Solid-Phase Extraction Methods for the Isolation of Natural Bioactive Substances of Plant Origin

A thesis presented to the Faculty of Chemistry and Pharmacy for the academic degree of Doctor of Natural Sciences

presented by

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accomplished at
the Institute of Analytical Chemistry and Radiochemistry
Center for Chemistry and Biomedicine
Leopold-Franzens-University of Innsbruck

Innsbruck, November 2014
Dedication

This thesis is dedicated to

My sweetest Mother (Syeda Tahseen Zahra) who brought me from heaven to earth and raise me up to heaven again, who always prays for my success and prosperity, under her feet is my paradise.

My loving Father (Syed Zafar Abbass late) whose inspiration towards knowledge served me as a beacon of light, to whom I owe all that is mine.

My caring Brothers (Fazal Abbas, Mujtaba and Hussain); my loving Sister (Sadaf) for their sublime love and whose heartily prays are always with me.

And to all those who Love me.
I hereby declare that this dissertation is my own original piece of work carried out under the supervision of O.Univ.Prof.Mag.Dr.Dr.h.c. Günther Karl Bonn.

Innsbruck, November 2014

Shah Hussain
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Abstract

Substances demonstrating activity on living cells are considered to be bioactive. These substances can occur naturally (natural bioactive substances) or they can be synthesized. Demonstrating numerous pharmacological activities, natural bioactive substances originate from plants, animals, and minerals. These can be found broadly as: proteins, carbohydrates, minerals, terpenes, flavonoids, phenolic acids, vitamins, lipids, saponins etc. As they are often found in plant or animal tissues, therefore, highly selective isolation methods are required to exploit their effectiveness. The thesis reports highly efficient solid-phase extraction (SPE) methods for some of the important bioactive substances i.e., phenolic acids and thionins from their natural sources. Moreover, bioanalysis of phenolic acids and their metabolites from human plasma has also been described. Phenolic acids were extracted from *Galphimia glauca* and * Arnicae flos* employing pure zirconium silicate and bismuth citrate powders as two novel sorbents. The efficiency of both the sorbents was compared to the most commonly applied commercially available stationary phases by using high performance liquid chromatography hyphenated to diode-array detection (HPLC-DAD). The nature of interaction between the carrier sorbent and the acidic target molecules was investigated by studying silica, C18, Oasis® MAX and zirconium silicate. The other bioactive substances studied were thionins, which are cysteine rich, small (~5 kDa) and basic proteins occurring in the plant kingdom. Efficient SPE methods for the selective isolation of thionins were developed. Hollow-monolithic extraction tips based on poly(styrene-co-divinylbenzene) with embedded zirconium silicate nano-powder and aluminum silicate powder in extraction columns were employed as two novel sorbents. European mistletoe, wheat and barley samples were used for selective isolation of
viscotoxins, purothionins and hordothionins, respectively. The isolated fractions were subjected to matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI/TOF MS). For peptide mass-fingerprint analysis tryptic digests of SPE eluates were performed. HPLC-DAD was employed for the purification of individual isoforms.

An efficient SPE clean-up method for the bioanalysis of phenolic acids and their metabolites from human plasma was also reported. Methanol deproteinization was applied, followed by zirconium silicate assisted exclusion of residual proteins. MALDI/TOF MS was used for monitoring the proteins during clean-up practice applied to human plasma samples. The proteins were quantified by colorimetric detection using the bicinchoninic acid (BCA) assay. The analytical strategy caused the depletion of >99.6% proteins from human plasma samples. Furthermore, HPLC-DAD and electrospray ionization mass spectrometric detection (ESI MS) was applied for the bioanalysis of the phenolic acids and their metabolites. The presented scheme improved the clean-up efficacy of the methanol deproteinization, significantly reduced the matrix effects and demonstrated high recoveries for analytes.

The developed SPE strategies provide an analytical platform for the selective isolation of natural bioactive substances from the complex samples. The presented techniques can aid the quest for the new sources of these pharmacological active compounds. Moreover, the presented clean-up scheme can be employed to study the pharmacokinetics of bioactive substances.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2’-azobis-2-methylpropionitrile</td>
</tr>
<tr>
<td>ALE</td>
<td>Artichoke leaves extract</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BDS</td>
<td>Base deactivated silica</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>Caffeic acid</td>
</tr>
<tr>
<td>CGA</td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>CN</td>
<td>Co-ordination number</td>
</tr>
<tr>
<td>CQAs</td>
<td>Caffeoylquinic acids</td>
</tr>
<tr>
<td>CV</td>
<td>Co-efficient of variance</td>
</tr>
<tr>
<td>CYN</td>
<td>Cynarin</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode-array detection</td>
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<tr>
<td>DCQA</td>
<td>Dicaffeoylquinic acid</td>
</tr>
<tr>
<td>DHCA</td>
<td>3,4-dihydroxyhydrocinnamic acid</td>
</tr>
<tr>
<td>DL</td>
<td>Desolvation line</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FLA</td>
<td>Ferulic acid</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
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<tr>
<td>GDE</td>
<td><em>Galphimia glauca</em> dry extract</td>
</tr>
<tr>
<td>GQA</td>
<td>Galloylquinic acid</td>
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<tr>
<td>HCCA</td>
<td>α-Cyano-4-hydroxycinnamic acid</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LLE</td>
<td>Liquid-liquid extraction</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge</td>
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<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption/ionization</td>
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<tr>
<td>MODQA</td>
<td>Methoxyoxaloyl-dicaffeoylquinic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>nOOGP</td>
<td>n-Octyl β-D-glucopyranoside</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PSDZ</td>
<td>Poly(styrene-co-divinylbenzene) embedded zirconium silicate</td>
</tr>
<tr>
<td>QC</td>
<td>Quality control</td>
</tr>
<tr>
<td>RE</td>
<td>Relative error</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>S/N</td>
<td>Signal to noise</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SA</td>
<td>Sinapinic acid</td>
</tr>
<tr>
<td>SALDI</td>
<td>Surface assisted lased desorption/ionization</td>
</tr>
<tr>
<td>SALLE</td>
<td>Salting-out assisted liquid/liquid extraction</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected ion monitoring</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid-phase extraction</td>
</tr>
<tr>
<td>STY</td>
<td>Styrene</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TGQA</td>
<td>Tetragalloyquinic acid</td>
</tr>
<tr>
<td>tGQA</td>
<td>Trigalloyquinic acid</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>XIC</td>
<td>Extracted ion chromatograms</td>
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CHAPTER 1

Introduction
1. Introduction

The dissertation describes research on solid-phase extraction (SPE) techniques for the isolation of pharmacologically active substances of plant origin. To put this work in perspective, the introduction formerly describes the significance of sample preparation for chemical analysis. The chapter then elucidates the basics of SPE and compares it to the conventional liquid-liquid extraction (LLE). Subsequently, it provides an overview about the biological activities of the selected analytes for the research (phenolic acids and thionins) and reviews some of the common methods for their extraction from complex samples. The final section of the chapter presents a brief outline of the dissertation.

1.1. The significance of sample preparation

Sample preparation is an exceptionally substantial part of the overall chemical analysis. Analytical laboratories are under enormous stress to provide rapid analysis at lower cost. This burden falls most profoundly on the sample preparation portion of the laboratory, which is asked to deliver more reproducible results, decrease the use of organic solvents and provide cleaner extracts for the subsequent instrumental analysis [1]. There has always been a need for the upgrading of sample preparation techniques because the majority of analysis time is consumed in preparing the sample. According to one report, more than 60% of the analysis time is spent in sample preparation compared to only about 7% for the actual measurement of the sample constituents [2].

LLE remained the method of choice for the clean-up and preconcentration of the analytes in past. In LLE, the sample is agitated in the presence of the extraction solvent that is not miscible with the sample. When the sample/solvent mixture settles after the agitation two layers are formed, one of which contains most of the analytes. However, the recovery of
analytes by LLE is rarely complete. It is also slow, labor-intensive and the disposal of the large volume of organic solvents is also an environmental concern. LLE is the cherished point from which to interpret SPE. In SPE the extraction solvent is replaced by the solid surface [3].

SPE is the increasingly popular sample preparation technique that was originally established as a complement or replacement for LLE. SPE isolates a sample into desired and undesired components using the affinity of analytes (in mobile phase) for a solid (stationary phase or sorbent) through which the mixture is passed. As a consequence, either the analytes or the impurities are retained on the sorbent. The portion that flows through the sorbent is discarded or collected, which depends on whether it encompasses the analytes or impurities. If the portion retained on the stationary phase contains the desired analytes, they can be detached from the sorbent by rinsing it with an appropriate eluent in a separate step called elution. In its simplest form, SPE employs a packing material such as bonded silica or polymeric media packed into a plastic or a syringe barrel. Similar to an HPLC column, porous polymeric or metallic frits enclose the packing in the cartridge format. Additional SPE formats are disks, pipette tips and 96-well plates.

The primary objectives of SPE are enrichment (concentration), sample clean-up and medium exchange (from the complex sample matrix to a cleaner solvent). SPE has now emerged as the most popular sample preparation technique in many areas of chemistry, including environmental, clinical, pharmaceutical, food and industrial chemistry.

1.2. General procedure for SPE

SPE has the following operational steps:
1. Conditioning

2. Loading or retention

3. Washing

4. Elution

1.2.1. Conditioning

The conditioning or wetting step activates the bonded phase so that it can readily accept the liquid sample. Conditioning of the sorbent (with appropriate solvents) is imperative to ensure the reproducible interactions with the analyte.

1.2.2. Loading or retention

The process of applying the sample to the stationary phase is called loading. The optimum flow rate is as important as the choice of the right sorbent for this step. The optimum flow rate should be controlled and recorded for the reproducibility. A decent SPE sorbent retains a higher amount of analyte compared to its amount in the mobile phase. This distribution can be defined by the coefficient factor $K$, which designates the fraction of analyte retained onto the sorbent and the portion of the analytes still remained in the mobile phase [3].

\[
K = \frac{\text{Concentration of analyte in sorbent}}{\text{Concentration of analyte in sample}}
\]

The higher the value for the $K$ the more efficient would be the sorbent.

1.2.3. Washing

During retention step many undesirable components from the complex samples are retained onto the sorbent along with the preferred substances. The purpose of washing is to
selectively eliminate the unwanted components without eluting the analytes. Usually, the good washing solvents are miscible with the sample matrix and the analytes are poorly soluble in it.

1.2.4. Elution

During elution the retained substances are released from the sorbent using a stronger solvent which disrupts analyte-sorbent binding. The choice of elution solvent also depends upon its compatibility with the subsequent instrumental analysis.

Fig. 1.1: Schematic view of a general SPE column and procedure.
1.3. Separation modes in SPE

1.3.1. Normal Phase SPE

Normal phase SPE is carried out with a hydrophilic sorbent and the mobile phase comprising of very hydrophobic solvent such as hexane as a “carrier” with changing amounts of a more hydrophilic organic solvent as the “modifier”. Silica gels, alumina, bonded phase silica materials containing highly polar functional groups are the sorbents of choice for normal phase SPE. Silica sorbents with cyanopropyl groups, diol groups or aminopropyl groups are frequently serve the purpose.

1.3.2. Reversed Phase SPE

Reversed phase SPE employs hydrophobic sorbents (e.g. silica with bonded octadecylsilane groups or organic polymers containing benzene rings) for the isolation of relatively non-polar analytes from a polar sample such as water. The analytes are retained on the stationary phase based upon their hydrophobicity, i.e., more hydrophobic the substance more strongly it is retained on the stationary phase, and vice versa. The retained analytes are eluted by a small volume of organic solvents.

