 22: HILIC separation of Green tea extract on HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10 mM ammonium acetate (A)/isopropanol (B) with isocratic elution 85/15 % v/v in 40 min with a flow rate of 0.5 ml/min at 45°C.UV detection at 254 nm was performed. The trace has been made by hand because of bad printer, and not possible to regenerate the data.

3.1.1.2 Effect of injection volume

The injection volume is directly related to the sensitivity and limit of detection of the system. It is important to use the correct injection volume on first dimension so that eluted first dimension fractions contain enough solute to produce signal or response on the second dimension detection. Green tea sample was again used as test sample and 10 mM ammonium acetate (A) AcN (B) was used as mobile phase. Isocratic elution was performed and the percentage of mobile phase 'B' was 85 %. The ZIC-HILIC column was used for separation and 50 µl, 100 µl, 150 µl of sample. Fig.23 shows that a 50 µl injection volume gave better results on first dimension as compared to 100 µl and 150 µl because these large volumes created very broad peaks. When eluted fractions of 50 µl (usual injection volume in chromatography) injected sample were examined on the RP second dimension system it produced quite high peak signals (shown in Fig.24) as compared to smaller injected volumes, which produced very low peaks (results not shown).

Hence 50 µl injection volume provided sufficient amount of sample so this injection volume was used in further experiments.

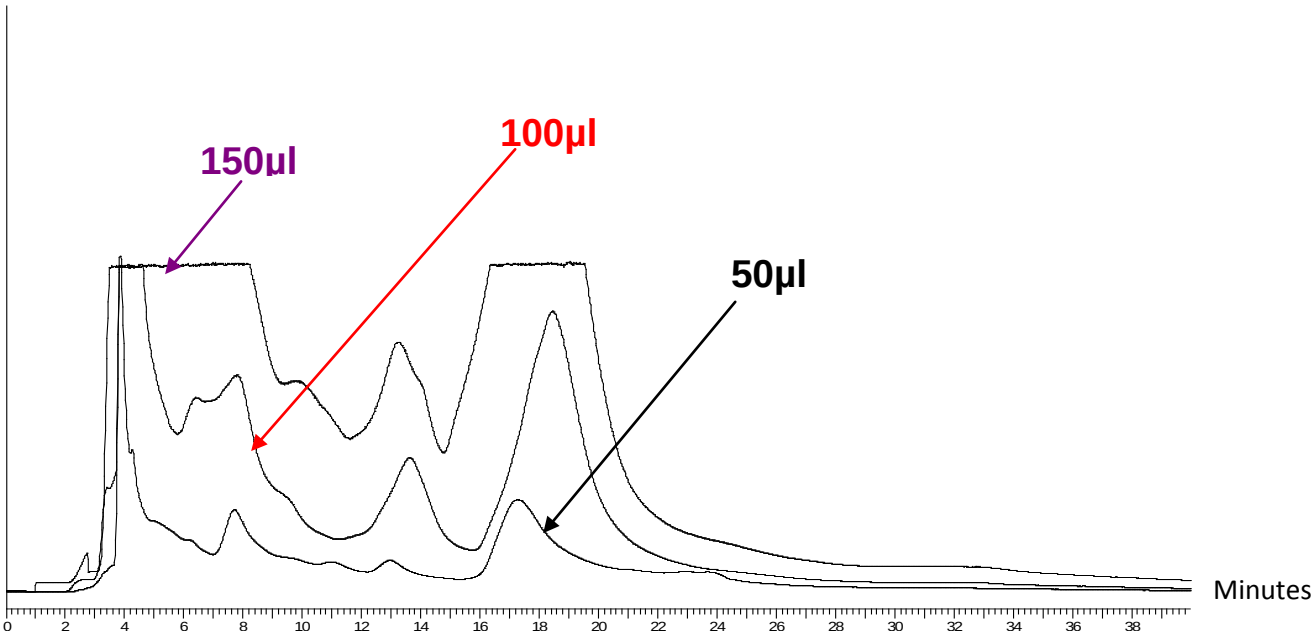


Fig.23: Overlapped chromatogram of HILIC separation of Green tea extract on HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10mM ammonium acetate (A)/AcN (B) with isocratic elution 85/15 % v/v in 30 min with a flow rate of 0.5 ml/min at 45 °C.UV detection at 254 nm was performed. Injection volumes used were 50 µl, 100µl, 150 µl.

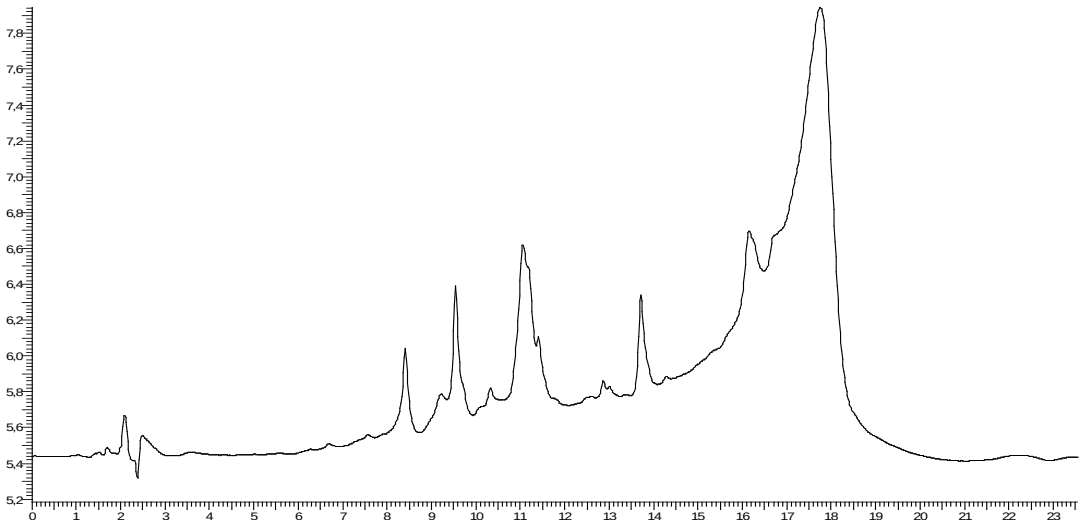


Fig.24: A fraction from the first dimension separation (HILIC) of Green tea extracts (50 µl injection volume) using HILIC column (150 mm × 4.6 mm i.d) was injected to second dimension column C18 column (50 mm×4.6 mm i.d.) with mobile phase consisting of (A) water with 0.1 % FA (B) acetonitrile with 0.1 % FA and gradient profile from 5 % to 100 % B in 20 min with a flow rate of 0.5 ml/min at 45°C. UV detection at 254 nm was performed.

3.1.1.3 Effect of temperature

Column temperature impact on HILIC phases is described in detail in section 1.2.1. To optimize the effect of temperature on the HILIC phase, different experiments were performed, using isocratic elution. The mobile phase consisted of 15 % 10 mM ammonium acetate and 85 % AcN. Green tea extract was used as test sample. Replicated analyses were performed at 5 °C, 15 °C, 25 °C, 35 °C, 45 °C and 60 °C. As shown in Fig 25 an increase in temperature resulted in retention time of the compounds. This is in accordance with another recent study [22-24]. These experiments showed that the effect of temperature on retention in HILIC is quite opposite to that in RP chromatography where the retention time most often decreases when the temperature is increased. To summarize and arrange the data of Fig. 25 the retention time of last eluting peak was plotted against temperature used (as shown in Fig. 26). If a of column temperature of 45 °C was used in the online 2D LC system then the analysis time would be very long hence a column temperature of 25 °C was used during further experiments. The flow rate used in these experiments was 10 times faster than that used in the online 2D LC system otherwise the analysis time became too long.

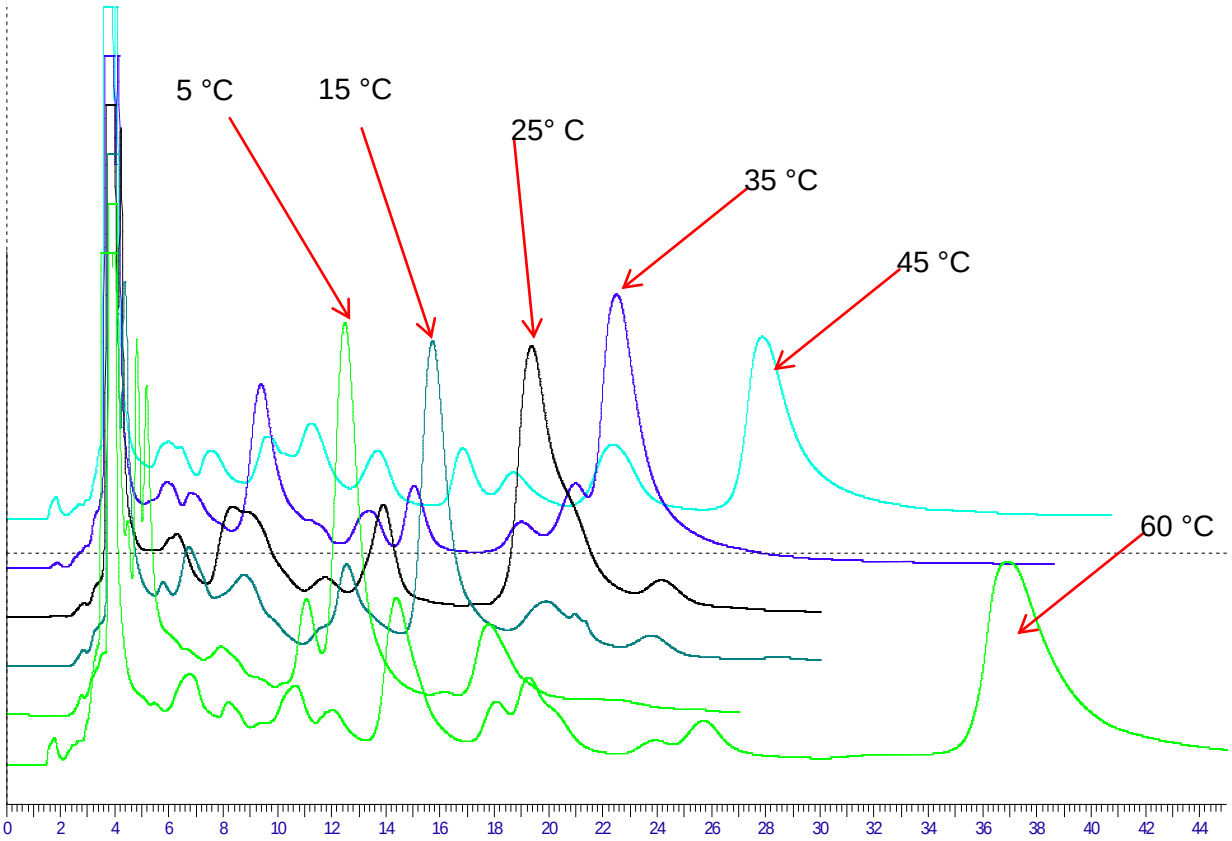


Fig. 25: HILIC separation of Green tea extract on HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10 mM ammonium acetate (A)/AcN (B) with isocratic elution 85/15 % v/v in 25 min with a flow rate of 0.5 ml/min at different temperature. UV detection at 254 nm was performed. The temperatures used were 5 °C, 15 °C, 25 °C, 35 °C, 45 °C and 60 °C. 50µl were injected.

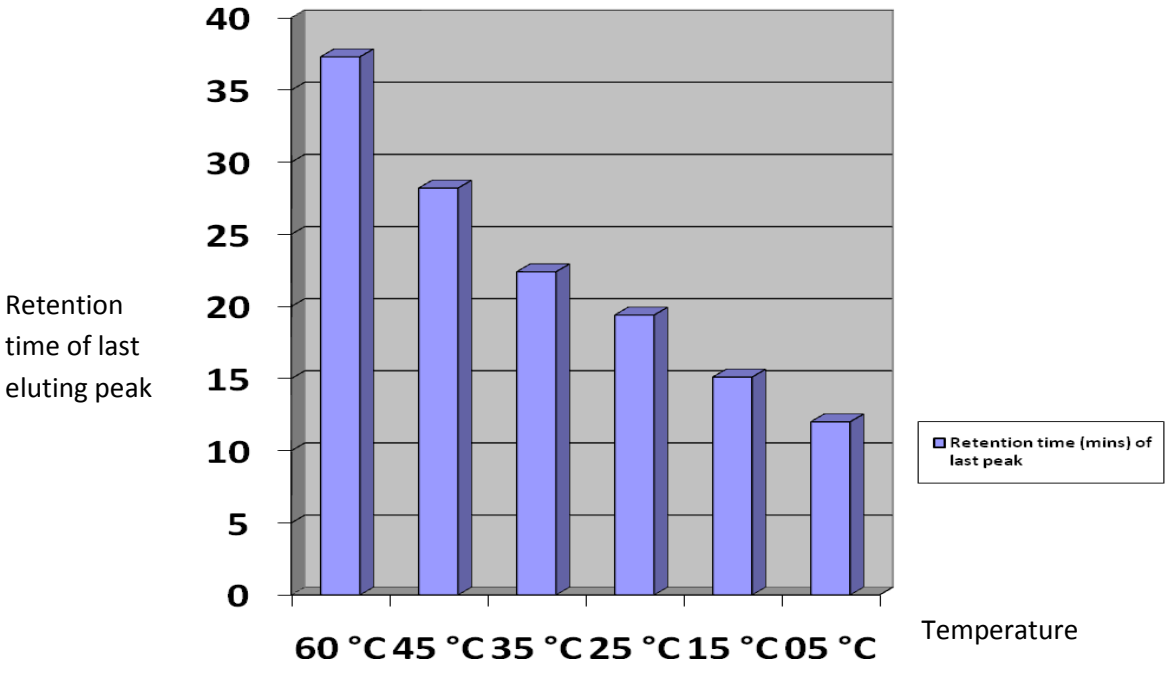


Fig. 26: Plot of retention time of last elution peak as function of temperature

3.1.2 Second dimension reversed phase liquid chromatography

In the previous chapter the optimization of HILIC first dimension experiments are including comparison between gradient and isocratic elution, use of column temperature and injection volume. In summary, the first dimensional (HILIC) experiments provided better performance by using isocratic elution with higher percentage of AcN, 50 µl of injection volume and an ambient column temperature. The second dimension must be compatible and orthogonal with the first dimension separation (HILIC). Another important requirement of the second dimension method is it that must be fast (as discussed earlier), and provide sufficient resolution.

To achieve second dimensional separation the orthogonal compared to HILIC, reversed phase stationary phase was initially selected. The literature studies in the introduction part provide the information of an orthogonal relationship between RP and HILIC phases. The eluted fractions collected during HILIC experiments were used in these experiments. With the optimized HILIC method 12 eluted fractions were collected. Of these 12 fractions, two randomly selected fractions (Fraction No. X and Y) were used as test samples for second dimension analysis. The binary mobile phase consisted of (A) 0.1 % formic acid in water (v/v) and (B) AcN with gradient from 5 % to 100 % B at a flow rate of 1.0 ml/min. A reversed phase C-18 column was used for separation. The chromatograms of Fraction X and Y are shown in Fig. 27. The second dimension reversed phase column did not give sufficient separation of Fraction X and Y and also the gradient response and resolution were poor (as shown in Fig. 27). When the flow rate was increased to achieve the fast second dimension separation the backpressure increased to unacceptably high levels. To overcome the backpressure problem a short second dimension column which could provide better separation at high flow rate and orthogonal to HILIC separation, was needed. RP also has limitations regarding high column temperature so high temperature could not be used as variable to reduce the backpressure.

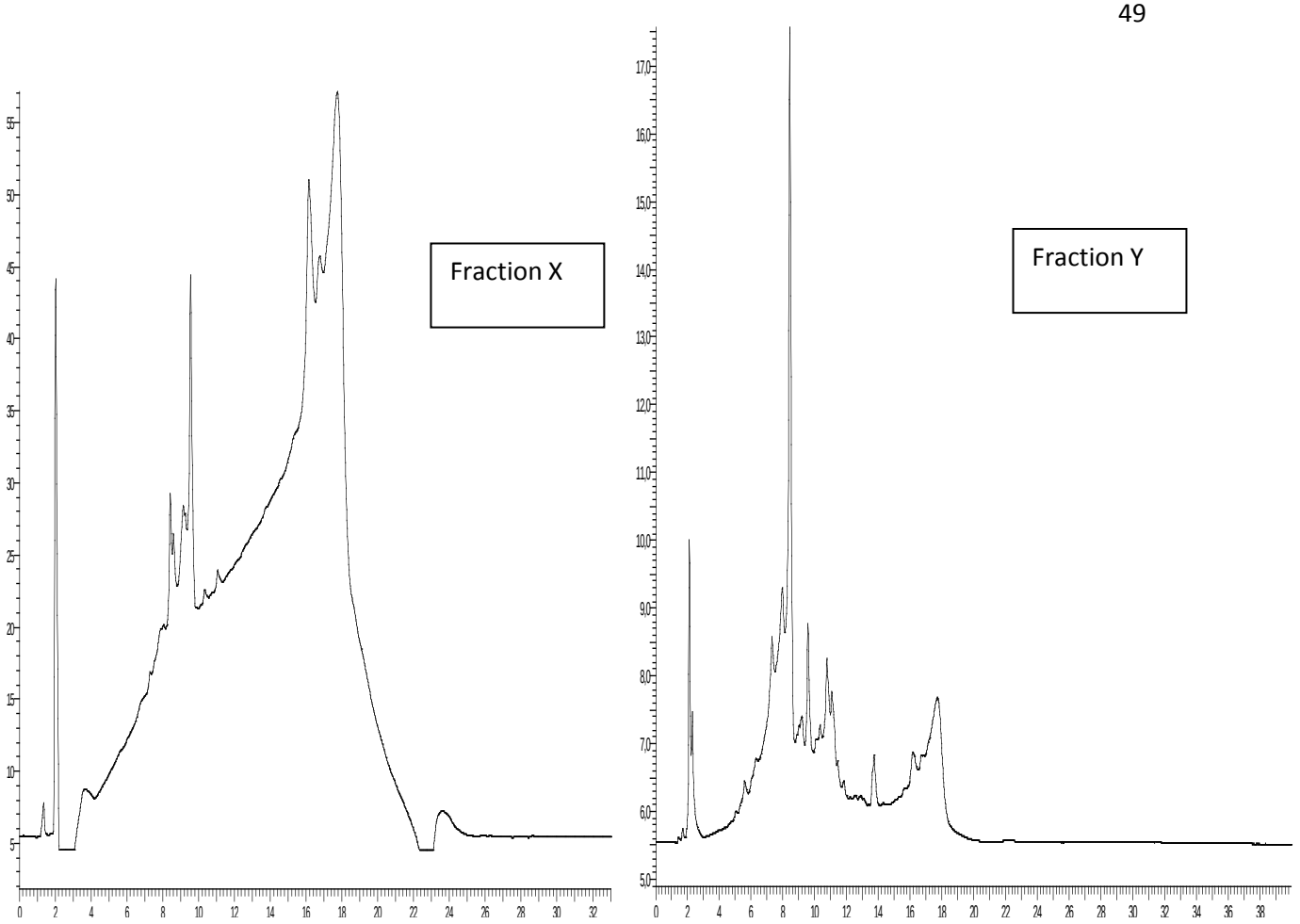


Fig.27: Second dimension RP analysis of Fractions x and y with mobile phase consisting of (A) water with 0.1 % FA (B) AcN and gradient from 5 % to 100 % B in 20 min. The injection volumes used were 50 µl. UV was recorded at 254 nm. The flow rate was set to 1.0 ml/min and 50 µL was injected. The dimension of column was a (150 mm X4.6mm i.d).

3.1.3 Hypercarb column for second dimension separation

As mentioned above, a medium length (150 mm X 4.6 mm i.d.) RP second dimension column didn't provide fast and sufficient separation of first dimension eluted fractions. The behavior of RP chromatography did not provide sufficient separation and poor performance hence other stationary phases that are orthogonal to HILIC and withstand high flow rate was searched. The porous graphite carbon "Hypercarb" material can be used with high temperature reducing the backpressure. Therefore the Hypercarb explored for use in the second dimension to achieve fast separation.

3.1.3.1 Method development on Hypercarb column

Second dimension method development for Hypercarb material was also divided into three categories. These categories involve comparison between gradient and isocratic elution, use of column temperature and mobile phase composition and flow rate. A number of experiments were performed in each category.

3.1.3.1.1 Gradient elution

The gradient elution was examined on the Hypercarb column with high flow rate. The binary mobile phase consisted of (A) 0.1 % formic acid in water (v/v) and (B) AcN. A linear gradient was performed from 0 % B to 100 % in 2 min and from 100 to 0 % B in 2 min. The fast gradient elution did not provide efficient separation of a simple mixture of toluene and 3-bromophenol as shown in Fig. 28. The gradient time was too short; our instruments could not handle a short gradient program when a large difference in mobile phase composition is used within short time interval.

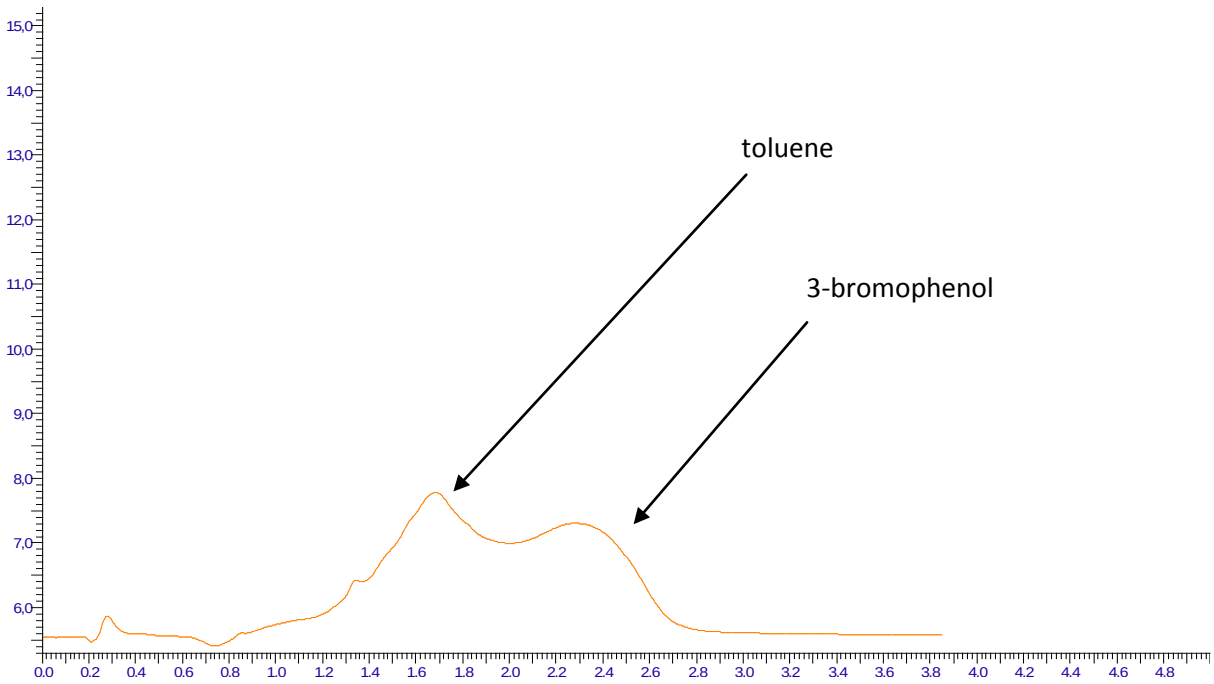


Fig. 28: Second dimension separation of toluene and 3bromophenol using Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1 % FA and AcN (B) The gradient profile was from 0 % to 100 % B in 2 min. The injection volumes used were 50 µl. UV was recorded at 254 nm.

3.1.3.1.2 Isocratic Elution

Because of the poor performance of fast gradient analysis on Hypercarb column with our instrumentation isocratic elution was examined with the same sample. The mobile phase consisted of water with 0.1% FA and AcN (B) 40/60 % (v/v). The isocratic elution gave better separation of toluene and 3-bromophenol in a short run time with high flow rate as shown in Fig. 29.

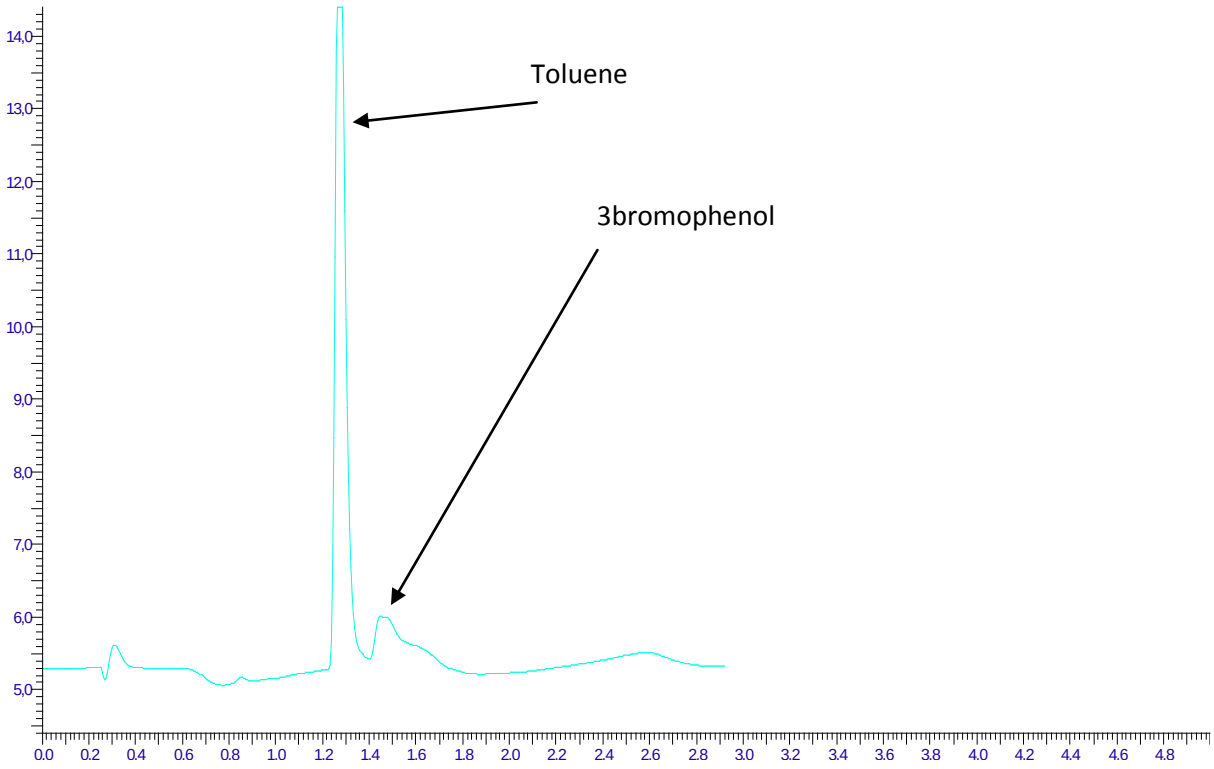


Fig. 29**:** Second dimension separation of toluene (1µg/ml) and 3bromophenol (1 µg/ml)using Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1% FA and AcN (B) 40/60 % (v/v). The injection volumes was 50 µl. UV was recorded at 254 nm. The flow rate was set to 3.0 ml/min and the analysis was carried out at 45 °C.