1.3.3. Ion-exchange SPE

Electrostatic interactions between the analyte of interest and the charged groups (positive or negative) on the stationary phase are the basis of separation in ion-exchange SPE. It can be sub divided into anion-exchange- and cation-exchange SPE. Anion-exchange SPE employs sorbents derivatized with quaternary ammonium groups which retains anionic analytes from the sample. On the contrary, cation-exchange SPE retain cationic analytes from the samples by employing the sorbents derivatized with sulphonyc or carboxylic groups.
1.4. History of SPE

SPE is the most prevalent sample preparation technique today. The history of SPE dates back to the early 1970s when the enrichment of organic pollutants from water was reported [4]. Disposable cartridges for SPE have been introduced for more than 35 years (first cartridges in 1978, syringe-format types in 1979, precolumns for the on-line coupling with liquid chromatography (LC) in the early 1980s) yet, SPE development has been sluggish for many years [5]. LLE remained the preferred practice for the sample preparation for numerous years especially in the environmental field. The substantial development of SPE has occurred during the past decade, with many enhancements in formats, automation and introduction of new sorbents. The need to decrease the consumption of organic solvents in laboratories, due to the growing environmental concerns over the disposal of excessive organic solvents, has greatly contributed to the growth of SPE at the expense of LLE procedures [6]. Moreover, LLE was found less effective for polar analytes [7]. At the same time, the accessibility of cleaner and more reproducible sorbents than in the past has also assisted in growing acceptance for SPE by regulatory agencies. Other reasons for the mounting interest in SPE techniques are the enormous choice of sorbents with the capability of retaining polar analytes. Novel formats have been also familiarized e.g., the 96-well SPE plates and the microfibers for solid-phase microextraction.

1.5. Advantages of SPE

SPE offers many significant advantages over LLE [1].
1. The steps involved in LLE are generally more complicated as compared to SPE and require a significant amount of time. SPE, on the other hand, is faster, easy to use and can be automated.

2. Much smaller amounts of organic solvents are used in SPE as compared to LLE.

3. Higher enrichment factors can be achieved with SPE.

4. SPE is suitable for both polar and non-polar analytes and provides cleaner extracts with higher recoveries.

1.6. SPE of natural bioactive substances

Substances showing activity on living cells or tissue are considered to be bioactive. A number of substances have been reported for biological activities in living cells. These bioactive substances can exist naturally (natural bioactive substances) or they can be synthesized. The naturally existing biologically active substances can be found broadly as: proteins, carbohydrates, minerals, terpenes, flavonoids, phenolic acids, vitamins, lipids, saponins etc. [8]. The origin of these bioactive substances from plant and animal tissues demands highly selective isolation methods in order to exploit their utility. The presented thesis reports the extremely efficient SPE of some of the very important bioactive substances namely, phenolic acids from their plant sources, phenolic acids and their metabolites from human plasma and thionins from their natural sources, as well.

1.6.1. Phenolic acids

Phenolic acids are organic compounds having phenolic ring and a carboxylic functional group. They are widely distributed in various plant species. Their ability to protect the human body from oxidative stress makes them enormously significant. They form a diverse
group that includes widely distributed benzoic acid derivatives (gallic acid, vanillic acid, etc.) and cinnamic acid derivatives (chlorogenic acid, cynarin, etc.).

The dissertation particularly focuses on the SPE of galloyl- and caffeoylquinic acids (GQAs and CQAs) from complex plant materials. GQAs and CQAs contain one free carboxylic group in the quinic acid moiety. The esters of quinic acids with caffeic, ferulic or p-coumaric acids are grouped under CQAs, whereas the esters of quinic acid with gallic acids are termed as GQAs. GQAs show advanced structural complexity over CQAs, because GQAs also have the tendency to form aryl esters. Such GQAs exhibiting aryl esterification are described in the literature as depsides [9, 10].

1.6.1.1. Natural sources of GQAs and CQAs

The foremost natural sources for GQAs include *Galphimia glauca* [11, 12], *Byrsonima fagifolia* [13], tera tannin, tannic acids [10], *Koelreuteria henryi* [14], *Pistacia lentiscus* [15] and *Hiraea reclinata* [16]. CQAs exist in a variety of plants and were extracted from *Cynara scolymus* L. [17], *Pimpinella anisum*, *Camellia sinensis* (green tea), *Cymbopogon citratus* [18], *Ilex Species* [19] and *Arnica montana* [20].

For the study, the aerial parts of *Galphimia glauca* and the flowers of *Arnica montana* (*Arnicae flos*) were used for the extraction of GQAs [11] and CQAs [20], respectively. *Galphimia glauca* is found in Middle- and South-America. It is used as a remedy against allergy by the local tribes. *Galphimia glauca* demonstrates antiasthmatic effects [11, 12] and serves as a homeopathic medicine against pollinosis [21]. It was also reported for anxiolytic [22] and antiprotozoal activity [23]. *Arnicae flos* is also employed in Ayurvedic, homeopathic, Unani and folk medicines. In Europe, It is used for the treatment of skin diseases and muscle pain. *Arnicae flos* can also be used as flavor additive in food materials.
Moreover, it is effective against oral pathogens [24]. The anti-inflammatory activity of *Arnicae flos* was also documented [25]. *Arnicae flos* extracts also have the potential to be applied as topical medication against inflammation due to good skin penetration ability [26].

1.6.1.2. Pharmacological importance

GQAs are very strong antioxidants [14]. They were also described to be reverse transcriptase inhibitors and can be applied as anti-HIV agents [9, 16]. Anti-allergic and anti-asthmatic effects have also been reported in the literature [11, 12]. CQAs are inhibitor of chemically induced carcinomas in animals [27]. Moreover, CQAs have a role in preclusion of type 2 diabetes [28, 29]. CQAs also denote antioxidant, hepatoprotective and antiviral agents [30-32]. CQAs are powerful anti-HIV agents because of their ability to constrain HIV integrase. This enzyme integrates the double stranded viral DNA (formed by the action of reverse transcriptase) to the host chromosomal DNA [33]. CQAs have also been reported for their neurotrophic and neuroprotective effects [34, 35], in preventing cell toxicity and the treatment of oxidative stress [36] along with antihypertensive activity [37].

1.6.1.3. SPE of phenolic acids

SPE of phenolic acids from their natural sources has generally been attained by silica based carriers derivatized with octadecyl groups or quaternary amines [38-41]. In this regard, the use of alumina powder was reported by Alonse *et al.* [42]. SPE of phenolic acids from plant material is imperative for eliminating sterols, chlorophyll, waxes and oils to avert destruction of an analytical column. In addition, their interference in the process of chromatography and in further analytical investigations can be avoided this way [38]. The thesis reports two novel sorbents (zirconium silicate and bismuth citrate) for the isolation of GQAs and CQAs from *Galphimia glauca* and *Arnicae flos, respectively* [43].
The prerequisite for a material to be employed as the sorbent in SPE is its stability under the experimental conditions. The configuration of bismuth citrate complex was illustrated in X-ray crystallography for potassium ammonium complexes of bismuth citrate by Asato et al. [44]. The bridging of citrate ions provides mechanical stability to bismuth citrate. The bismuth reveals co-ordination number (CN) of nine in the complex. Carboxylic ligands from tetra ionized citrate ions occupy Seven out of nine coordination sites and the remaining two sites are occupied by water molecules. These two sites occupied by water molecules can be replaced by the ligands of analytes.

Zirconium silicate is an excellent refractory material which is extremely stable under acidic and alkaline conditions. Jameson et al. reported the structure of zirconium silicate [45]. Electrostatic interactions in zirconium silicate can be explained by coordination chemistry. Zirconium exhibits CN of eight in zirconium silicate, oxygen atoms occupy four of the co-ordination sites, whereas remaining four are assumed to be surrounded by water molecules. These water molecules can be exchanged by the ligands of analytes during sorption.

1.6.2. Thionins

Thionins are low molecular weight (~5 KDa), cysteine-rich, basic proteins with a polypeptide chain of 45 to 48 amino acids and 3 to 4 internal disulfide bridges. Thionins are found in the endosperms of Gramineae, e.g. wheat and barley, as well as in various plant species, including leaves and stems (e.g. mistletoe, pyrularia and rosids). Thionins can be divided into two groups, namely α/β-thionins and γ-thionins (plant defesins). α/β-Thionins can be subdivided into the types I, II, III, IV and V [46, 47].
1.6.2.1. Structure of thionins

All the five types of α/β-thionins appear to be exceptionally homologous at the amino acid level. Primary structure determinations revealed that about 12 to 17% of the amino acids are cysteine residues. These cysteine residues are extremely conserved and are involved in disulphide bond formation [48, 49]. Among thionins, the crystal structure of crambin (type IV thionin) was the first to be resolved through anomalous scattering of sulphur.

Crambin posses the shape of the Greek capital letter gamma Γ, antiparallel pair of helices constitutes the stem of gamma and the two antiparallel β-strands establishes the cross-arm, an irregular strand and a classic β-turn [50]. A number of X-ray structure determinations of different thionins, along with NMR studies clearly confirmed a distinct architectural feature for these small proteins. Irrespective of small variations in length (45-48 amino acids) α/β-thionins share similar three dimensional structure with minor exceptions [46].

1.6.2.2. Pharmacological importance of thionins

Thionins are toxic to certain yeast strains [51] and insect larvae [52], antibacterial [53] and show in vitro protein synthesis inhibition [54]. Thionins are described to be effective against plant pathogenic bacteria [55] and actively obstruct endogenous mRNA translation [56]. They are also powerful antifungal agents [57] and a synergistic improvement of the antifungal activity of thionins by 2S albumins and trypsin inhibitors is also reported [58]. Enhanced natural killer cell-mediated killing of tumor cells [59], immunomodulatory effect on human granulocytes [60, 61], and induced apoptosis in human lymphocytes [62] has also been the prominent pharmacological activities of thionins.
1.6.2.3. SPE of thionins

SPE was proved to be very valuable for isolating polypeptides from complex biological tissues [63] and from food samples [64]. Herraiz et al. assessed different SPE sorbents based upon non-polar and ionic interactions employing small synthetic peptides and casein enzymatic hydrolysates [65]. Different SPE sorbents covering cyanopropyl, ethyl, cyclohexyl, phenyl, octyl or octadecyl functional groups can be used providing an extensive range of possibilities for pre-concentration of peptides. The sorbent must be selected keeping in view the polarity, hydrophobicity and length of the peptide [66].

Selective extraction of thionins from their complex natural sources is often complicated and requires several purification steps. This burdensome practice can be significantly simplified by an effective SPE of the thionins using a selective SPE sorbent. The presented thesis unveils two novel sorbents for the extremely convenient and selective isolation of plant thionins i.e. poly(styrene-co-divinylbenzene) (poly(STY-co-DVB)) embedded zirconium silicate nano powder hollow monolithic sorbent (PSDZ) and aluminum silicate powder.

PSDZ constitutes a stationary phase having hydrophobic and electrostatic interactions. Poly(STY-co-DVB) framework provides the mechanical embedding of zirconium silicate to the stationary phase. Zirconium silicate nano powder offers the electrostatic interactions to the analytes and consequently enhances the selectivity of the stationary phase, whereas poly(STY-co-DVB) network gives mechanical strength and hydrophobicity to the phase.

Aluminum silicate (mullite) is an excellent ceramic material with extreme chemical stability. The composition of mullite varies from 3Al₂O₃.2SiO₂ (3:2) to 2Al₂O₃.1.5SiO₂ (2:1). The average structure of mullite is analogous to closely related, but structurally simple sillimanite. In mullite, distorted edge-sharing octahedral (AlO₆) chains run parallel to the crystallographic c
axis. These chains are cross linked by Si-O and Al-O corner sharing tetrahedra. As the alumina content increases Si$^{4+}$ is replaced by Al$^{3+}$, to maintain the charge neutrality oxygen vacancies are created that are randomly dispersed throughout the structure [67-70]. The IR absorption frequencies for mullite was reported by Mackenzie et al. [71] and Rüsher et al. [72]. The IR spectrum of mullite gives a perfect indication about the composition of mullite i.e., 3:2 mullite can be distinguished from 2:1 mullite on the basis of IR spectral comparisons especially from 1100 cm$^{-1}$ – 1200 cm$^{-1}$ [72].