3.1.3.2 Effect of temperature on Hypercarb

Column temperature impact on Hypercarb is described in detail in section 1.5 with Hypercarb the retention time usually decreases by increasing the temperature [23, 33]. To optimize the effect of temperature on the Hypercarb phase, different experiments were performed using isocratic elution. The mobile phase was consisted of water

with 0.1 % FA and AcN (B) 40/60% (v/v). Toluene and 3-bromophenol were used as test compounds. Two series of Analysis were performed at 20 °C, 40 °C, 60 °C, 80 °C, 90 °C, 110 °C and 150 °C as shown in Fig 30 and 31. In the first set of experiments the column temperature was gradually increased in each experiment from 20 °C to 90 °C. When the temperature was increased, the retention time of eluting peaks decreased as shown in Fig. 31(overlapped chromatograms). In the second set of experiments the column temperature gap between 1st & 2nd experiments was 70 °C as shown in Fig. 31. The patterns of these experiments were similar with first set of experiments. These experiments showed that when the column temperature was higher than 110 °C the separation became unrepeatable and unstable. Hence, the best temperature for separation on Hypercarb was 110 °C because of short retention times and improved resolution.

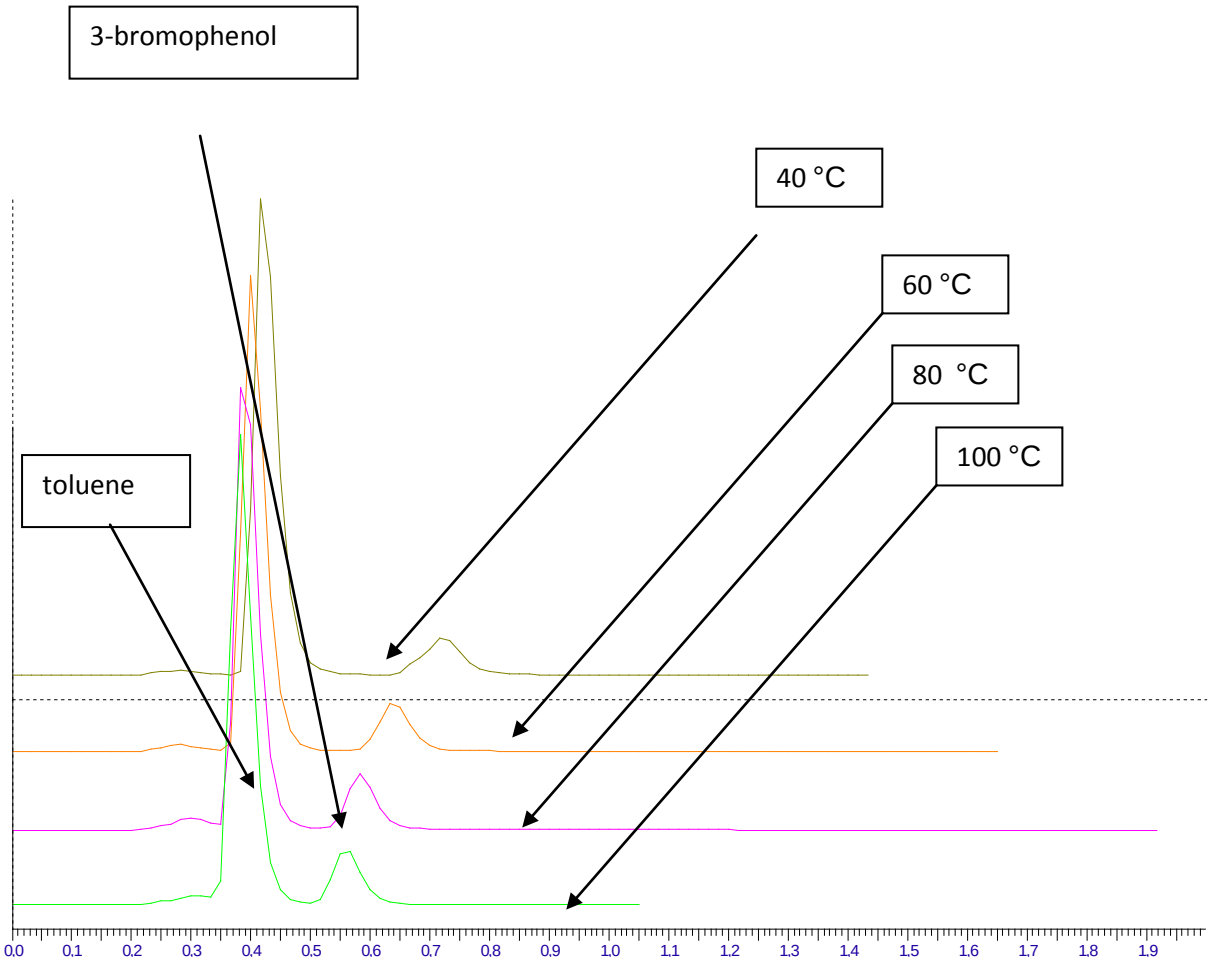


Fig. 30: Second dimension separation of toluene (1 µg/ml) and 3bromophenol (1 µg/ml)using Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1 % FA and AcN (B) 40/60 % (v/v) . The injection volume used was 50 µl. UV was recorded at 254 nm. The flow rate was set to 2.0 ml/min and the analysis were carried out at 40 °C, 60 °C, 80 °C and 100 °C.

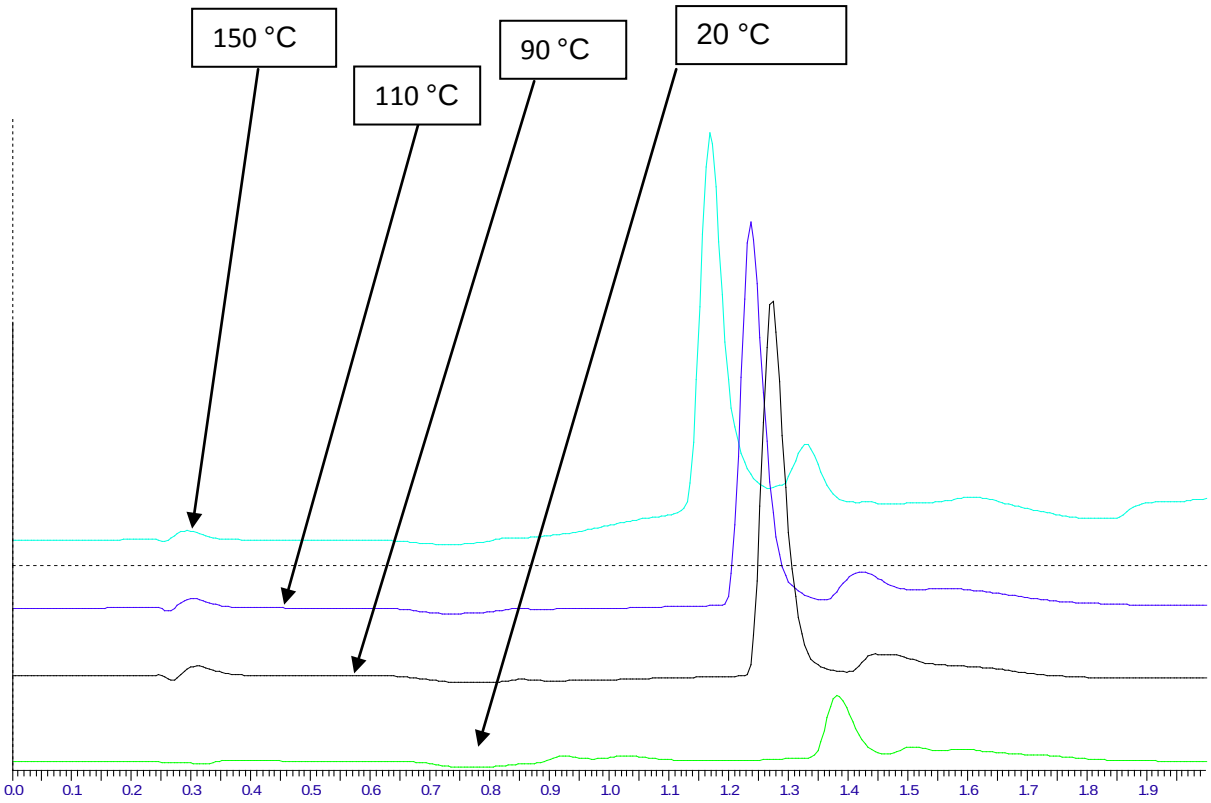


Fig.31: Second dimension separation of toluene (1 µg/ml) and 3-bromophenol (1 µg/ml) using Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1 % FA and AcN (B) 40/60 % (v/v). The injection volumes used was 50 µl. UV was recorded at 254 nm. The flow rate was set to 1.0 ml/min and the analysis were carried out at 20 °C, 90 °C, 110 °C and 150 °C.

3.1.3.3 Effect of flow rate and acetonitrile composition

To develop a robust second dimension method different flow rates and mobile phase compositions were examined to obtain as short time as possible. In the optimized HILIC experiment 12 eluted fractions were collected. Out of these 12 fractions three randomly selected fractions (Fraction No. 1, 2 and 3) were used as sample for second dimension analysis. The binary mobile phase consisted of 0.1 % formic acid in water (A) and AcN (B) (v/v) and a column temperature of 110 ◦C was chosen. Isocratic elution performed at 50 %, 60 % and 70% B. The column was washed for 5.00 min before the next analysis. Flow rates of 1.0, 2.0 and 3.0 ml/min were investigated. For each flow rate 50, 60 and 70 % of AcN were examined using fraction 1, 2 and 3 as sample. When the flow rate was 3.0 ml/min and the composition of AcN was 70 %, overlapped chromatograms (Fig. 32) of fraction No 1,

2 and 3 illustrate that the separation was completed within 2 min, but with broad peaks. This flow rate and composition of (3.0 ml/min, 70 % AcN) was not explored further due to broad peaks and poor separation.

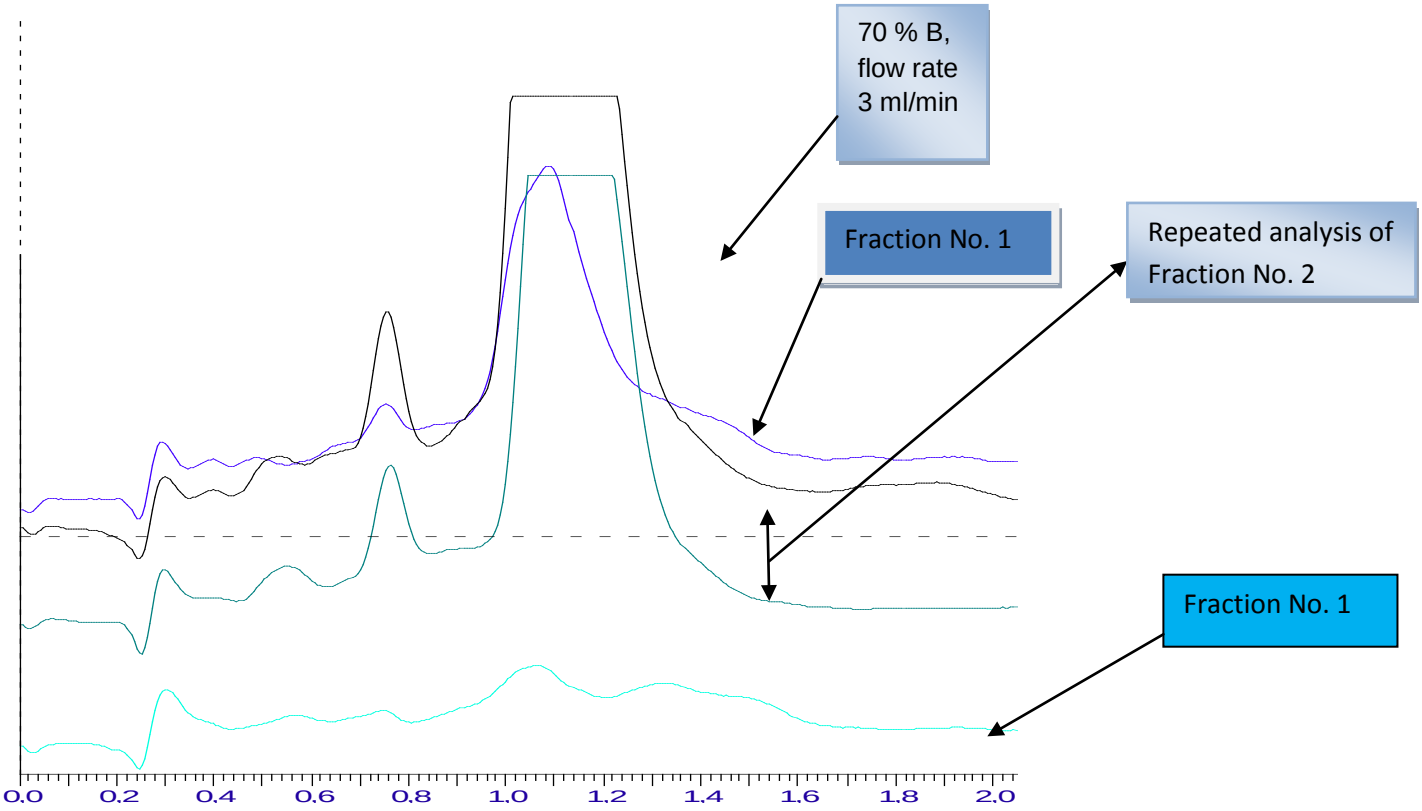


Fig. 32: Second dimension separation of randomly selected 1st dimension fractions (Fraction No. 1, 2 and 3) on Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1 % FA (A)/AcN (B) with isocratic elution 30/70 % (v/v) in 8 min with a flow rate of 3 ml/min at 110° C.UV detection at 254nm was performed. 50 µl were injection.