The greater affinity of cysteine containing peptides to the different metal atoms has been described in the literature. Xu et al. found augmented retention of cysteine encompassing peptides by using gold nano-particles in the stationary phase [73]. The interaction of aluminum with cysteine and the stability constant of their co-ordination complex has also been well documented [74, 75].

1.7. Bioanalysis of phenolic acids

Isolation of drugs and their metabolites from biological fluids such as blood, plasma or urine is complicated due to the existence of proteins or other macromolecules in the samples. The sample preparation techniques largely used for the bioanalysis of small molecules include protein precipitation, liquid-liquid extraction (LLE), SPE and ultrafiltration [76, 77]. Each of these techniques is associated with certain advantages and disadvantages.

Protein precipitation is the most convenient procedure for purifying biological fluids. It includes denaturation of proteins by the use of organic solvents, strong acid/base, salts or heat and subsequently unsettles protein-drug binding [78, 79]. However, not all protein precipitation techniques are appropriate for eliminating the proteins for the analysis of small molecules. The protein denaturation based on acids, salts and heat generally trap the
analytes in the protein aggregates and results in the loss of recovery. Therefore, for the bioanalysis of small molecules, water miscible organic solvents are employed for protein depletion [76, 80]. However, the use of organic solvents can achieve only about 90-96% removal of proteins from human plasma [81]. The residual proteins can generate matrix effects and interfere with the chromatographic procedures. Therefore, it is absolutely imperative to remove the remaining proteins after organic solvent deproteinization. Salting-out assisted liquid/liquid extraction technique (SALLE) has recently been reported for the additional clean-up of the biological samples after organic solvent protein precipitation. SALLE includes double protein precipitation i.e., the addition of salts into the resulting supernatant after protein precipitation by the organic solvent. The approach results in the elimination of more than 99% plasma proteins [82, 83].

The bioanalysis based on LLE has the advantage of cleaner extracts but it is quite time consuming due to slow drying of solvents. Moreover, LLE is inappropriate for hydrophilic compounds [77]. SPE is an efficient sample preparation technique for the bioanalysis of small molecules, but rather expensive compared to protein precipitation. Furthermore, it requires the selection of an appropriate sorbent, development and optimization of the method for selective extraction of various classes of compounds from the biological samples [76]. Ultrafiltration is the least common sample preparation technique for isolating small molecules from the complex biological samples. More than 99% of proteins can be removed from human plasma using ultrafiltration [84]; but the ultrafiltrate only provides the analysis of drug not bounded to proteins as opposed to total drug. Therefore, it is highly unsuitable for sturdily protein-bound drugs. In addition, separate experiments must be executed to determine the extent of drug-membrane binding. The selection of membrane is also crucial
for the analysis, as membrane contaminants and structure can disturb the quantitation and hence results in the loss of recovery [85, 86].

The dissertation reports an effective SPE based clean-up method of human plasma for the bioanalysis of phenolic acids and their metabolites. Methanol deproteinization was applied to human plasma, followed by zirconium silicate assisted exclusion of residual proteins. The binding of zirconium (IV) silicate to the proteins caused the removal of lingering proteins after solvent deproteinization through a rapid SPE procedure.

1.8. Research aims

The existence of natural bioactive substances in the complex plant and animal tissues demands highly selective isolation techniques in order to exploit their effectiveness. The purpose of this research was the development of new SPE methods for the efficient isolation of pharmacologically active substances of plant origin. More specifically the thesis covers:

1. The development of new SPE sorbents for the selective isolation of phenolic acids from their natural sources and to compare these with the most commonly applied commercially available sorbents (chapter 2).

2. The synthesis of a novel stationary phase for the highly efficient and convenient SPE of plant thionins from their complex sources for the subsequent instrumental analysis (chapter 3).

3. The comparison of the developed stationary phases in order to determine the best SPE sorbent for extracting thionins (chapter 4).
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4. Development of a SPE based clean-up method for the bioanalysis of phenolic acids and their metabolites from human plasma samples (chapter 5). Finally, chapter 6 presents the summary and future prospects of the research.
1.9. References


CHAPTER 2

Solid-Phase Extraction of Galloyl- and Caffeoylquinic acids from Natural Sources (*Galphimia glauca and Arnicae flos*) Using Pure Zirconium Silicate and Bismuth Citrate Powders as Sorbents inside Micro Spin Columns*

2. Solid-Phase Extraction of Galloyl- and Caffeoylquinic acids from Natural Sources (Galphimia glauca and Arnicae flos) Using Pure Zirconium Silicate and Bismuth Citrate Powders as Sorbents inside Micro Spin Columns


2.1. Introduction

Galloyl- and caffeoylquinic acids (GQAs and CQAs) can be characterized by one free carboxylic group in the quinic acid moiety. CQAs are the esters of quinic acids with caffeic, ferulic or p-coumaric acids, whereas GQAs are the esters of quinic acid with gallic acids. GQAs show higher structural complexity over CQAs, because not only gallic acids residues can be attached to hydroxyl groups of quinic acids, but there is also the possibility of aryl ester formation between additional gallic acid residues. Such GQAs displaying aryl esterification are reported in literature as depsides [1,2].

Group members of these compounds can be found in a wide range of plant species, they are well known for their biological activities and their pharmacological importance. Three representative compounds, namely 1,3,4,5-tetragalloylquinic acid (TGQA, see Fig. 2.1A, a), chlorogenic acid (3-caffeoylquinic acid, CGA, see Fig. 2.1B, a) and cynarin (1,5-dicaffeoylquinic acid, CYN, see Fig. 2.1B, b) were used as model compounds for this study.
TGQA is a very strong antioxidant [3]. It is reported to be a reverse transcriptase inhibitor and can be applied as an anti-HIV agent [1,4]. Furthermore, anti-asthmatic and anti-allergic effects have been proved by Neszmelyi et al. and Dorsch et al. [5,6]. TGQA along with other GQAs have been isolated from different plant sources (Byrsonima fagifolia [7], Pistacia lentiscus [8], tera tannin, tannic acids [2], Koelreuteria henryi [3], Galphimia glauca [5,6] and Hiraea reclinata [4]).

![Chemical structures of studied GQAs and CQAs](image)

**Fig. 2.1:** (A): Chemical structures of studied GQAs. A: TGQA, B: tGQA (B): Chemical structures of studied CQAs. A: CGA, B: CYN, C: MODQA.
CQAs, the second class of pharmacologically active molecules investigated in this study, are also present in a variety of plants and were isolated from *Cynara scolymus* L. [9], *Ilex Species* [10], *Pimpinella anisum*, *Camellia sinensis* (green tea), *Cymbopogon citratus* [11], and *Arnica montana* [12].

CGA is a well-known inhibitor of chemically induced carcinomas in animals [13]. Moreover, an influence on insulin sensitivity and fat metabolism could be proved, and therefore it may play a role in prevention of type 2 diabetes [14,15]. CGA also represents an antioxidant, hepatoprotective and antiviral agent [16-18].

Dicaffeoylquinic acids (DCQAs) are potent anti-HIV agents because of their capacity to inhibit HIV integrase. This enzyme helps to integrate the double stranded viral DNA (produced by the action of reverse transcriptase) to the host chromosomal DNA [19]. They also have been studied in terms of their neurotrophic and neuroprotective effects [20,21]. DCQAs have a potential role in preventing cell toxicity and the treatment of oxidative stress [22] along with antihypertensive activity [23].

For this study we used the aerial parts of *Galphimia glauca* and the flowers of *Arnica montana* (*Arnicae flos*) as the natural sources of GQAs [5] and CQAs [12], respectively. *Galphimia glauca* originates from Middle- and South-America and is applied by indigenous tribes as an antiallergic drug. *Galphimia glauca* is famous for its antiasthamatic effects [6,5] and is used as a homeopathic medicine against pollinosis [24]. It is also known for its anxiolytic [25], and antiprotozoal activity [26]. Similarly, *Arnicae flos* is used in Ayurvedic, homeopathic, Unani and folk medicines. In Europe, it is used for treatment of skin infections, bruises, muscle pain and flavor additive in food materials. Furthermore, it shows activity against oral pathogens [27]. The anti-inflammatory activity of *Arnicae flos* was
studied successfully by Klaas et al. [28]. Wagner et al. reported a good skin penetration ability of *Arnicae flos* extract and demonstrated its role as a topical medication against inflammation [29].

Solid phase extraction (SPE) of free phenolic acids from natural sources has usually been achieved by silica based carriers derivatized with octadecyl groups or quaternary amines [30-33]. Alonse et al. reported the isolation of a DCQA from plasma samples using alumina powder [34]. SPE of phenolic acids from plant material is important for excluding chlorophyll, sterols, waxes and oils to prevent damage of an analytical column. In addition, their interference in the process of chromatography and in further analytical investigations can be avoided this way [30]. The purpose of this work was to develop and to optimize SPE methods for GQAs and CQAs. To the author’s knowledge, the highly selective isolation of GQAs and CQAs from natural sources using pure zirconium silicate and bismuth citrate powders as sorbents inside micro spin columns is reported for the first time.

### 2.2. Materials and methods

#### 2.2.1. Chemicals and materials

Acetonitrile HPLC-grade (ACN), methanol, ethanol, formic acid (FA), trifluoroacetic acid (TFA), acetic acid, sodium chloride (NaCl), sodium acetate and water HPLC-grade were purchased from Merck KGaA (Darmstadt, Germany). Dihydroxybenzoic acid (DHB), bismuth citrate (325 mesh 99.99% metal basis) and zirconium (IV) silicate (325 mesh) were purchased from Sigma Aldrich (St. Louis, USA). Micro-Spin Columns (200 μl sample capacity, polypropylene frit with a pore size of 20 μm, pH stable within the used range) were purchased from The Nest Group, Inc. (Southborough MA, USA). Sep-Pak (C18) cartridges and Oasis® MAX cartridges were provided by Waters (Milford, USA). Davisil chromatographic
silica media (40-63 Microns) were purchased from Grace GmbH and Co. KG (Worms, Germany). Disposable PTFE syringe filters (pore size 0.2 µm, hydrophobic, ø 30 mm) were received from Carl Roth Gmbh + Co. KG (Karlsruhe, Germany).

2.2.1.1. Plant materials and standards

Aerial parts of Galphimia glauca plant (Galphimiae herba), dry extract of Galphimia glauca (GDE; extract from Galphimiae herba enriched with TGQA) and pure TGQA acid were provided by Bionorica SE (Neumarkt/Oberpfalz, Germany). CGA was purchased from Sigma Aldrich (St. Louis, USA), CYN was from Extrasynthese (Genay, France). Arnicae flos samples were purchased from a local pharmacy in Innsbruck.

2.2.2. Preparation of plant extracts

The Galphimia glauca extract was prepared by extracting 60 mg of plant material five times for 15 minutes with 4 ml H₂O/ACN (1/1, v/v) by using ultra-sonication. The final volume was adjusted to 20 ml with H₂O/ACN (1/1, v/v). Arnicae flos extract was prepared in two concentration levels by extracting 60 mg and 20 mg of plant material separately, five times with 4 ml H₂O/Methanol (1/1, v/v) by ultra-sonification. The final volumes were adjusted to 20 ml with H₂O/Methanol (1/1, v/v). GDE was also prepared in two aliquots; 0.5 mg and 0.1 mg per ml of H₂O/ACN (1/1, v/v). The extracts were stored at -20 °C after filtration by disposable PTFE syringe filters for further qualitative and quantitative studies. The plant extracts for recovery studies of a particular compound were prepared in consideration with the breakthrough concentration levels or point of overload for sorbents. For the recovery studies of GQAs from the sorbents, 0.1 mg/ml GDE and 60 mg/20 ml Galphimia glauca extract was examined, while for CQAs 60 mg/20 ml Arnicae flos extract was used. For the
selectivity studies of GQAs and CQAs (and for the quantification of TGQA and CYN) 60 mg/20 ml of *Galphimia glauca* and *Arnicae flos* extracts were used respectively. CGA was quantified from 20 mg/20 ml of *Arnicae flos* extract. 0.5 mg/ml GDE enabled the quantification of TGQA.

2.2.3. **MALDI-TOF-MS**

For qualitative analysis, a Bruker Daltonics Ultraflex I MALDI-TOF/TOF instrument (Billerica, USA) was used after manual collection of fractions from HPLC-DAD. CQAs were analyzed by SALDI-MS using a stainless steel MALDI target (Applied Biosystems, Vienna, Austria) coated with an amorphous TiO$_2$ layer (100 nm) [35], whereas GQAs were measured on stainless steel target (Bruker Daltonics GmbH, Billerica, USA) using DHB as a matrix. For MALDI-TOF-MS measurements, 1 µl of the sample was spotted on a stainless steel target followed by 1 µl of DHB solution (40 mg/ml DHB in H$_2$O/ACN (1/1, v/v) containing 0.1% TFA). All measurements were recorded in reflectron mode. Mass spectra were recorded by summing up 500 laser shots. Laser power was set between 60% and 70% of its maximal intensity, using a 337 nm nitrogen laser at 50 Hz. The Flex Analysis version 2.4 software provided by the manufacturer was used for data processing. For SALDI-MS measurements, 2 µl of the sample solutions were spotted on the TiO$_2$ coated target and allowed to dry before measurement. Centrifugation was carried out with an Eppendorf Centrifuge 5415 D (Hamburg, Germany) and ultra-sonication was performed on a Transsonic T820/H from Elma (Singen, Germany). An Eppendorf Concentrator 5301 was used after collection of fractions for evaporating solvents.
2.2.4. **HPLC-DAD**

A Shimadzu (Tokyo, Japan) HPLC-DAD was used for the analysis comprising an online degasser unit (DGU-14A), two solvent delivery pumps (LC-10ADvp), an auto-injector (SIL-10ADvp), column oven (CTO-10Avp), system controller (SCL-10Avp) and a DAD (SPD-M10Avp). System control and data analysis were performed by using the manufacturer’s software packages (LCMS-Solution, version 3 and LCMS-post run, version 3-H2).

A (BDS) Hypersil C18 column (250 × 4 mm, 5 µm particle size, Thermo Scientific, Berlin, Germany) with a guard column of the same phase was used for chromatographic separations. The solvents were water containing 0.1% TFA (A) and acetonitrile (B).

2.2.4.1. **HPLC method for Galphimia glauca and Arnicae flos**

For *Galphimia glauca* samples a linear gradient from 3% B to 28% B in 50 minutes, a flow rate of 1 ml/min and an injection volume of 10 µl was used. For *Arnicae flos* samples, a linear gradient from 5% B to 30% B in 50 minutes was deployed at a flow rate of 0.8 ml/min and with an injection volume of 20 µl. Column temperature was set to 25 °C for both runs. These methods were used for quantification of the compounds of interest from *Galphimia glauca extract*, GDE and *Arnicae flos* extracts as well as for selectivity testing of different sorbents.

2.2.4.2. **HPLC short run for TGQA, CYN and CGA**

For the recovery studies of TGQA, CYN and CGA standards, short runs were employed. Gradient from 13% B to 30% B in ten minutes was used for TGQA with the flow rate of 1 ml/min. CYN and CGA standards were measured at the flow rate of 0.8 ml/min, the gradient for CYN was from 10% B to 30% B in 20 minutes, whereas, for CGA a gradient from 5% B to
20% B in 15 minutes was used. 10 µl injection volume and 25 ºC column temperature was maintained for all three runs.

2.2.5. **SPE optimization steps and work-flow**

First, the conditions for SPE using bismuth citrate as sorbent were optimized to reach high recovery rates. Different flow rates, sorption recycles, elution steps and eluent compositions were tested using TGQA standard as a model compound. Once the SPE-spin column method was optimized, a different sorbent (zirconium silicate) and different standards (CGA, CYN) were subjected to the same protocol, but the ratio of particle-amount/sample-volume was varied to investigate the point of overload (breakthrough). After optimization of the SPE method, different commercially available sorbents were tested and their performance towards the individual representatives of GQAs and CQAs (TGQA and CYN) was compared.

2.2.6. **Solvents used for standards**

Stock solutions of each standard (1000 ppm) were prepared. TGQA was dissolved in H2O/ACN (1/1, v/v), whereas for CGA and CYN a H2O/Methanol (1/1, v/v) mixture was used.

2.2.7. **Activation, washing and elution**

Activation of bismuth citrate, zirconium silicate and silica was performed by washing two times with 200 µl ACN for TGQA, in case of CQAs the activation was carried out by washing two times with 200 µl methanol, each. 1 ml methanol was used for activating Oasis® MAX and C18. All solid phases were equilibrated with water (same volume as was used for their activation) twice, followed by a sorption step with 200 µl standard solution.
**Table 2.1:** Optimization of SPE conditions for standard compounds, bold italic and underlined data show the effect of a particular parameter on recovery. Recovery data are reported as an average of three (n=3) replicate measurements (%RSD <5%).

<table>
<thead>
<tr>
<th>Sorbent</th>
<th>Amount mg</th>
<th>Compd./conc. ppm</th>
<th>Centrifugation rpm</th>
<th>Sorption recycles</th>
<th>Eluent FA %</th>
<th>Elution steps</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bi-Citrate</td>
<td>90</td>
<td>TGQA/30</td>
<td>10,000</td>
<td>5</td>
<td>5</td>
<td>1 (200 µl)</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/60</td>
<td>10,000</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/90</td>
<td>10,000</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/120</td>
<td>10,000</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>29.7</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/150</td>
<td>10,000</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>29.8</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/180</td>
<td>10,000</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/120</td>
<td>5,000</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/120</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>2 (100+100 µl)</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/120</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3 (70+70+60 µl)</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/120</td>
<td>5,000</td>
<td>10</td>
<td>20</td>
<td>3</td>
<td>67</td>
</tr>
<tr>
<td>Zr-Silicate</td>
<td>90</td>
<td>TGQA/120</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>TGQA/120</td>
<td>5,000</td>
<td>10</td>
<td>40</td>
<td>3</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CGA/30</td>
<td>5,000</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CGA/30</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>CGA/30</td>
<td>5,000</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>CGA/30</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>CGA/30</td>
<td>5,000</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CGA/10</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CYN/30</td>
<td>5,000</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CYN/30</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CYN/20</td>
<td>5,000</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>CYN/20</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>TGQA/30</td>
<td>5,000</td>
<td>10</td>
<td>30</td>
<td>3</td>
<td>84</td>
</tr>
</tbody>
</table>

Washing steps were performed using 200 µl ACN (for Oasis® MAX washing was made with methanol to remove substances binding to the sorbent through hydrophobic interactions).

Finally, elution was performed by using an aqueous 5-30% FA solution.
2.2.8. Optimization of SPE method using bismuth citrate

Bismuth citrate (325 mesh) powder was filled in micro-spin columns having a volume of 200 µl. TGQA was selected as starting compound and in a first experiment, 90 mg of bismuth citrate was used. After activation and equilibration, 200 µl of a TGQA solution was loaded on the columns, followed by elution with 200 µl water containing 5% FA (five recycles at 10000 rpm). Different concentrations of TGQA (30, 60, 90, 120, 150 and 180 ppm) were tested (five recycles at 10000 rpm). The TGQA amount in the eluate was quantified by HPLC-DAD and from this recovery data the breakthrough concentration was determined. From here onwards the concentrations within the breakthrough concentration were used for further optimization of the method.

2.2.8.1. Optimization of flow rate

Experiments were made at centrifugation speeds of 5000 and 10000 rpm and recoveries were monitored at these flow rates, keeping parameters like sample concentration, eluent strength, number of recycles at sorption and elution steps constant. A considerable increase in recovery rates at 5000 rpm could be observed.

2.2.8.2. Effect of sorption recycles and elution steps

Optimization experiments were performed at 5000 rpm with five and ten recycles respectively and elution was done with water containing 5% FA. By performing ten sorption recycles higher recoveries could be achieved. Elution step optimization was performed by dividing the elution volume into two parts (100 and 100 µl) or into three parts (70, 70 and 60 µl), each having 5 recycles. Three elution steps displayed better results and were therefore used for further optimization.
2.2.8.3. Effect of eluent strength

The effect of different eluents (water containing FA varying from 5 to 40%) was tested. By using 5 to 30% FA the recoveries increased gradually, whereas the use of 40% FA did not demonstrate further improvement, therefore 30% FA acid was used for the following experiments.

2.2.8.4. Optimized SPE-spin column protocol

According to the experiments presented in 2.2.8.1-2.2.8.3 a reproducible SPE-spin column protocol was developed. After filling up the micro-spin columns with the certain amount of the sorbent the activation and equilibration was performed as described in 2.7. 200 μl of the standards or plant samples were loaded onto the sorbent by ten times recycling of the solution in the sorption step, subsequently, washing was performed with 200 μl ACN (two times washing for standards and four for plant samples). The elution was brought about by 30% FA by dividing the 200 μl elution volume into three parts (70, 70 and 60 μl), each having 5 recycles. The centrifugation speed was fixed at 5000 rpm for the whole of the procedure.

2.2.8.5. Effect of pH, solvents and ionic strength

The effects of pH, organic solvents and ionic strength on sorption of the representative compounds of GQAs and CQAs (TGQA and CYN, respectively) to zirconium silicate and bismuth citrate have been investigated using the abovementioned SPE-spin column protocol.
The recoveries of TGQA and CYN achieved on sorbents with different retention mechanisms.

The recovery rates were recorded at pH 2, 4 and 6. Standard solutions (30 ppm) with pH 2 were prepared by diluting the stock solutions (see 2.2.6) with 1 M acetic acid while acetate buffer was used for the solutions having pH 4 and 6. The effect of ionic strength on retention of TGQA and CYN was investigated by diluting their respective stock solutions in 1, 0.1, and 0.01 M NaCl solutions. ACN, methanol and ethanol were used to elaborate the solvent effects on sorption behavior of both the phenolic acids on zirconium silicate and bismuth citrate.
Table 2.2: Quantification of TGQA, CGA and DCQA from Galphimia glauca and Arnicae flos respectively.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Regression eq.</th>
<th>R²</th>
<th>Conc. range ppm</th>
<th>% in dry extract/plant extract</th>
<th>LOD µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGQA</td>
<td>Y=21737x-31779</td>
<td>0.9992</td>
<td>30-180</td>
<td>25% in GDE</td>
<td>0.05</td>
</tr>
<tr>
<td>TGQA</td>
<td>Y=21737x-31779</td>
<td>0.9992</td>
<td>30-180</td>
<td>9.5% in Galphimia glauca</td>
<td>0.05</td>
</tr>
<tr>
<td>CGA</td>
<td>Y=189571x-35796</td>
<td>0.9989</td>
<td>2-10</td>
<td>0.28% in Arniceae flos</td>
<td>0.01</td>
</tr>
<tr>
<td>DCQA</td>
<td>Y=100478x-239548</td>
<td>0.9943</td>
<td>10-50</td>
<td>0.58% in Arniceae flos</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 2.3A: Complete profile of stationary phases to find best possible interactions for CQAs and GQAs. Recovery data are reported as an average of three (n=3) replicate measurements with %RSD <5%.