When a flow rate of 3.0 ml/min and 60 % AcN were used for the analysis of fraction No 1, 2 and 3 the overlapped chromatograms (Fig. 33) illustrate that the separation was completed within 2 min with good resolution. These conditions provided improved separation but further online experiments demonstrated incompatibility with the HILIC mobile phase because the elution strength of the optimized mobile phase of first dimension was too high.

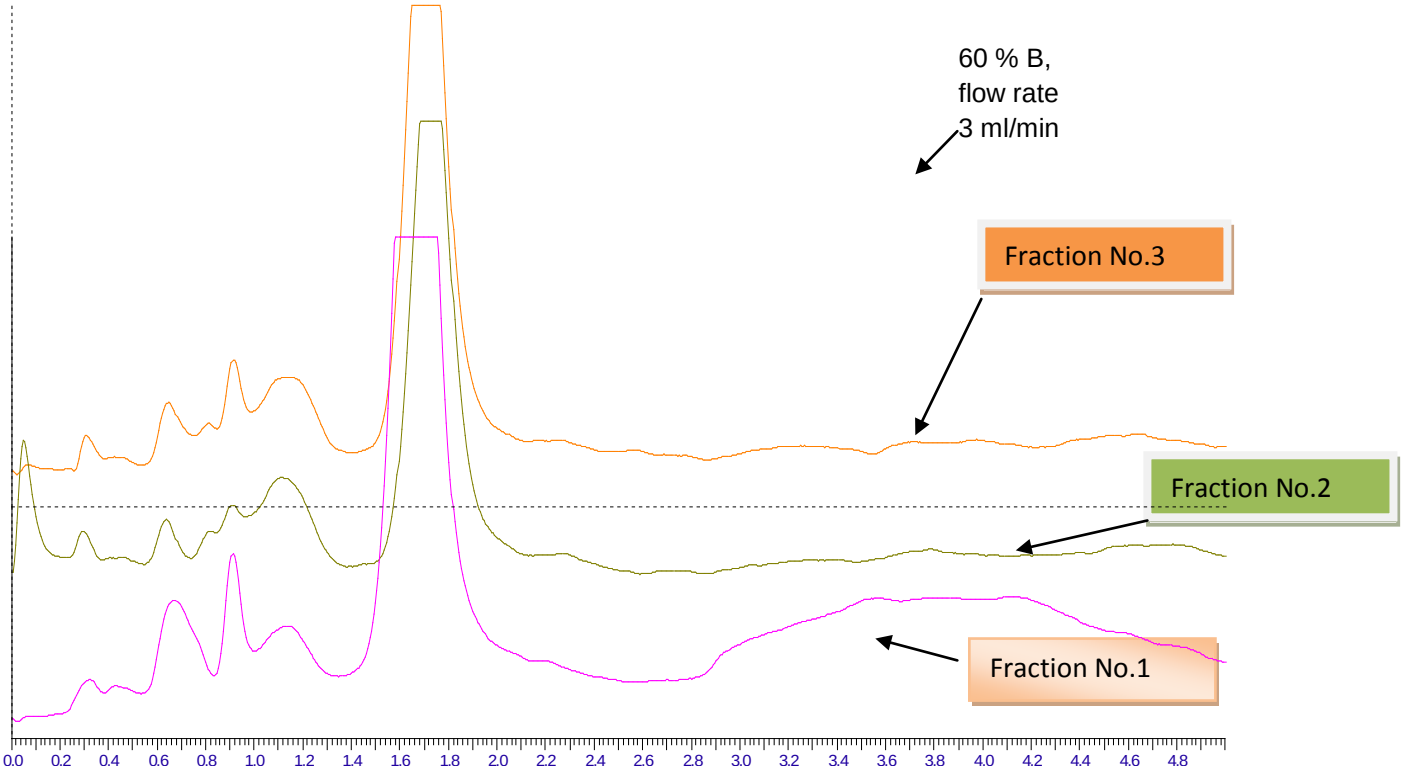


Fig.33: Second dimension separation of randomly selected fractions (Fraction No. 1, 2 and 3) on Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1 % FA (A)/AcN (B) with isocratic elution 40/60 % v/v in 8 min with a flow rate of 3 ml/min at 110°C.UV detection at 254 nm was performed. The 50 µl injection volumes were used.

When the flow rate was 2.0 ml/min and composition of AcN was 70 % then the overlapped chromatogram (Fig. 34) of fraction No 1, 2 and 3 illustrate that the separation completed within 2 min with good separation. These conditions provided good separation and would also show compatibility with online first dimension experiments. The optimized mobile phase of first dimension was slightly similar (explained in next section of online 2D LC).

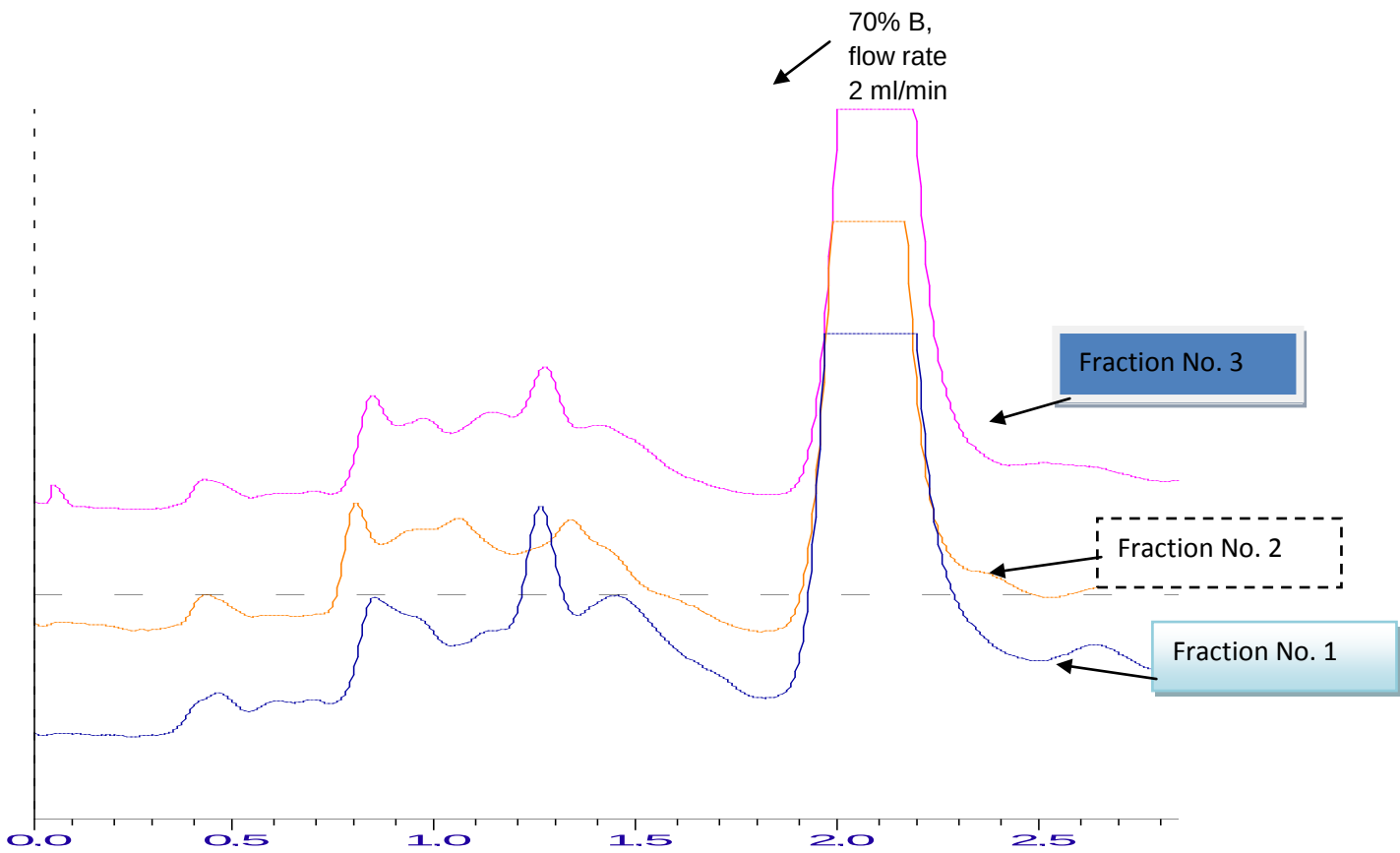


Fig.34: Second dimension separation of randomly selected fractions (Fraction No. 1, 2 and 3) on Hypercarb column (50 mm× 4.6 mm i.d) with mobile phase consisting of water with 0.1 % FA (A)/AcN (B) with isocratic elution 30/70 % (v/v) in 8 min with a flow rate of 2 ml/min at 110 °C.UV detection at 254 nm was performed. 50 µl were injected.

All other conditions of flow rate and composition did not provide sufficient separation within a short time. At lower flow rates (1ml/min with 50, 60, 70 % AcN and 2 ml/min with 50, 60 % AcN) the separation was not completed within the 2 min that was required for the online HILIC system as shown in Fig. 41 (APPENDIX.III). A, B, C, D, and F. The chromatogram in Fig 41 E shows that the peaks were broad when 3 ml/min of flow rate and 50 % of AcN was used.

3.1.4 Conclusions on individual dimensions

The optimized HILIC method can be coupled to an RP/Hypercarb method in order to identify compounds in complex green tea extracts. Isocratic elusion has been found to be efficient in both first dimension HILIC separation and second dimension Hypercarb separation. In this study, better 2nd dimension separation was achieved at higher temperature than ambient. The best (but slow) HILIC separation was obtained with an isocratic mobile phase composition of 85 % AcN/15 % 10 mM ammonium acetate at 45° C of temperature on column. 25° C was used instead for faster 1st dimension separation. The Hypercarb column gave better separation with fast second dimension analysis compared to silica based RP column. A flow rate of 2 or 3 ml/min with isocratic elution between 60 to 70% AcN and 30 to 40 % water and 1 % formic acid at 110° C provided a good second dimension separation.

3.2 Online 2D LC system

In off-line 2D LC experiments method development of the first dimension separation on HILIC columns and second dimension separation on RP and Hypercarb columns were performed. These developed methods and systems were combined in the online 2DLC system. Peak capacity of off-line 2D LC separation was recorded and compared with the online system and used to evaluate the best method (comparison is discussed in last part of online section). The off-line 2D LC required more operator attention and time while online 2D LC can reduce the time and attention of operator. In online 2D LC, the dimensions are connected via an interface and the eluent of first dimension moves directly to the second dimension. The only need of the online 2D LC system is that first dimension should have low flow rate so the second dimension can manage to separation one fraction while another is being (slowly) collected. The performance of the online 2D LC system was initially evaluated using a simple standard mixture of uracil, toluene and benzoic acid. The chromatographic conditions are given in the experimental section. These three standard compounds were first injected separately to check the orthogonality of the system. Due to very

small difference between retention times, these standards were not separated properly but the 2D plot provides the related information and as shown in the beginning of the results and discussion part in Fig. 20 and 21. The online system was not fully optimized because the working power (RAM) of the computer was not sufficient to accommodate two HPLC systems working simultaneously. This defect creates some variation on online chromatogram.

3.2.1 Online 2D LC Serbian plant extracts analyses

After examining the online 2D LC system with standards (e.g. the results shown in Fig 21), analyses of Serbian plant samples were carried using the same system. The plant samples were grinded and then extracted with AcN/water 50/50 (v/v), methanol and AcN as detailed in the experimental section. All the samples were not examined due to limited time. The details of these analyses are given below.

3.2.1.1 Ruta Graveolens

The developed conditions from the off-line 2D LC experiments were utilized in this experiment. The methanol extracted chromatograms (as shown in Fig. 35) of first and second dimension separation of Ruta graveolens. In the first dimension the run time was 250 min with very broad peak due to very slow flow rate (0.05 ml/min).

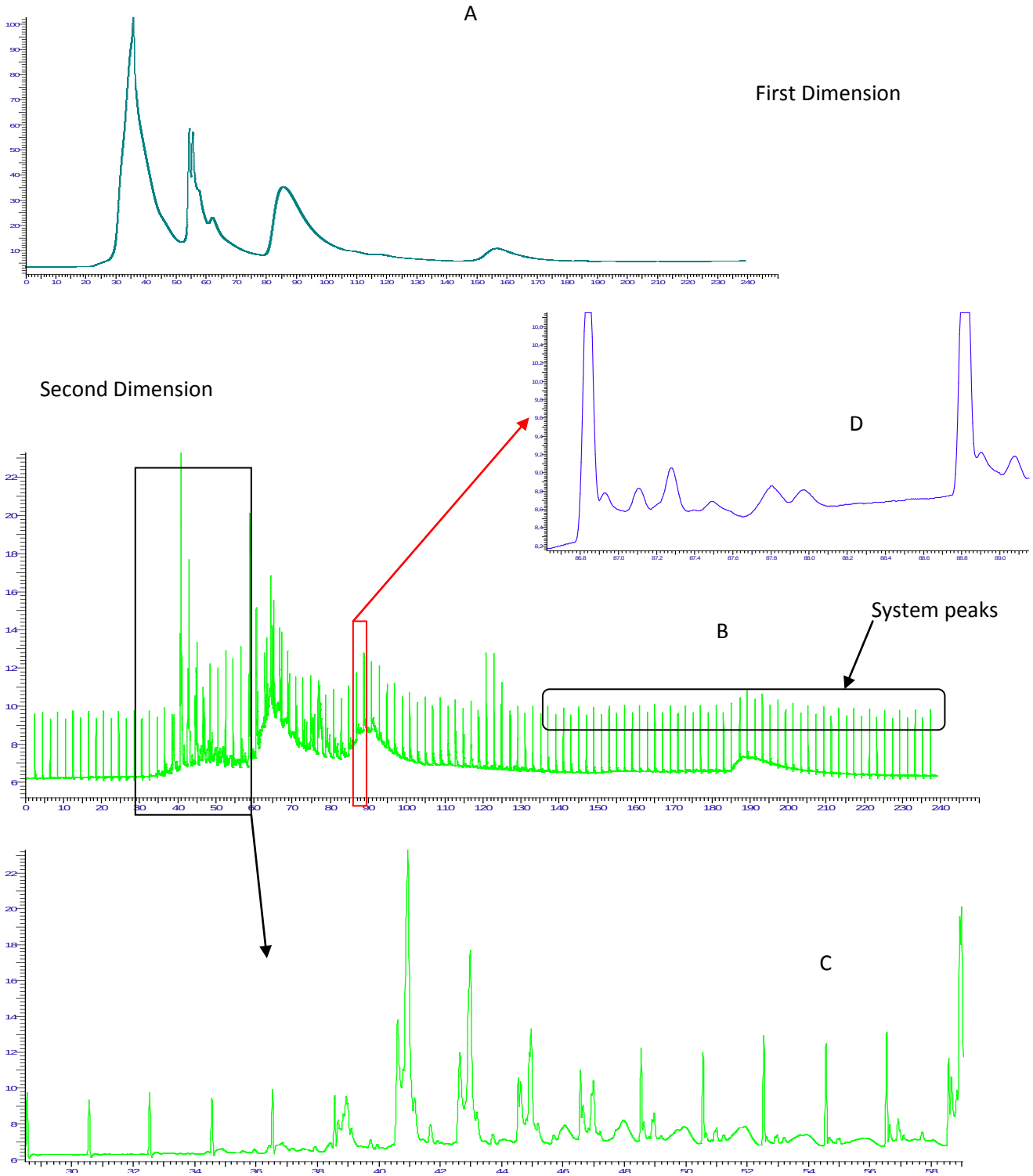


Fig. 35: Chromatograms first and second dimension separation of (methanol extracted) Ruta Graveolens (250mg) (A) on HILIC column (150mm× 4.6mm i.d) with mobile phase consisting of 10mM ammonium acetate (A)/AcN (B) with isocratic elution 85/15% v/v min with a flow rate of 0.05ml/min. UV detection of first detector was performed at 254nm. (B) Second dimension separation performed on Hypercarb column (50mm× 4.6mm i.d) with isocratic elution 10mM ammonium acetate (A)/AcN (B) 30/70 % v/v with a flow rate of 2/min at 100°C.UV detection of second detector was also performed at 254nm. 50 µl were injected. (C) The expansion of second dimension and (D) the expansion of chromatogram of B.

The chromatograms of the first and second dimension were plotted as 2D and shown in Fig.36. The chromatograms of first and second dimension look poor as separate are and not able to predict or show the final results or resolved peaks, but the combined 2D contour plot shows comprehensive and resolved peaks.

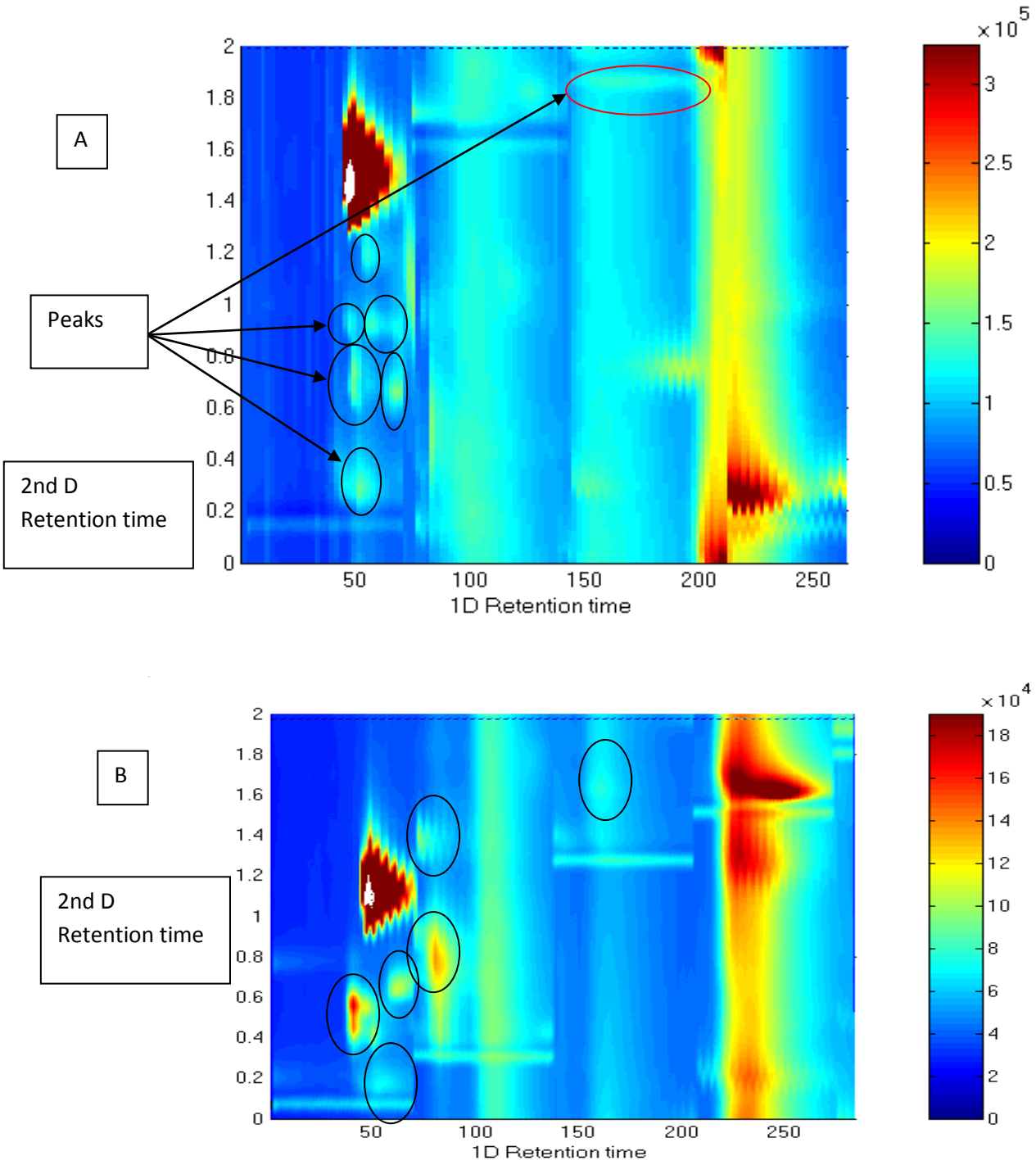


Fig.36. 2D contour plots obtained for the HILIC×Hypercarb-LC analysis of Serbian plant (Ruta Graveolens) with the long 1.5 ml/min (A) and short 2.5 ml/min (B) second dimension.

3.2.1.2 Satureja Montana III

The sample of Satureja Montana III was also extracted with methanol and analyzed by online system. The chromatograms of the first and second dimension are shown in Fig. 37.

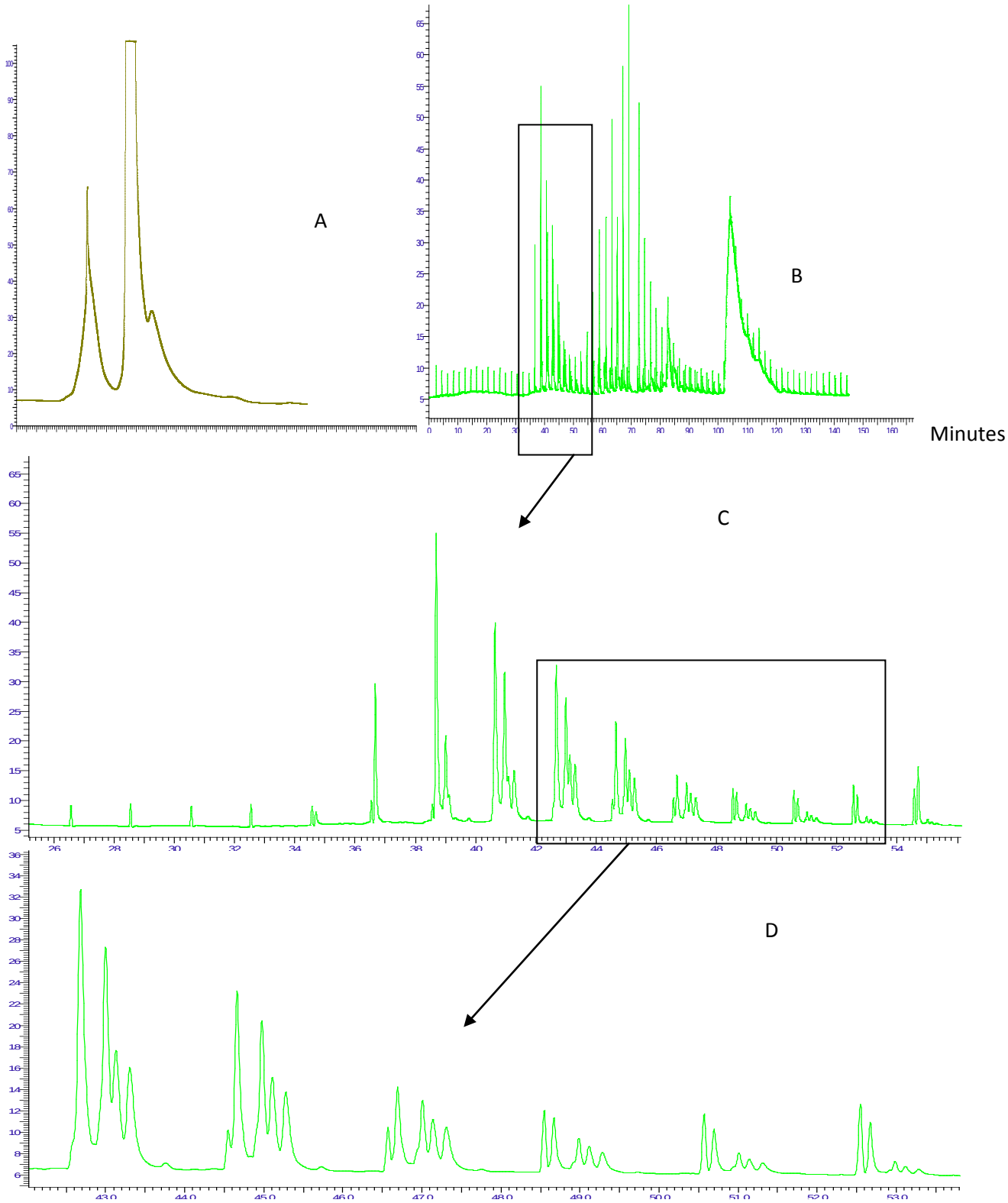


Fig 37: Chromatograms first and second dimension separation of (methanol extracted) satureja Montana III (250mg) on (A) HILIC column (150mm× 4.6mm i.d) with mobile phase consisting of 10mM ammonium acetate /AcN with isocratic elution 85/15% (v/v) with a flow rate of 0.05ml/min. UV detection of first detector was performed at 254nm. Second dimension separation performed on Hypercarb column (50mm× 4.6mm i.d) with isocratic elution with 10mM ammonium acetate /AcN 30/70 % (v/v) with a flow rate of 2/min at 100°C.UV detection (second detector) was also performed at 254nm. 50 µl were injected. (C) expansion of second dimension (D) the expansion of chromatogram of C.

In 2DLC systems, different chromatographic modes are combined to increase the number of separated compounds in complex samples. Peak capacity significantly increases in 2D LC, where the total theoretical peak capacity of 2D LC system should be ideally equal to the product of the peak capacities of each dimension. Considering orthogonal 2D LC system, the resulting peak capacity, is equal to the product of the peak capacities in the individual dimensions.

The real increase in the peak capacity is usually lower than predicted from Eq. (1) (from introduction part) [4].

The peak capacities obtained by the different principles, techniques and conditions in this study of comprehensive online and off-line 2D LC are summaried in table 4. Peak capacities presented in table 4 are the maximum number of peaks that can practically be resolved in a separation window between the first and the last eluting peak in first and second dimension chromatogram. Since the two separation mechanisms are orthogonal, the peak capacity (nc 2D) is the product of the peak capacity of each dimension. 1nc and 2nc are the peak capacities contributed from the first and second LC dimension[4], respectively. In this study the peak capacity was calculated by multiplying the numbers of resolved peaks in the first dimension chromatogram (from first to last eluted peak) and the number of peaks of one fraction of second dimension chromatogram.