<table>
<thead>
<tr>
<th>Sorbent</th>
<th>Amount/size</th>
<th>Interaction type</th>
<th>Compd./conc.</th>
<th>Unbound %</th>
<th>Wash 1 %</th>
<th>Wash 2 %</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica</td>
<td>30 mg</td>
<td>Hydrophilic</td>
<td>TGQA/30</td>
<td>88</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>C18</td>
<td>30 mg/1 cc</td>
<td>Hydrophobic</td>
<td>TGQA/30</td>
<td>57</td>
<td>8</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>Hydrophobic and electrostatic</td>
<td>TGQA/30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Zr-Silicate</td>
<td>30 mg</td>
<td>Hydrophilic and electrostatic</td>
<td>TGQA/30</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>Silica</td>
<td>30 mg</td>
<td>Hydrophilic</td>
<td>CYN/20</td>
<td>94</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>C18</td>
<td>30 mg/1 cc</td>
<td>Hydrophobic</td>
<td>CYN/20</td>
<td>43</td>
<td>8</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>Hydrophobic and electrostatic</td>
<td>CYN/20</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>70</td>
</tr>
<tr>
<td>Zr-Silicate</td>
<td>30 mg</td>
<td>Hydrophilic and electrostatic</td>
<td>CYN/20</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>94</td>
</tr>
</tbody>
</table>

2.2.8.6. Quantification of TGQA, CGA and DCQA

TGQA was quantified in Galphimia glauca, using 60 mg/20 ml extract, and from 0.5 mg/ml GDE. TGQA standards from 30-180 ppm were used for external standard calibration. Injection volume was 10 µl. CYN was used as a standard compound for quantifying the major peak belonging to a DCQA observed in Arnicae flos. The compounds in Arnicae flos extract were quantified through 10-50 ppm CYN for calibration. Injection volume was 20 µl.

CGA was quantified out of 20 mg/20 ml Arnicae flos. 2-10 ppm standards were used for calibration with injection volumes of 50 µl. Limit of detection (LOD) is defined as three times
signal to noise and was determined for CGA, CYN and TGQA standards by three replicate measurements.

2.2.8.7. Recovery studies

The recovery rates were determined by comparison of the standard-compound peak-areas obtained by HPLC-DAD analysis before and after subjection to SPE. Different sorbents (C18 (30 mg, 1cc), Oasis® MAX (30 mg, 1cc), silica (30 mg in spin column) and zirconium silicate (30 mg)) were tested to compare the recovery rates by using TGQA and CYN as model compounds for phenolic acids. For Oasis® MAX and C18 cartridges the sample volumes and recycle steps during the sorption and elution steps were the same as in the SPE spin-column protocol like explained in 2.2.8.4. Furthermore, the sorbents with electrostatic interactions (zirconium silicate, bismuth citrate and Oasis® MAX) were compared for recoveries of CGA, CYN and TGQA standards and in their respective plants along with trigalloylquinic acid (tGQA) (Fig. 2.1A, b) and methoxyloxaloyl-dicaffeoylquinic acid (MODQA) (Fig. 2.1B, c) (Exact IUPAC names cannot be assigned due to availability of mass data alone).

2.2.8.8. Selectivity studies

200 µl of Galphimia glauca extract or 200 µl Arnicae flos extract was subjected to SPE-spin column protocol (2.2.8.4). Galphimia glauca extract (60 mg/20 ml) was tested for selectivity of GQAs against bismuth citrate, zirconium silicate, C18 (1 ml cartridge) and Oasis® MAX (1 ml cartridge). Plant samples were measured with HPLC-DAD before and after SPE. Similarly, Arnicae flos extract (60 mg/20 ml) was investigated for selectivity of CQAs against zirconium silicate, bismuth citrate, Oasis® MAX and C18.
Table 2.3B: Effect of pH, organic solvents and ionic strength on the retention behavior of zirconium silicate and bismuth citrate. Sorbent amounts: 100 mg, standards concentration: 30 ppm. Recovery data are reported as an average of three (n=3) replicate measurements with %RSD <5%.

<table>
<thead>
<tr>
<th>Sorbent</th>
<th>pH/recovery %</th>
<th>Solvent/recovery %</th>
<th>Ionic strength (M)/recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYN</td>
<td>TGQA</td>
<td>CYN</td>
</tr>
<tr>
<td>Zirconium silicate</td>
<td>2/12</td>
<td>ACN/94</td>
<td>0.01/92</td>
</tr>
<tr>
<td></td>
<td>4/78</td>
<td>Ethanol/78</td>
<td>0.1/92</td>
</tr>
<tr>
<td></td>
<td>6/94</td>
<td>Methanol/80</td>
<td>1/88</td>
</tr>
<tr>
<td>Bismuth citrate</td>
<td>2/15</td>
<td>ACN/28</td>
<td>0.01/35</td>
</tr>
<tr>
<td></td>
<td>4/23</td>
<td>Ethanol/28</td>
<td>0.1/25</td>
</tr>
<tr>
<td></td>
<td>6/35</td>
<td>Methanol/29</td>
<td>1/15</td>
</tr>
</tbody>
</table>

Table 2.4: Comparison of Recoveries for standard compounds and plant samples from electrostatic stationary phases. Recovery data are reported as an average of three (n=3) replicate measurements and %RSD <5%.

<table>
<thead>
<tr>
<th>Sorbent</th>
<th>Amount/size</th>
<th>Compd./conc. ppm</th>
<th>Sample</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5% FA</td>
<td>30% FA</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>30 mg</td>
<td>TGQA/30</td>
<td>Standard</td>
<td>40</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>30 mg</td>
<td>TGQA/30</td>
<td>Standard</td>
<td>66</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>TGQA/30</td>
<td>Standard</td>
<td>0</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>30 mg</td>
<td>CYN/20</td>
<td>Standard</td>
<td>20</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>30 mg</td>
<td>CYN/20</td>
<td>Standard</td>
<td>84</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>CYN/20</td>
<td>Standard</td>
<td>20</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>30 mg</td>
<td>CGA/10</td>
<td>Standard</td>
<td>28</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>30 mg</td>
<td>CGA/10</td>
<td>Standard</td>
<td>72</td>
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<td>30 mg/1 cc</td>
<td>CGA/10</td>
<td>Standard</td>
<td>54</td>
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<tr>
<td>BiCitrate</td>
<td>30 mg</td>
<td>TGQA, TGQA</td>
<td>GDE 0.1 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>30 mg</td>
<td>TGQA, TGQA</td>
<td>GDE 0.1 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>100 mg</td>
<td>TGQA, TGQA</td>
<td>GDE 0.1 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>100 mg</td>
<td>TGQA, TGQA</td>
<td>GDE 0.1 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>TGQA, TGQA</td>
<td>GDE 0.1 mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>100 mg</td>
<td>TGQA, TGQA</td>
<td>Goliathina globosa 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>100 mg</td>
<td>TGQA, TGQA</td>
<td>Goliathina globosa 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>TGQA, TGQA</td>
<td>Goliathina globosa 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>30 mg</td>
<td>CQA, CQA, MODQA</td>
<td>Arnisiae flos 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>ZrSilicate</td>
<td>30 mg</td>
<td>CQA, CQA, MODQA</td>
<td>Arnisiae flos 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>BiCitrate</td>
<td>100 mg</td>
<td>CQA, CQA, MODQA</td>
<td>Arnisiae flos 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>ZrSillicate</td>
<td>100 mg</td>
<td>CQA, CQA, MODQA</td>
<td>Arnisiae flos 60 mg/20 ml</td>
<td>-</td>
</tr>
<tr>
<td>Oasis® Max</td>
<td>30 mg/1 cc</td>
<td>CQA, CQA, MODQA</td>
<td>Arnisiae flos 60 mg/20 ml</td>
<td>-</td>
</tr>
</tbody>
</table>
2.2.8.9. Identification of HPLC-DAD peaks

*Galphimia glauca* extract was subjected to HPLC-DAD. The compounds were identified by their UV spectra, the retention time of the standard (in case of TGQA), and the mass-spectra of the collected HPLC-DAD fractions. Two major peaks belonging to GQAs were collected as separate fractions and measured off-line via MALDI-TOF-MS applying DHB as a matrix in negative ion mode. The same procedure was performed using *Arnicae flos* extract to determine peaks corresponding to CQAs in HPLC-DAD chromatogram. The peaks were identified by their UV spectra, the comparison of the retention time of the standards and molecular mass obtained from SALDI-MS measured in positive ion mode.

2.3. Results and discussion

The development and optimization of the SPE method using micro-spin columns filled with zirconium silicate and bismuth citrate particles is reported. Based on the affinity of carboxylic group towards metal surfaces two new sorbents for phenolic acids are described. Table 2.1 presents the step by step optimization of the method including centrifugation speed, elution procedure, eluent strength, number of recycles and breakthrough concentration of a particular compound towards a certain sorbent amount. The retention time for TGQA was 7.60±0.03 min, 13.22±0.04 min for CYN and 14.57±0.03 min for CGA. LOD for CGA, CYN was 0.01 μg/ml and TGQA showed a LOD of 0.05 μg/ml. TGQA, CGA and DCQA (a structural isomer of CYN observed in *Arnicae flos*) \[36\] were quantified through the calibration curve of the standards. TGQA quantity was 25% in GDE and 9.5% in *Galphimia glauca* plant extract. 0.28% CGA and 0.58% DCQA could be detected in *Arnicae flos*. (Table 2.2)
A comparison was also made among the different kind of sorbents based on the type of interactions with the target molecules. The aim of this comparative study was to find the optimal sorbent for free phenolic acids. Recovery studies of the standards from different sorbents were monitored using shorter runs (2.2.4.2). Four stationary phases with different interaction properties, C18 (hydrophobic), silica (hydrophilic), Oasis® MAX (hydrophobic and electrostatic), zirconium silicate (predominantly electrostatic and hydrophilic) were tested and compared. A whole profile of these phases was observed for SPE (% unbound, % loss in wash and % recovery) of TGQA and CYN (Table 2.3A).

As can be seen in Table 2.3A, C18 and silica presented low affinities to TGQA and CYN. Most of the loaded compounds remained unbound after the sorption step. Oasis® MAX is binding TGQA too strong and the elution cannot be done efficiently, while an acceptable recovery could be achieved for CYN (70%). Zirconium silicate displayed promising results with TGQA (84%) and CYN (94%) (Fig. 2.2). SPE is a combination of retention and elution procedures and the strength of interaction has an impact on the final recovery. The magnitude of binding energies between sorbents and analytes are described in the literature as follows: hydrophobic interactions 1-10 kcal/mol, hydrogen bonding involving polar groups 5-10 kcal/mol, ionic or electrostatic 50-200 kcal/mol and covalent bonding interactions 100-1000 kcal/mol [37]. Too strong interactions during sorption can cause problems with the elution, therefore, a balance between the binding strength and the ease of elution is the key to success for an ideal sorbent.

Table 2.3B elaborates the effect of pH, organic solvents and ionic strength on the recovery rates of TGQA and CYN, deploying the optimized SPE-spin column protocol using zirconium silicate and bismuth citrate. Three organic solvents (ACN, methanol and ethanol) were
chosen to investigate the effects of different solvents on the sorption behavior of TGQA- and CYN-standards on zirconium silicate and bismuth citrate. For zirconium silicate, ACN was found to be the most suitable solvent for sorption of both TGQA (83%) and CYN (94%), displaying the optimum recovery rates of the compounds. Zirconium silicate experienced a slight decrease of retention for both phenolic acids when using methanol and ethanol as solvents. Bismuth citrate demonstrated no significant variation in the recovery rates of CYN for all three solvents. This remained true for the sorption of TGQA on bismuth citrate as well, till a sizeable loss of recovery was observed for methanol as the sorption solvent, the recovery drastically decreased to about 50%. The pH of the solution has a significant role in the retention of both TGQA and CYN on either of the sorbents. The recovery rates were recorded at pH 2, 4 and 6. CYN recovery on zirconium silicate was supreme at pH 6 (94%), decreased at pH 4 (78%) and almost all of the CYN remained unbound at pH 2. Similarly, TGQA disclosed maximum recovery on zirconium silicate at pH 6 (85%), no decline was witnessed at pH 4, while a considerable diminution in the retention was observed at pH 2 (56%). The changes of recovery rates by variations in the pH are ascribed to the increased affinity of phenolic acids to the metal surface at higher pH values. At pH > 4 the phenolic acids exist in ionized form which in turn augments the attraction towards metals. The effect of ionic strength was scrutinized for the retention of TGQA and CYN at 0.01, 0.1 and 1 M NaCl solutions. Generally, an increase in ionic strength depicted a decreasing trend of recovery rates. In case of zirconium silicate, increased ionic strength of the solution from 0.01 to 0.1 M found not to affect the sorption, while a further increase from 0.1 to 1 M recorded a downward trend in retention. Bismuth citrate also offered a gradual decrease (0.01 > 0.1 > 1) on the retention of TGQA and CYN with the intensification of ionic strength.
This trend can be attributed to the presence of large numbers of ions may limit the sorption process due to competition in the binding sites.