As an example, in the first row of table 4, the peak capacity was calculated by multiplying the 10 resolved peaks of the chromatogram shown in Fig.20 with the 9 resolved peaks of fraction X in Fig. 27 resulting in a total peak capacity of 90. In isocratic off-line 2D LC analysis the peak capacity was calculated by using the of peak number of first dimension (from table 2) and second dimension fraction (Fig. 27).

Table 4: Peak capacities of off-line and online systems.

Application	Mode	Condition	First Dimension Column	Second Dimension Column	Peak capacities nc,2D= 1nc × 2nc from Equation 1	Analysis time
Green tea extracts	Off-line 2D LC (A)	Gradient first dimension = 85-45% B. (B) AcN (A) 10mM amonium acetate. Second dimension =A water+FA 70%, B =can	ZIC-HILIC phase	Reversed Phase	90 (from Fig. 20 and 27)	First dimension = 35min and second dimension = 25min
Green tea extracts	Off-line 2D LC (B)	Isocratic first dimension= 85% B. (B) AcN (A) 10mM amonium acetate 15%. Second dimension=A water+FA 70%, B =AcN 30%	ZIC-HILIC phase	Reversed Phase	121 (from table 2 and Fig. 27)	First Dimension = 35min and second dimension = 25min
Other tea extracts	Off-line 2D LC (C)	Isocratic first dimension= 85% B. (B) AcN (A)10mM amonium acetate 15% Second dimension=A water+FA 70%, B =AcN 30%	ZIC-HILIC phase	Hypercarb Phase	66 (from table 2 and Fig. 34)	First Dimension = 35min and second dimension = 4min
Serbian plant extracts (Ruta Graveolens)	Online 2D LC (D)	Isocratic first dimension=second dimension 85% B. (B) AcN (A)10mmol Amonium Acetate 15%	ZIC-HILIC phase	Hypercarb Phase	36 (from Fig. 35)	First Dimension = second dimension = 180min
Serbian plant extracts (satureja montanaIII)	Online 2D LC (E)	Isocratic first dimension=second dimension 85% B. (B) AcN (A)10mmol Amonium Acetate 15%	ZIC-HILIC phase	Hypercarb Phase	30 (from Fig. 37)	First Dimension = second dimension =200min

3.2.2 Repeatability of the online 2D LC

After developing the extraction method, the Serbian plant extracts were used to check the repeatability of the online 2D LC system. The available samples were injected three times each to check the retention time and separation repeatabilities Fig. 41. From these experiments it was concluded that the online 2D LC system shows satisfactory repeatability of retention time and chromatographic profile.

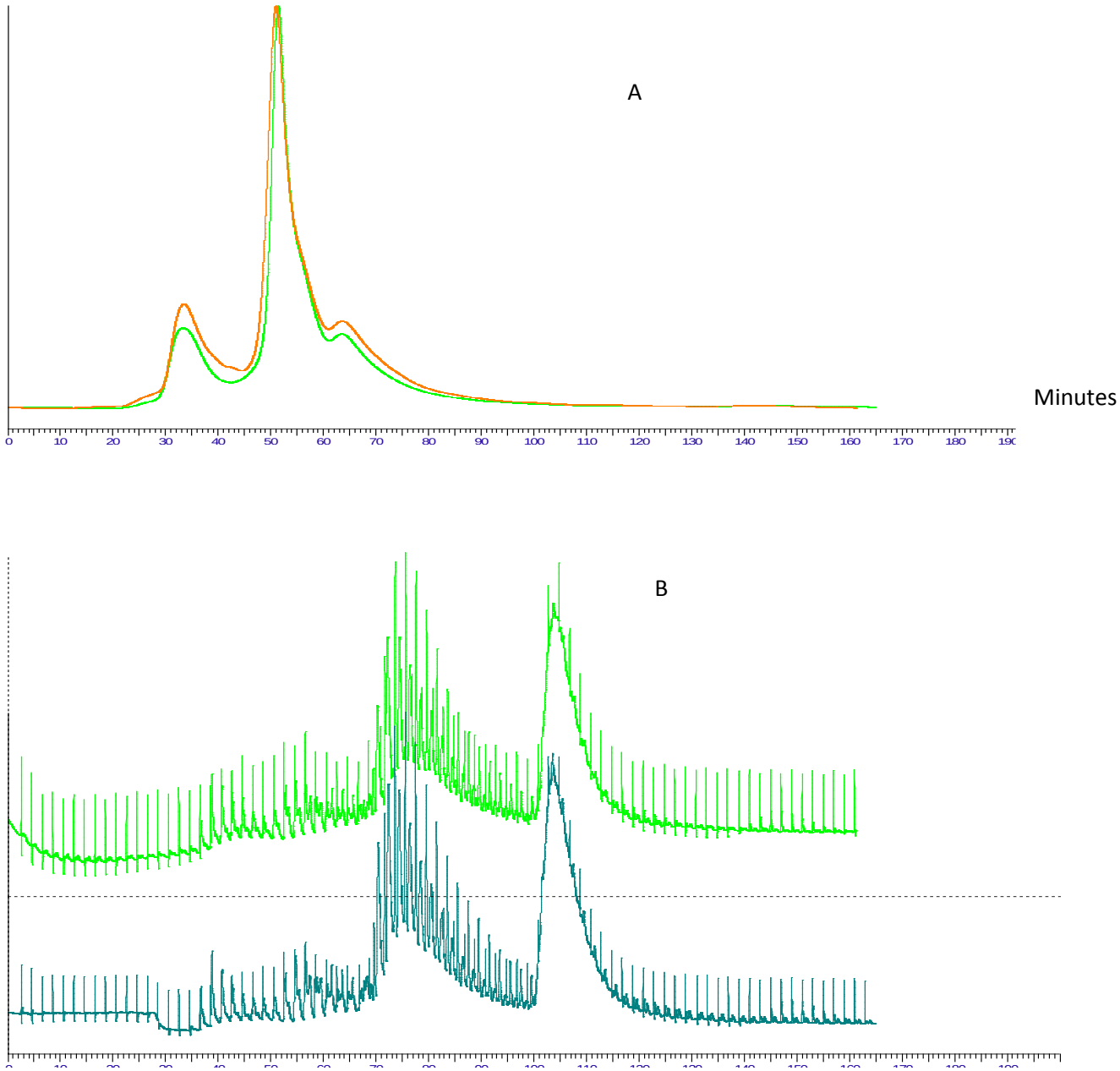


Fig 41: (A) Overlaid chromatograms of the first dimension separation (B) overlaid chromatograms of the second dimension separation of calamintha sample. Other condition as in Fig. 37

4.0 Conclusions

Online comprehensive two dimensional LC method for the analysis of plants extracts has been developed. Serbian plants extracts were separated by using a reversed phase/Hypercarb column in the second and a ZIC HILIC column in the first dimension. These two separation mechanisms proved to give orthogonal separation and improved resolution of plants extracts compared to one dimensional separation. The 2D LC system is characterised by sufficient practical peak capacities. Moreover, for the online approach utilised shown virtually no additional band broadening.

The online HILIC×Hypercarb method therefore represents a powerful separation strategy for the detailed investigation of complex plants extracts. The developed methodology should be equally suitable for the analysis of other complex samples.

5.0 References

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6.0 APPENDIX I

6.1 Packing columns

Most of the packing procedures of columns in liquid chromatography are using slurry techniques [88]. This slurry packing procedure usually involves: a packing solvent reservoir, a high pressure pump, a slurry reservoir and the HPLC column body (Fig. 42) [89]. The slurry is made by sonication of a mixture of the particles and slurry-liquid. The reservoir is filled with the slurry-liquid and connected to the pump. The slurry is forced into the empty column by the packing liquid. At the outlet side of the column a porous frit is placed that allows liquid to flow through while retaining the packing material. When the packing is finished, the column is detached and another porous frit is placed on the open inlet of the column [57]. Several packing- and slurry-liquids have been studied in order to make reproducible and stable high performance columns [88].

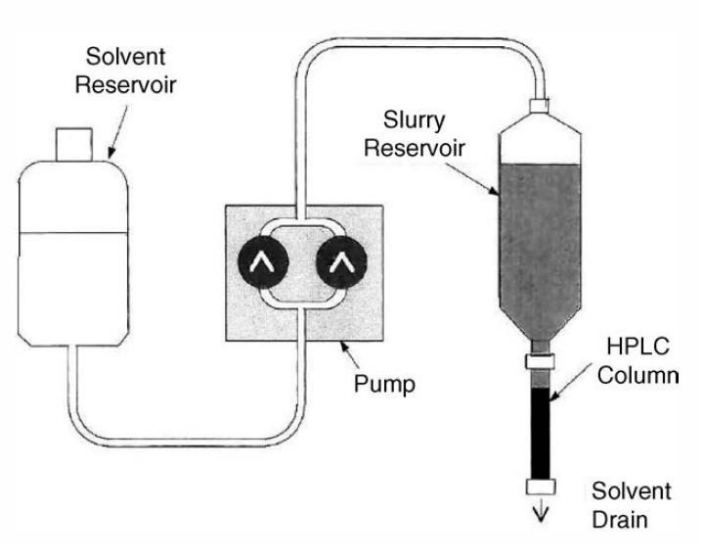

Fig. 42**:** A standard setup for column filling using the slurry technique. Figure reprinted from [88].

6.2 Experimental

Also see main thesis for location of instrument suppliers.

6.2.1 Procedure for slurry packing columns

Columns of 15 cm × 0.3 mm i.d. were packed in-house with a downward high pressure liquid slurry method, using a mixture of AcN/water (70/30, v/v) as packing liquid. The slurry liquid was carbon tetrachloride (99%) (VWR, Oslo, Norway) if not otherwise stated, and the packing material was either Kromasil C18 (G&T Septech, Kolbotn, Norway) or Halo C18 (Advanced Materials Technology, Wilmington, DE), ZIC HILIC. A union (Valco) with a steel frit (Valco) was connected to one end of the steel column, and the other end was connected to the packing chamber. The slurry liquid was ultrasonicated for 10 minutes and then transferred to the packing chamber with a 1 ml plastic syringe coupled to a fused silica tubing (~10 cm × 320 µm i.d.). An ISCO 100 DM syringe pump (Lincoln, NE) with an ISCO series D pump controller was subsequently connected to the packing chamber. The pressure was increased to 100 bars by opening a valve, and then increased up to 650 bars at a rate of 100 bars/min. The column was subjected to 650 bars for 15 minutes, before it was depressurized for 30 minutes. The column was then detached from the packing chamber, and a filter (1 and 2 µm used to pack Halo C18 and Kromasil C18, HILIC particles respectively) (Valco) and a union were connected to the free end of the steel column.

7.0 APPENDIX II

7.1 Determination of oxysterol by LC-ELSD

7.1.1 Introduction

Recent studies have shown that certain oxysterols are activators of the Hh pathway.

How these activate the pathway is unclear, but they are thought to work on the level between the pathway receptors PTCH and SMO as shown in Fig.42

Hh pathway

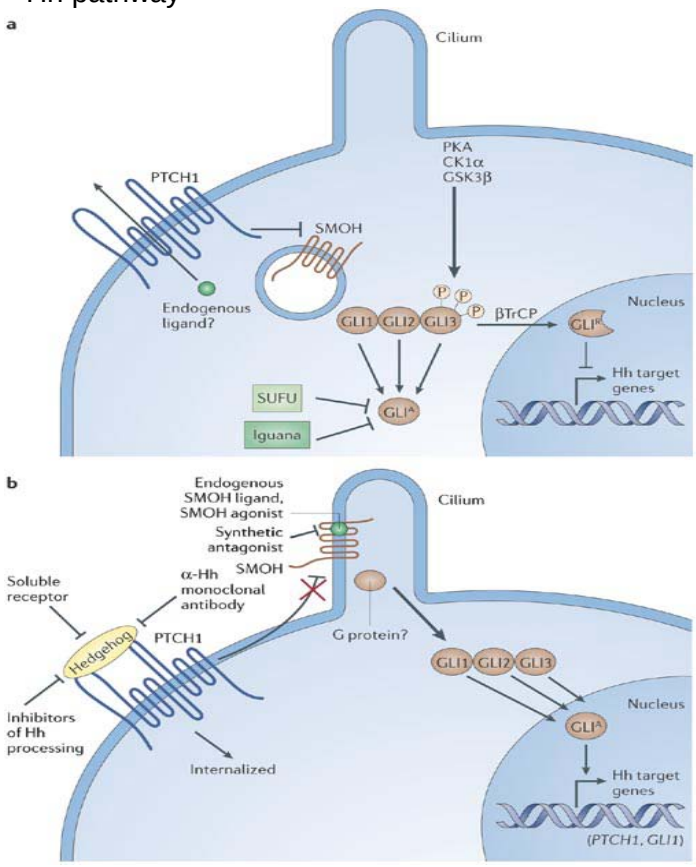

Fig. 42: Naturally occurring oxysterols, previously shown to direct pluripotent mesenchymal cells toward an osteoblast lineage, exert their osteoinductive effects through activation of Hedgehog signaling pathway.