Table 2.4 demonstrates a comparison of recoveries for phenolic acids from their standards and plant extracts. Sorbents with electrostatic interactions displayed better recoveries for the compounds of interest, therefore zirconium silicate, bismuth citrate and Oasis® MAX were selected for this comparison exclusively. It was found that zirconium silicate is the best sorbent among the tested sorbents for both GQAs and CQAs (from 83% to 94% for standards and about 48% to 79% from the mixture of complex matrices) whereas bismuth citrate showed good recoveries for GQAs (75% for standard, 60-64% from GDE, 39-42% from plant extract), but rather weak recoveries for CQAs. Oasis® MAX revealed good recoveries for CQAs (between 70-79% for standards) but is not suitable for the investigated GQAs, especially TGQA (4% in GDE, 7% in plant extract). In case of TGQA a basic eluate with 10% NH₃ in water/methanol, 1/1 (v/v), was tried as alternative but results could not be improved.

Oasis® MAX exhibited a decreasing trend of recoveries (monocaffeoyl- > dicaffeoyl-, > trigalloyl- > tetragalloylquinic acids) in this method. This can only be explained by a gradual increase in molecular sizes and therefore difficult elution procedure for Oasis® MAX especially for TGQA with Mw 800 Da. Monitoring the recovery rates of compounds from complex plant mixtures, the sorbent amount was adjusted from 30 mg to 100 mg for better recoveries of an individual compound. Plant extracts contain many CQAs or GQAs as well as other compounds competing at once for an adsorption site of the sorbent material, therefore surplus sorbent sites are needed to recover an individual compound from complex mixtures as compared to the pure standards.
In Fig. 2.3, chromatograms from *Galphimia glauca* before and after the SPE procedure are displayed. Chromatograms were recorded at 280 and 325 nm to embrace the broad range of compounds present in plant extract. GQAs showed maximum absorption at 280 nm, hence, this wavelength was used for their recovery study (standards and from plant extracts) using different sorbents. Chromatogram from *Galphimia glauca* at 280 nm (chromatogram A) depicted two major GQAs labeled 1 (tGQA) and 2 (TGQA). The compounds were identified by their UV spectra ($\lambda_{\text{max}} = 218$ nm and 274 nm for peak 1 and 2), the retention time of the standard in case of TGQA, and the mass-spectra of the collected HPLC-DAD fractions. Retention times for tGQA and TGQA were 23.93±0.04 min and 25.76±0.02 min respectively. The comparison chromatograms at 280 nm pictured on the left column in Fig. 2.3 ascertained an efficiency of zirconium silicate (Chromatogram B) and bismuth citrate (Chromatogram C) towards tGQA and TGQA, while Oasis® MAX and C18 (Chromatogram D and E) turned out to be unsuitable for the retention of tGQA and TGQA. This could be ascribed to a lower affinity of C18 towards GQAs and a very strong binding of Oasis® MAX towards GQAs (because of much larger size of tested GQAs) consequently making elution more difficult. The comparisons of HPLC-DAD chromatograms (Fig. 2.3, A1-E1) at 325 nm show the selectivities of different sorbents towards GQAs. Our sorbents bared a decreased interaction towards compounds of different chemical classes (displaying different UV spectra than GQAs, peaks are focused by arrows in A1) as compared to Oasis® MAX and C18. The wavelength of 325 nm was selected for selectivity studies because most of the compounds belonging to other chemical classes were visible at this wavelength along with tGQA and TGQA in *Galphimia glauca* extract. Zirconium silicate was the best sorbent for recoveries and selectivities of GQAs followed by bismuth citrate.
**Fig. 2.3:** Chromatograms A-E (280 nm) and A1-E1 (325 nm), recovery and selectivity comparison of different sorbents tested, respectively. A, A1: 60 mg/20 ml *Galphimia glauca* prior to SPE (arrows represent the undesired compounds belonging to other chemical classes). B, B1: SPE with 100 mg zirconium silicate. C, C1: SPE with 100 mg bismuth citrate. D, D1 SPE with Oasis® MAX 1cc cartridges. E, E1: SPE with C18 1cc cartridges. B and C also show mass spectra of (1, 2) peaks.
Fig. 2.4 illustrates chromatograms of *Arnicae flos* before and after SPE. Again two wavelengths were scanned to visualize a broader range of compounds present in the plant extract. Chromatogram from *Arnicae flos* at 325 nm (Chromatogram A) registered 3 major CQAs labeled 1 (CGA), 2 (DCQA) and 3 (MODQA). The peaks were identified by their UV spectra ($\lambda_{\text{max}}$ peak 1: 221 nm, 243 nm, 325 nm, peak 2: 222 nm, 245 nm, 327 nm and peak 3: 222 nm, 244 nm, 327 nm), the comparison of the retention time of the standard in case of CGA, and the molecular mass obtained from SALDI-MS measured in positive ion mode. Retention times of CQAs were observed to be 18.28±0.03 min for CGA, 37.23±0.02 min for DQCA and MODQA had the retention at 38.77±0.02 min. Chromatograms at 325 nm demonstrated zirconium silicate (Chromatogram B) to be a better sorbent for recovering CQAs from complex sample as compared to C18, Oasis® MAX and bismuth citrate (chromatograms E, D and C respectively). The selectivities of different sorbents towards CQAs were examined at 250 nm (chromatograms A1-E1), at this wavelength compounds belonging to different classes could also be seen along with CQAs and are pointed by arrows in A1, again zirconium silicate was the stand out sorbent regarding the selectivity of CQAs.

2.3.1. Proposed binding mechanism

The increased interactions of phenolic acids observed with our stationary phases are proposed to be based upon the coordination between metal atom and electron donor groups from the particular compounds.

Fig. 2.5 (A and B) illustrates a proposed binding of the carboxylic group of phenolic acids to the stationary phases. At the neutral pH at which all the experiments were brought about the only group which could be ionized is carboxylic group, hence reduces non-specific electrostatic interactions with phenolic groups ($pK_a \geq 9$).
**Fig. 2.4:** Chromatograms A-E (325 nm) and A1-E1 (250 nm), recovery and selectivity comparison of different sorbents tested, respectively. A, A1: 60mg Arnicae flos before subjecting to SPE (arrows represent the undesired compounds belonging to other chemical classes). B, B1: SPE with 100 mg zirconium silicate. C, C1: SPE with 100 mg bismuth citrate. D, D1: SPE with Oasis® MAX 1cc cartridge. E, E1: SPE with C18 1cc cartridges. G and H are SALDI mass spectra from peak 2 and 3. Mass spectra CGA not shown.
Fig. 2.5A: Proposed binding of bismuth citrate with carboxylic group of phenolic acid R-COO⁻. 1, 2 and 3 explains activation, sorption and elution respectively.

The structure of bismuth citrate complex was described in X-ray crystallography for potassium ammonium complexes of bismuth citrate by Asato et al. [38]. The mechanical stability of bismuth citrate can be explained by the bridging of citrate ions (Fig. 2.5A). The binding-mechanism of bismuth citrate is assumed to be based on the co-ordination number (CN) of nine. Seven out of nine coordination sites are occupied by carboxylic ligands from tetra ionized citrate ions and the remaining two sites are occupied by water molecules. In bismuth citrate these two sites occupied by water molecules are responsible for coordinating carboxylic group of the phenolic acids. Fig. 2.5A (1, 2 and 3) is the diagrammatic sketch of activation, sorption and elution steps respectively. During sorption, the water molecules are replaced by carboxylates from phenolic acids and provide a
selective isolation of phenolic acids in the complex mixture. Washing removes the non-specifically attached compounds. Finally elution by formic acid replaces phenolic acids for formate ions. Similarly the electrostatic interactions in zirconium silicate can be explained with the help of coordination chemistry. The structure of zirconium silicate is revealed in Fig. 2.5B as proposed by Jameson et al. [39]. Zirconium has the CN of eight in zirconium silicate, four of the co-ordination sites are occupied by oxygen atoms and the remaining four are assumed to be surrounded by water molecules. These water molecules are exchanged by the carboxylic ligands of phenolic acids during sorption. Elution is carried out by formic acid which exchanges phenolic acids with formates (Fig. 2.5B 1, 2 and 3).

![Diagram of zirconium silicate and carboxylic group interaction]

**Fig. 2.5B:** Proposed binding of zirconium silicate with carboxylic group of phenolic acid (R-COO⁻). 1, 2 and 3 illustrates activation, sorption and elution respectively.
The higher recoveries of the zirconium-based stationary phase compared to bismuth citrate-based stationary phase can be described as the number of available coordination sites for zirconium and the sterically hindered bismuth atoms for coordination.

2.4. Conclusions

A SPE method for the selective and efficient isolation of phenolic compounds containing carbon acid moieties by using inorganic particles as sorbent in spin columns has been developed, optimized and compared to commercial products. Sorbents with electrostatic interactions were found to be more selective and presented better recovery rates than sorbents with pure hydrophilic or hydrophobic interaction sites. It was demonstrated that zirconium silicate exhibited best suitability among the tested sorbents. The SPE method described in this publication is unique due to the ease it can be operated and tuned. The amount of sorbent particles can be adjusted depending on concentration of the samples. Zirconium silicate, in particular, is stable under the whole pH range from 1 to 14, in contrary to bismuth citrate, which dissolves in basic pH. The developed SPE method also excludes chlorophyll and waxes from complex mixtures to protect the analytical columns. Furthermore, these particles can turn out to be helpful for screening phenolic acids from still uninvestigated plants in a very short time. This SPE technique may help to find new sources of these important biologically active substances and the search for low abundant novel isomers can be optimized with the SPE method using zirconium silicate. Moreover, by using the introduced conditions, other metal particles can bring further improvement of the reported SPE method and the present work can be a valuable base for future investigations of medicinal plants.
2.5. Acknowledgement

Special Thanks to Higher Education Commission of Pakistan and OeAD for Author’s PhD scholarship. For financial support we also thank the Federal Ministry of Health and the Ministry of Science and Research (Project Novel Analytical Tools for Quality Control), Vienna, Austria.
2.6. References


CHAPTER 3

Solid-Phase Extraction Method for the Isolation of Plant Thionins from European Mistletoe, Wheat and Barley Using Zirconium Silicate Embedded in Poly(Styrene-co-Divinylbenzene) Hollow-Monoliths*


The results described in chapter 3 have been published in: Hussain S, Güzel Y, Schönrichler SA, Rainer M, Huck CW, Bonn GK (2013) Solid-phase extraction method for the isolation of plant thionins from European mistletoe, wheat and barley using zirconium silicate embedded in poly (styrene-co-divinylbenzene) hollow-monoliths. Analytical and Bioanalytical Chemistry 405 (23):7509-7521.

3.1. Introduction

Thionins (derived from the Greek word for sulphur) are small basic sulphur rich proteins (~5 KDa) with a polypeptide chain of 45 to 48 amino acids and 3 to 4 disulfide bridges. Thionins are usually found in the endosperms of Gramineae e.g. wheat and barley, as well as leaves and stems of different plants (mistletoe and Pyrularia pubera) and rosids. They are divided into two groups namely α/β-thionins and γ-thionins (plant defensins). α/β-Thionins can be sub-divided into the types I, II, III, IV and V [1, 2].

Type I thionins are present in the endosperm of grains such as wheat and barley [1, 3] (α-, β-purothionins and hordothionins repectively). They are highly basic, and consist of 45 amino acids, 8 of which are cysteines. α/β-Purothionins are antibacterial [4], they show in vitro protein synthesis inhibition in cell-free systems derived from wheat germ or rabbit reticulocytes [5]. Furthermore, they inhibited the synthesis of macromolecules in baby hamster kidney (BHK) cells in vivo [6], they showed toxicity to some yeast strains [7] and
insect larvae [8]. $\alpha/\beta$-Hordothionins are reported to be active against plant pathogenic bacteria [9]. Highly purified $\alpha$- and $\beta$-hordothionins are active in promoting the inhibition of endogenous mRNA translation in cell-free systems derived from rabbit reticulocytes, Artemia embryos or mouse liver. Inhibition of endogenous translation in a cell-free system from rat brain was also observed [10]. $\alpha$-Hordothionins are reported to be antifungal agents through rupturing the fungal membrane triggered by increased calcium ion uptake and permeabilization of the fungal membrane [11]. Franky et al. proved a synergistic enhancement of the antifungal activity of purothionins and hordothionins by 2S albumins and trypsin inhibitors [12]. Type II thionins were isolated from leaves and nuts of the parasitic plant *Pyrularia pubera* [13] and from the leaves of barley (*Hordeum vulgare*) [14]. They are slightly less basic than type I thionins and consist of 46 to 47 amino acids. Both, type I and II thionins have four disulfide bonds.

Type III thionins (viscotoxins) have been extracted from leaves and stems of mistletoe species such as *Viscum album* L. [15]. They consist of 45 to 46 amino acids, contain three disulfide bonds and are as basic as type II thionins. The European mistletoe (*Viscum Album L.*) is a semi-parasite growing on coniferous and leafy trees. Mistletoe extracts are used as complementary medicine for cancer therapy [16].

To date, seven different viscotoxin isoforms have been reported, namely A1, A2, A3, B, B2, 1-PS and C1 [17-19]. There are appreciable differences in toxicity between the diverse viscotoxins despite their sequence similarities. Viscotoxin A3 is the most cytotoxic whereas B is the least cytotoxic [20]. The overall shape of viscotoxins is very similar to that found for the other members of the thionin family comprising two antiparallel alpha helices and a short beta sheet [17, 21]. Their biological activity is related to plant defense against
pathogens [22]. Viscotoxins enhance the natural killer cell-mediated killing of tumor cells [23]. Furthermore, they exert a strong immunomodulatory effect on human granulocytes [24, 25], induce apoptosis in human lymphocytes [26] and in addition, they form complexes with negatively charged DNA [27]. Antifungal effects of viscotoxin A3 were reported by Giudici et al. [28]. Type IV thionins, which consist of 46 amino acids with three disulfide bonds are found in seeds of Abyssinian cabbage [29, 30]. Type V thionins are neutral thionins present in some grains like wheat without toxic activities [31].

Solid-phase extraction (SPE) is an indispensable tool in many areas of research. It is a rapid and effective way to clean-up and pre-concentrate the analytes of interest for subsequent instrumental analysis. SPE was proved to be very useful for isolating polypeptides from complex biological tissues [32] and from food samples [33]. Herraiz et al. evaluated different SPE sorbents based upon non-polar and ionic interactions using small synthetic peptides and casein enzymatic hydrolysates [34]. Different SPE sorbents containing cyanopropyl, ethyl, cyclohexyl, phenyl, octyl or octadecyl functional groups can be employed providing a wide range of possibilities for pre-concentration of peptides. The sorbent must be selected with respect to the polarity, hydrophobicity and length of the peptide [35].

The aim of this study was to design a highly selective sorbent for plant thionins. Hollow-monolithic extraction tips based on poly(styrene-co-divinylbenzene) (poly(STY-co-DVB)) with embedded zirconium silicate nano-powder were synthesized, which demonstrated an excellent selectivity for sulphur-rich proteins. To investigate the basic principle of binding between the monolithic sorbent and the thionins, additionally the pure zirconium-free hydrophobic sorbent (poly(STY-co-DVB) and the pure zirconium silicate nano-powder was tested.
To the author’s knowledge for the first time a selective SPE method for thionins (of mistletoe, wheat and barley) was developed and optimized using a poly(STY-co-DVB) hollow-monolithic extraction column containing zirconium silicate nano-powder. The sorbent offers a combination of hydrophobic and electrostatic interactions and demonstrated highest selectivity for plant thionins.

3.2. Materials and methods

3.2.1. Chemicals and reagents

Acetonitrile HPLC-grade (ACN), methanol ultra LC-MS grade and water HPLC-grade were purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Formic acid (FA) was received from Merck KGaA (Darmstadt, Germany). n-Octyl β-D-glucopyranoside (nOGP, 98%), iodoacetamide (IAA, ≥98.0%), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (HCCA), divinylbenzene (DVB), styrene (STY), 2,2’-azobis-2-methylpropionitrile (AIBN), decanol and zirconium (IV) silicate nano-powder (100 nm, 98.5% trace meals basis) were purchased from Sigma Aldrich (St. Louis, USA). Phosphate buffer saline (PBS, 0.01 M Na₂HPO₄/NaH₂PO₄ and 0.15 M NaCl, pH=7.4) PhyNexus, Inc., San Jose, CA (US). Trifluoroacetic acid (TFA, for protein sequence analysis), dithiothreitol (DTT, ≥99.0%) and ammonium bicarbonate (ultra, ≥99.5%) were purchased from Fluka (Buchs, Switzerland). Trypsin (sequencing grade modified) was obtained from Promega Biosciences (San Luis Obistpo, CA, USA). The peptide calibration standard (bradykinin 1-7, angiotensin I, angiotensin II, substance P, bombesin, renin substrates, ACTH clip 1-17, ACTH clip 18-39, somatostatin) and protein calibration standards (insulin, ubiquitin I, cytochrome C, myoglobin (mass range: ~5-17.5 KDa) were from Bruker Daltonics Care (Bremen,
Germany). Viscum album L. (European mistletoe) was received by a local pharmacy. Wheat
flour and barley seeds were purchased from a local market in Innsbruck.

3.2.2. Preparation of extracts

Mistletoe and barley seeds were milled with a Retsch ZM 200 mill (Retsch, Hann, Germany)
to a particle size less than 0.5 mm. Then, 200 mg of mistletoe powder, wheat flour and
barley flour were separately extracted in 5 ml PBS for 60 minutes in ultra-sound. After
extraction, samples were centrifuged and the supernatants were stored at -20 °C.

3.2.3. Synthesis of poly(STY-co-DVB) embedded zirconium silicate hollow-monolith

The polymerization mixture contained 50 µl of distilled DVB (cross-linker), 100 µl of STY, 4
mg AIBN (radical initiator) and 150 µl decanol (porogen). After thorough mixing, the mixture
was transferred into a vial containing 90 mg ultra-sonicated zirconium silicate nano-powder
and vortexed for two minutes. 20 µl of this mixture was taken into 200 µl pipette tips and
dispensed off. Subsequently, the tips were placed at 75 °C into an oven for 16 hours. The
total mass of dried packing in filled monolithic tips was ~4 mg, the total amount of zirconium
silicate in the extraction tip was ~2.70 mg and the height of the extraction tip was 1.60 cm ±
0.1 cm. The monolithic extraction tips were used only for one extraction.

3.2.3.1. Synthesis of Poly(STY-co-DVB) powder

2.5 ml of DVB, 120 ml of ACN and 62.5 mg AIBN were added into a round-bottom flask
equipped with a magnetic stirrer, a condenser, a nitrogen inlet and a thermometer. The
mixture was first purged with nitrogen for ten min under stirring. With continuous stirring
and nitrogen purge, the mixture was heated at 60°C in an oil bath for one hour. Then, 2.5 ml
of styrene and 100 mg AIBN were added and the mixture was heated at 70 °C in an oil bath
for 16 hours with continuous stirring and nitrogen purge. Finally, the mixture was cooled to room temperature, filtered and washed thoroughly in a sintered-glass filter with ACN and methanol. The obtained beads were dried under vacuum at room temperature for four hours.

3.2.3.2 Scanning electron microscopic analysis of hollow-monolith

Scanning electron microscopy (SEM) was carried out by Electron Micro Probe (JEOL 8100, Japan). Before examination under the scanning electron microscope, the sorbent was sputtered with gold. SEM pictures were taken with an acceleration voltage of 15 kV and currents of 5 - 10 nA.

3.2.4. SPE method

The hollow-monolithic extraction tip columns were activated by washing with 20 µl methanol (aspirating and expelling twice). Equilibration was performed in the same manner using water. 20 µl of sample was loaded onto the monolithic tip by aspirating and dispensing several times. Washing was done three times with deionized water in case of mistletoe and with 30% FA in methanol/water (1/1, v/v) for wheat and barley samples. Elution was performed by 2% TFA in ACN/water (1/1, v/v).

3.2.5. Protein Digestion

For protein digestion, the method of Güzel et al. was used with slight modifications [36]. SPE eluents (from mistletoe, wheat and barley containing viscotoxins, purothionins and hordothionins, respectively) were evaporated (to dryness) in an Eppendorf Concentrator 5301 (Hamburg, Germany). Reconstitution was achieved in 40 µl of 0.5 M ammonium bicarbonate (pH < 8.0), 8 µl 40 mM nOGP and 8 µl 45 mM DTT. The samples were placed on
a thermomixer (Eppendorf AG) for 30 min at 37 °C and 900 rpm in order to perform
denaturation. After cooling down to room temperature, the denaturized proteins were
alkylated by incubating them 30 min under light exclusion by adding 8 µl 100 mM IAA to
each fraction. In a next step 8 µl of 0.1 µg/µl trypsin solution (diluted in 50 mM acetic-acid
buffer pH 3.0) were added to the samples and placed on a thermomixer for enzymatic
digestion at 37 °C for 16 hours. In order to stop the digestion process, 5 µl of 5% TFA
solution (pH < 3.0) were added. Afterwards, the digested protein solution was spotted on a
stainless steel target followed by the addition of saturated HCCA solution (HCCA in
ACN/water (1/1, v/v) containing 0.1% TFA)) for further MALDI/TOF MS analysis.

3.2.6. MALDI/TOF MS and MS/MS analysis including database search

For MALDI/TOF MS measurements 1 µl of sample was spotted on a stainless steel target
(Bruker Daltonics GmbH, Bremen, Germany) followed by the addition of 1 µl of saturated SA
solution (SA in ACN/water (1/1, v/v) containing 0.1% TFA) for protein analysis or 1 µl of
saturated HCCA solution in case of peptide analysis. All measurements were recorded on an
Ultraflex I (Bruker Daltonics, Germany) MALDI/TOF MS in linear and reflectron mode. An
external calibration was performed by spotting 0.5 µl of protein or peptide calibration
standard (Bruker Daltonics, Bremen, Germany). All mass spectra were recorded by summing
500 laser shots. Laser power was adjusted between 50% and 70% of its maximal intensity,
using a 337 nm laser at 50 Hz. The Flex Analysis version 2.4 and BioTools 3.0 software
packages provided by the manufacturer were used for data processing. Database searching
analysis was performed with Mascot software (http://matrixscience.com) and SwissProt as
database. For peptide mass-fingerprint (PMF) database searching analysis, the parameters
were set as following: C-carbamidomethyl (fixed modification), M-oxidation, mass value
Chapter 3  

SPE of thionins employing PSDZ sorbent

(monoisotopic), peptide mass tolerance (200-300 ppm), mass tolerance (0.6 Da), missed cleavage (1 to 3) and taxonomy (other green plants).

3.2.7. HPLC-DAD

A Shimadzu (Tokyo, Japan) HPLC-DAD was used for the analysis comprising an online degasser unit (DGU-14A), two solvent delivery pumps (LC-10ADvp), an auto-injector (SIL-10ADvp), column oven (CTO-10Avp), system controller (SCL-10Avp) and a diode-array detector (SPD-M10 Avp). System control and data analysis were performed by using the manufacturer’s software packages (LCMS-Solution, version 3 and LCMS-post run, version 3-H2).