This was demonstrated by 1) oxysterol-induced expression of the Hh target genes *Gli-1* and *Patched*, 2) oxysterol-induced activation of a luciferase reporter driven by a multimerized Gli-responsive element, 3) Inhibition of oxysterol effects by the hedgehog pathway inhibitor, cyclopamine, and 4) Unresponsiveness of *Smoothened/* mouse embryonic fibroblasts to oxysterols. Using *Patched/* cells that possess high baseline Gli activity, it is found that oxysterols did not dramatically shift the IC50 concentration of cyclopamine needed to inhibit Gli activity in these cells. Furthermore, binding studies showed that oxysterols did not compete with fluorescently labeled cyclopamine, BODIPY-cyclopamine, for direct binding to Smoothened. These findings demonstrate that oxysterols stimulate hedgehog pathway activity by indirectly activating the seven trans membrane pathway component Smoothened. The structure of one oxysterol is shown in Fig 43

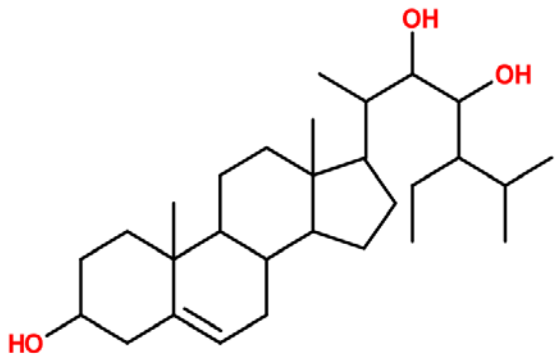

Fig. 43: Structure of oxysterol 11

7.1.2 Experimental

Preliminary separation of the oxysterols standards was obtained on a column using an Agilent 1100 series (Agilent, Palo, Alto, CA, USA) pump with online vacuum degasser for mp delivery and an ELSD detector. Nitrogen gas flow of detector was 2.75 (SLPM). The chromatographic instrument was controlled by Ez chrome software. Chromatography was carried out using a mobile phase consisting of 50% methanol/ 50% water at a flow rate of 0.2ml/min. The chromatogram of uracil standard with given condition is shown in Fig. 44.

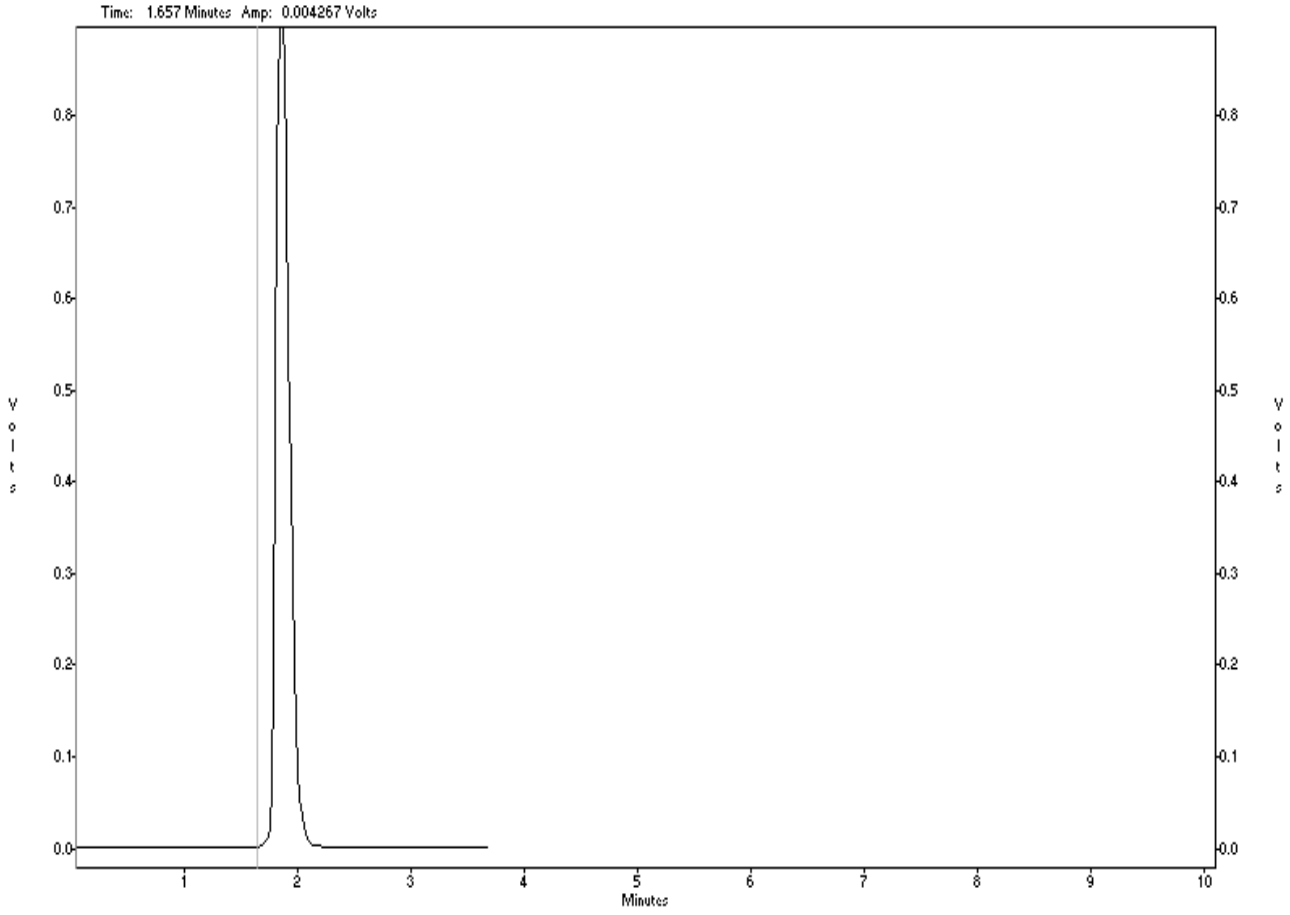

Fig. 44: Chromatogram of toluene standard with concentration 1 mg/ml with detection ELSD detector. Drift tube temperature of detector was 80 °C and nitrogen gas flow of detector was 2.75(SLPM). The mobile phase consisting of 50 % methanol/ 50 % water at a flow rate of 0.2 ml/min.

The chromatogram of oxysterol 11 is shown in Fig. 45 with the same condition as given above. The compound was possible detected but with very low intensity on given instrument.

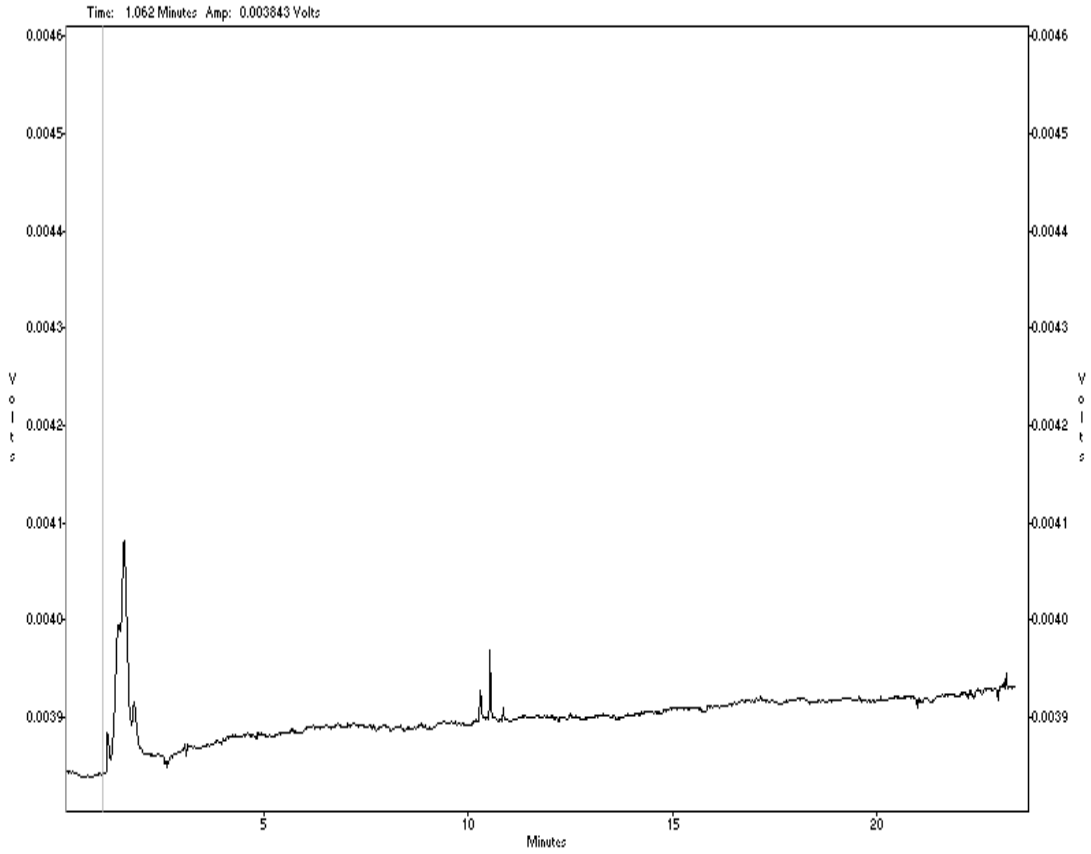

Fig. 45: chromatogram of 0.1mg/ml of oxysterol11 dissolved in dimethyl sulfoxide(DMSO) with detection ELSD detector. Drift tube temperature of detector was 80 °C and nitrogen gas flow of detector was 2.75(SLPM). The mobile phase consisting of 50 % methanol/ 50 % water at a flow rate of 0.2 ml/min.

7.1.3 Conclusion

By changing the composition of mobile phase no effect on the retention time of the apparent oxysterol 11 peak was observed. The retention time of toluene on the other hand changed with the composition of mobile phase. No peaks appeared when oxysterol 20 was injected and it was concluded that the oxysterol could not be detected at the investigated conditions. This project was not proceeded further because of lack of sample.

8.0 Appendix III

8.1 Effect of flow rate and acetonitrile composition

With lower flow rate (1ml/min with 50, 60, 70 % AcN and 2 ml/min with 50, 60 % AcN) the separation was not completed within 2 min and, that was not compatible with the online HILIC system as shown in Fig. 46. A, B, C, D, and F. The chromatogram in Fig 46 E shows that the peaks shapes were broad when 3 ml/min of flow rate and 50 % of AcN were used.

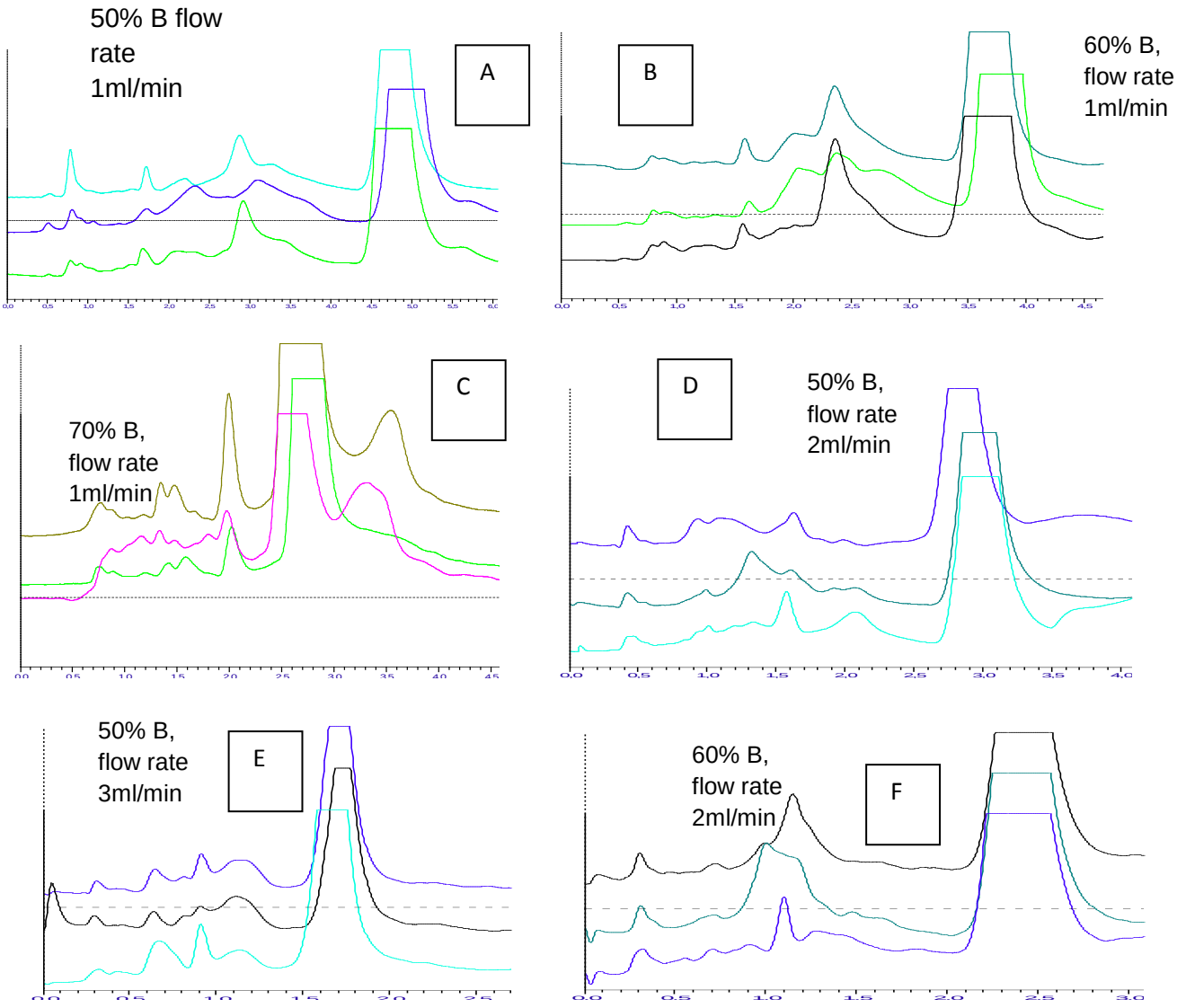

Fig.46 A, B, C, D, E, F: Second dimension separation of first three HILIC fractions on Hypercarb column (50mm× 4.6mm i.d) with mobile phase consisting of water with 0.1 % FA (A)/AcN (B) with isocratic elution 50, 60, 70% AcN in 8 min with a flow rate of 1/min and 50, 60% AcN with flow rate 2ml/min and 50 % Acn with 3ml/min at 110°C.UV detection at 254nm was performed. The 50 µl injection volumes were used.

9.0 Appendix IV

9.1 Examination of extraction solvent

The plant samples were grinded and then extracted with AcN/water (50/50 v/v), methanol and AcN as detailed in the experimental section, and analysed by the online 2D LC system.

9.1.1 AcN/Water extracted plant material

The sample which was extracted with water/AcN mixture was not well separated in the first dimension as shown in Fig. 47.

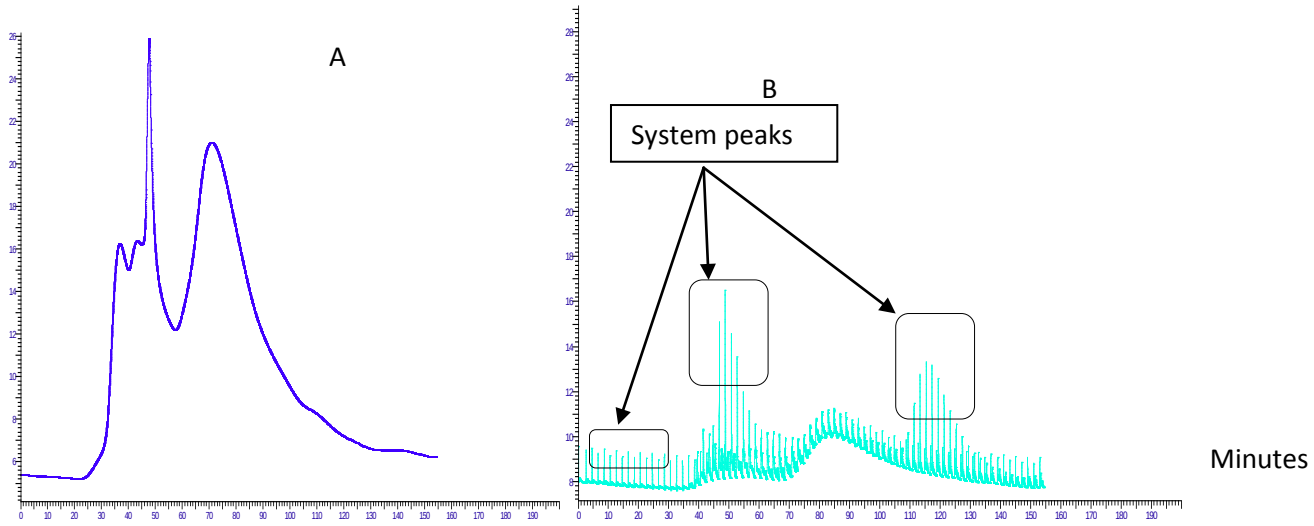

Fig.47: Chromatogram first and second dimension separation of (AcN/Water extracted) satureja montana1 on (A) HILIC column (150mm× 4.6mm i.d) with mobile phase consisting of 10mM ammonium acetate/AcN 85/15% (v/v) min with a flow rate of 0.05ml/min. UV detection of first detector was performed at 254nm. (B) Second dimension separation performed on Hypercarb column (50mm× 4.6mm i.d) with isocratic elution with 10mM ammonium acetate/AcN 30/70 % (v/v) at a flow rate of 2/min at 100°C.UV detection of second detector was also performed at 254nm. 50 µl of sample were injected in the first dimension.

9.1.2 ACN extracted plant material

The same plant sample was extracted with AcN, and analysed by the online 2D LC system as shown in Fig.48. .

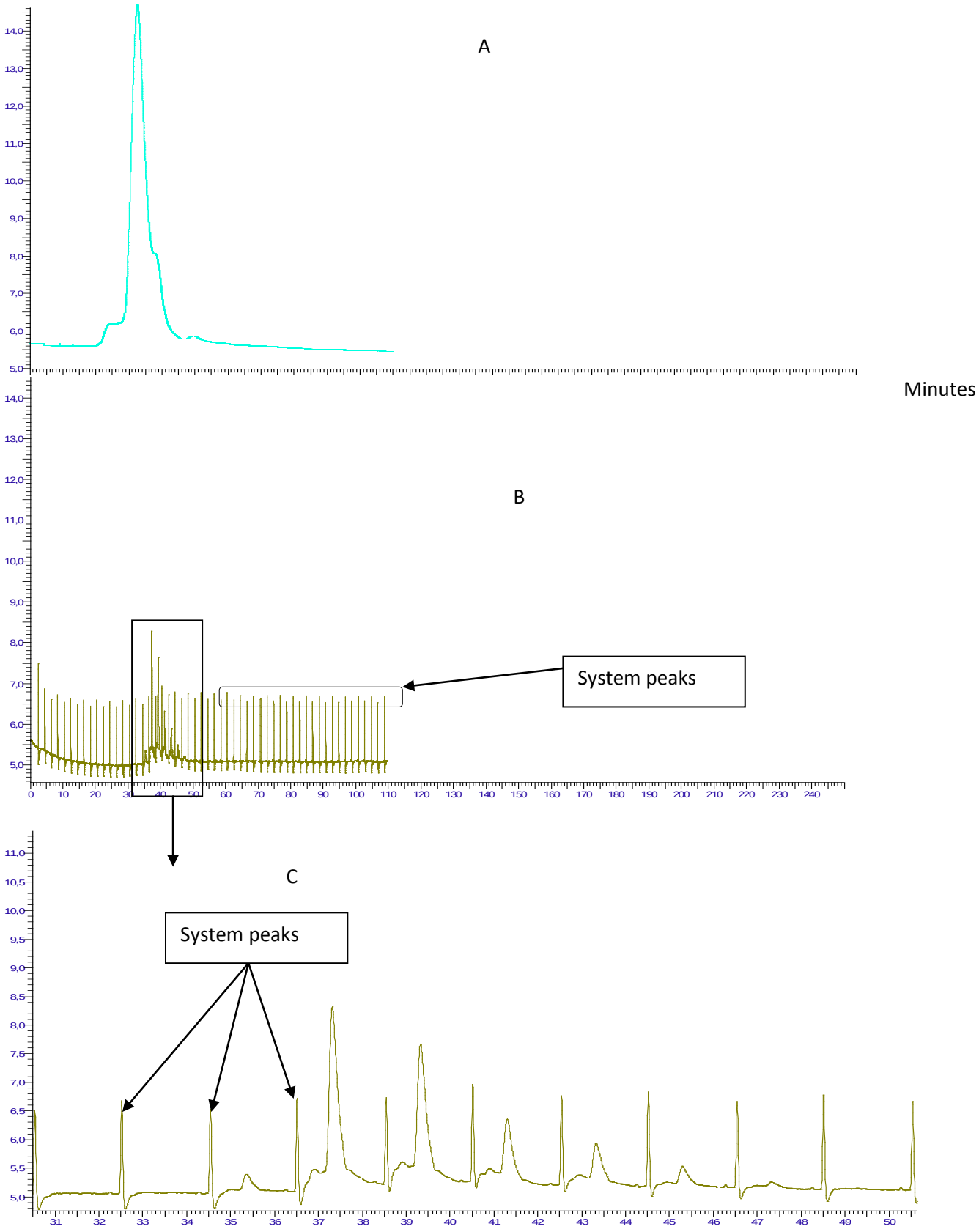

Fig. 48: Chromatogram first and second dimension separation of (AcN extracted) satureja montana I on (A) HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10 mM ammonium acetate/AcN 85/15 % (v/v) with a flow rate of 0.05 ml/min. UV detection of first detector was performed at 254 nm. (B) Second dimension separation performed on Hypercarb column (50 mm× 4.6 mm i.d) with isocratic elution with 10mM ammonium acetate /AcN 30/70 % (v/v) at a flow rate of 2 ml/min at 100 °C.UV detection of second detector was also performed at 254 nm. 50 µl of sample were injected in the first diemsnion.

9.1.3 Methanol extracted plant material

Methanol extracted plant sample has been found to give quite similar first dimension chromatogram profile like the AcN extracts. In the second dimension chromatogram more peaks were observed and orthogonal separation was obtained large number of compounds can be separated in relatively short analysis time as shown in Fig. 49.

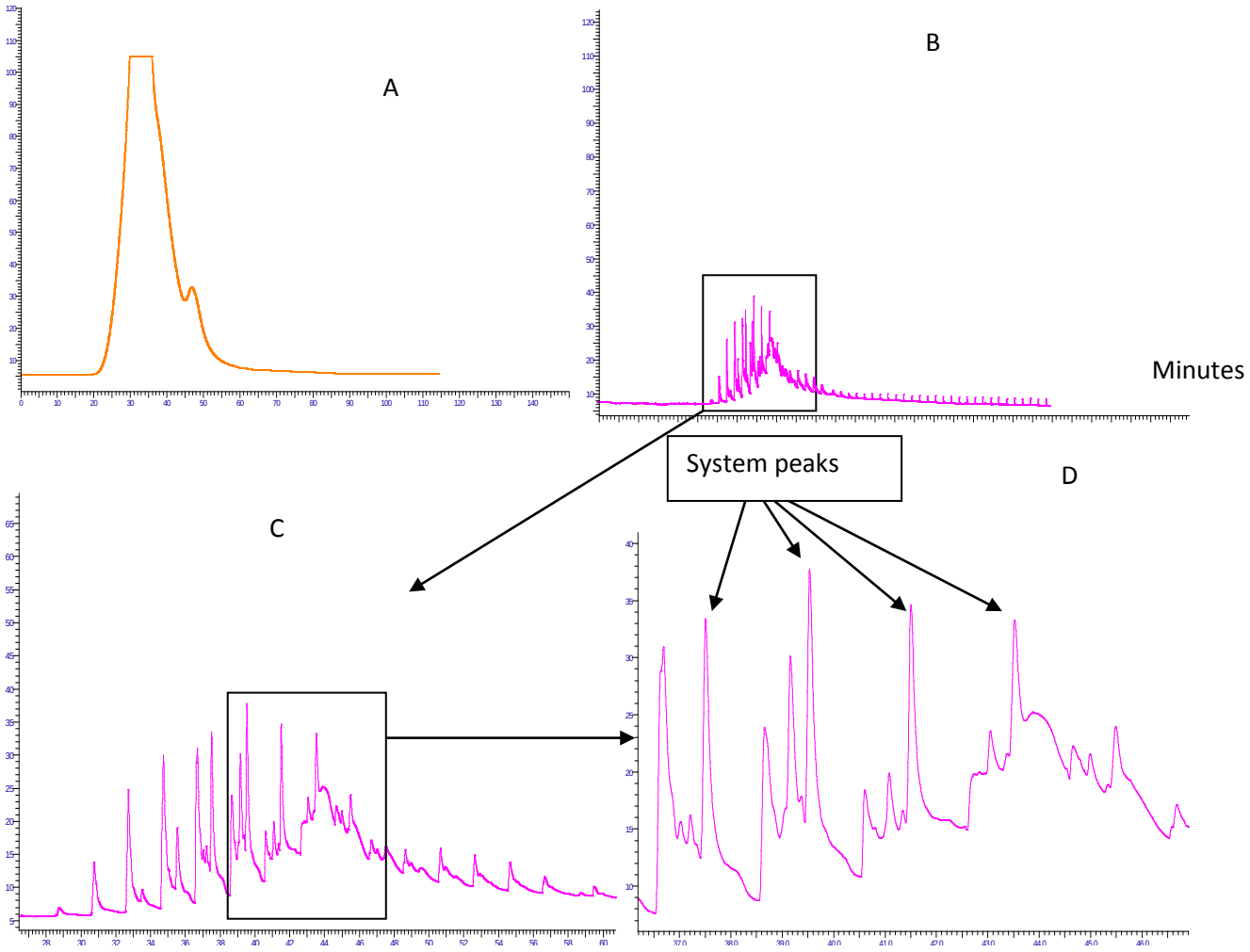

Fig.49: Chromatograms first and second dimension separation of (methanol extracted) satureja Montana I on (A) HILIC column (150 mm× 4.6 mm i.d) with mobile phase consisting of 10mM ammonium acetate /AcN with isocratic elution 85/15 % (v/v) min with a flow rate of 0.05 ml/min. UV detection of first detector was performed at 254 nm. (B) Second dimension separation performed on Hypercarb column (50 mm× 4.6 mm i.d) with isocratic elution with 10mM ammonium acetate/AcN 30/70 % (v/v) with a flow rate of 2 ml/min at 100 °C.UV detection of second detector was also performed at 254 nm. 50 µl of sample were injected. (A) first dimension chromatogram of satureja montanaIII (B) second dimension, (C) expansion of second dimension and (D) expansion of chromatogram of C.