A Hypercrab S graphitised carbon HPLC-column (100 × 4.6 mm, 250 Å, 7 µm particle size, (Shandon Scientific Ltd. Astmoor, Great Britain)) was used for chromatographic separations. The mobile phase was a combination of water containing 0.1% TFA (A) and ACN (B).

Following gradient was used: (min/%B) 0/25, 20/35, 21/60, 30/65, 31/95, 40/95, 42/5 and 47/5. Flow rate was 1 ml/min and the injection volume was 20 µl.

3.2.8. Identification of HPLC-DAD peaks

The peaks corresponding to the major isoforms were assigned after manual collections of fractions. The fractions were dried in a concentrator and reconstituted in 5 µl 2% TFA in ACN/water (1/1, v/v) for further MALDI/TOF MS analysis.

3.2.9. Investigation of binding mechanism

To elaborate the binding mechanism between the monolithic sorbent and the sulphur rich thionins, wheat purothionins were investigated exclusively for the retention behavior on
poly(STY-co-DVB) (1:1) polymer powder (see 3.2.3.1) and the zirconium silicate nanopowder. 100 mg of each sorbent was taken separately into a 1.5 ml vial, activated with 500 µl methanol and equilibrated with 500 µl water. 100 µl of wheat extract was loaded in each vial and vortexed for two minutes. Washing was performed three times with 500 µl 30% FA in methanol/water (1/1, v/v). Elution was performed by using 2% TFA in ACN/water (1/1, v/v). The supernatants from the individual steps were subjected to MALDI/TOF MS analysis.

3.3. Results and discussion

Isolation of thionins from complex samples is often complicated and needs many purification steps. This cumbersome procedure can be considerably simplified by a prior enrichment of the thionins using a selective SPE resin. The aim of this study was to design a sorbent which possesses a high selectivity to sulphur rich proteins. Xu et al. observed an increased binding of cysteine containing peptides by incorporating gold nanoparticles in the stationary phase [37]. This characteristic property of thiol-groups to co-ordinate with gold metal atoms is now also proved for zirconium atoms and their ability to enrich and isolate thionins was demonstrated. Mistletoe, wheat and barley extracts were loaded on the sorbent and enriched in a pipette tip. Then, the selectively retained thionins were subjected to instrumental analysis.

Fig. 3.1 displays the SEM micrographs of the poly(STY-co-DVB) embedded zirconium silicate sorbent before activation. Fig. 3.1 (A-F) depicts the micrographs recorded at different resolutions. The embedded nano-particles (<100 nm) are clearly visible below the resolution power of 30 µm. Fig. 3.1 B shows the thickness of the polymer bed (~30 µm). MALDI/TOF MS measurements were carried out to prove the selectivity of the SPE method towards the enrichment of thionins.
Fig. 3.1: SEM micrographs of monolithic poly(STY-co-DVB) embedded zirconium silicate nano-powder.

In Fig. 3.2 the selective isolation of viscotoxins from mistletoe sample is demonstrated and the mass spectra of the mistletoe extract (prior SPE) (A), the wash from the sorbent (B) and the eluted thionins (C) is depicted. Non-specifically bound proteins were easily washed away with water. No signal from the interested viscotoxins was observed in the mass spectra obtained from the washing fractions, ensuring a strong binding of the viscotoxins to the sorbent. The viscotoxins which could be detected in the eluted fraction are listed in Table 3.1 with their amino acid sequences [38-43]. The reported molecular masses are based upon their known amino acid sequences. Protein Information Resource (PIR) software
http://pir.georgetown.edu/pirwww/search/comp_mw.shtml was used for the determination of the exact mass.

**Fig. 3.2:** MALDI/TOF MS analysis of the mistletoe sample. A) Mass spectra of mistletoe extract before SPE, B) wash from the sorbent and C) eluate-strongly retained viscotoxins inset D is the expanded view of retained viscotoxins.
Fig. 3.3 shows the MALDI/TOF MS spectra of a wheat sample. Wheat has a diverse range of proteins which makes the isolation of thionins extremely difficult without a prior enrichment step. The presented hollow-monolithic extraction tip exhibited a strong interaction for wheat purothionins which allowed the removal of the weaker bound non-specific proteins by using 30% FA in methanol/water (1/1, v/v). Mass spectrum A reveals the entire range of proteins up to 14 KDa in wheat flour extract before subjecting to SPE. Spectrum B represents proteins removed by the washing step. Again, no known purothionin signal could be observed in the washing fraction. Lastly, spectrum C depicts the eluted purothionins from the sorbent. The purothionins detected in the eluate are listed in Table 3.2 including their known amino acid sequences [44, 45].

Table 3.1: Detectable viscotoxins and their reported amino acid sequences from the SPE eluate from European mistletoe.

<table>
<thead>
<tr>
<th>Viscotoxins</th>
<th>Amino acid sequence</th>
<th>Theoretical Mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>KSCCPNTTGRNINYACRLTGAPRPT</td>
<td>4835.53</td>
<td>4835.7</td>
</tr>
<tr>
<td></td>
<td>CAKLSGCKIIISGCSTVPSD[38]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>KSCCPNTTGRNINYNTCRCFGGGSRE</td>
<td>4834.42</td>
<td>4835.7</td>
</tr>
<tr>
<td></td>
<td>VCASLSGCKIIISASTCPSYPDK [39]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>KSCCPSTTGRNINYNTCRTGSSRETC</td>
<td>4889.53</td>
<td>4890.6</td>
</tr>
<tr>
<td></td>
<td>AKLSGCKIIISASTCPNYPK [40]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>KSCCPNTTGRNINYNTCRLGGGSRER</td>
<td>4857.45</td>
<td>4857.3</td>
</tr>
<tr>
<td></td>
<td>CASLSGCKIIISASTCPSYPDK [41]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-PS</td>
<td>KSCCP8TTGRBYDRCFRFVGGGSRZV</td>
<td>4904.02</td>
<td>4904.5</td>
</tr>
<tr>
<td></td>
<td>CARIS GCKIIISASTCPYBK [42]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>KSCCKNNTGRNINYNTCRFAGGSRSERP</td>
<td>4977.65/4967*</td>
<td>4967.7</td>
</tr>
<tr>
<td></td>
<td>CAKLSGCKIIISASTCPSYDK [43]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Kong et al. reported viscotoxin B2 to show the molecular mass at 4967 Da in MALDI/TOF MS [43]*
Fig. 3.3: MALDI/TOF MS analysis of wheat flour. A) Mass spectra of wheat extract prior to SPE, B) wash from the sorbent and C) eluate—strongly retained purothionins.

Table 3.2: Detectable purothionins and their reported amino acid sequences from the SPE eluate of wheat flour.

<table>
<thead>
<tr>
<th>Purothionins</th>
<th>Amino acid sequence</th>
<th>Theoretical Mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_1^+$</td>
<td>KSCCRSTLGRNCYNLCRARGAQKL CAGVCRCCKISSGLSCPGBKFK [44]</td>
<td>4825.70</td>
<td>4825.4</td>
</tr>
<tr>
<td>$\alpha_2^+$</td>
<td>KSCCRTTLGRNCYNLCRSGAQKLC STVCRCKLTSGLSCPGBKFK [44]</td>
<td>4929.82</td>
<td>4925.4</td>
</tr>
<tr>
<td>$\beta^-$</td>
<td>KSCCKSTLGRNCYNLCRARGAQQL CANVCRCKLTSGLSCPGBKFK [45]</td>
<td>4926.81</td>
<td>4925.4</td>
</tr>
</tbody>
</table>
Fig. 3.4 unveils the efficiency of the previously mentioned protocol. Fig. 3.4 (A, B and C) presents the mass spectra obtained from the barley extract, the wash and the eluate, respectively. The barley sample displayed an almost identical diversity of proteins as described for the wheat sample. The washing step allowed the removal of most undesired proteins. The hordothionins detected in eluate are reported together with their known amino acid sequences [46, 47] in Table 3.3.

![Fig. 3.4: MALDI/TOF MS analysis of barley. A) Mass spectra of barley extract before subjecting to SPE, B) wash from the sorbent C) eluate-strongly retained hordothionins.](image-url)
Table 3.3: Detected hordothionins and their reported amino acid sequences from the SPE eluate of barley.

<table>
<thead>
<tr>
<th>Hordothionins</th>
<th>Amino acid sequence</th>
<th>Theoretical Mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-</td>
<td>KSCCRSTLGRNCYNLCRVRGAQKL</td>
<td>4855.75</td>
<td>4856.4</td>
</tr>
<tr>
<td></td>
<td>CAGVCRCRLTSGLCPFQPK [46]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-</td>
<td>KSCCRSTLGRNCYNLCRVRGAQKL</td>
<td>4926.81</td>
<td>4926.2</td>
</tr>
<tr>
<td></td>
<td>CANACRCKLTSGLCPQSPFPK [47]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Identification of thionins from peptide mass-fingerprints.

<table>
<thead>
<tr>
<th>Mistletoe</th>
<th>Seq. coverage [%]</th>
<th>Mascot search score</th>
<th>Average error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscotoxin-A2</td>
<td>65</td>
<td>37</td>
<td>258</td>
</tr>
<tr>
<td>Viscotoxin-A3</td>
<td>20</td>
<td>35</td>
<td>215</td>
</tr>
<tr>
<td>Viscotoxin-B</td>
<td>27</td>
<td>40</td>
<td>64</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wheat</th>
<th>Seq. coverage [%]</th>
<th>Mascot search score</th>
<th>Average error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2-purothionin</td>
<td>12</td>
<td>21</td>
<td>126</td>
</tr>
<tr>
<td>α2-purothionin</td>
<td>39</td>
<td>34</td>
<td>177</td>
</tr>
<tr>
<td>β-purothionin</td>
<td>37</td>
<td>44</td>
<td>87</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Barley</th>
<th>Seq. coverage [%]</th>
<th>Mascot search score</th>
<th>Average error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-hordothionin</td>
<td>47</td>
<td>39</td>
<td>155</td>
</tr>
<tr>
<td>β-hordothionin</td>
<td>21</td>
<td>28</td>
<td>133</td>
</tr>
</tbody>
</table>

3.3.1. Confirmation of thionin identity by protein digestion

Furthermore, a tryptic digest was performed with the SPE eluates as described in 3.2.5. The eluted proteins with the most intensive signals were confirmed through their peptide mass-fingerprint. The minor constituents could not be confirmed. Table 3.4 describes the results of the peptide mass-fingerprint analysis of the enriched and digested mistletoe, wheat and barley proteins using Mascot as search engine. Viscotoxin A2, A3 and B exhibited the
sequence coverage of 65, 20 and 27% respectively obtained from mistletoe extract. \(\alpha_1\), \(\alpha_2\), and \(\beta\)-purothionins revealed a sequence coverage of 12, 39 and 37%, respectively. \(\alpha\)-hordothionin disclosed a sequence coverage of 47% while 21% could be achieved for \(\beta\)-hordothionin from barley.

**Fig. 3.5:** HPLC-DAD purification of individual isoforms of thionins. A) Chromatogram from mistletoe eluate: 1-viscotoxin A2, 2-viscotoxin A3 and 3-viscotoxin B. B) Chromatogram from wheat eluate: 1-\(\alpha_1\)-purothionin, 2-\(\beta\)-purothionin. C) Chromatogram from barley eluate: 1-\(\alpha\)-hordothionin, 2-\(\beta\)-hordothionin.
3.3.2. HPLC-DAD separation of isolated thionins

After the selective isolation of thionins from their respective samples, the purification of individual isoforms was carried out by HPLC-DAD, the chromatograms are shown in Fig. 3.5. The fractions were collected manually and measured off-line with MALDI/TOF MS. In Fig. 3.5 A the chromatogram of the enriched viscotoxins are shown. For peak 1 (retention time (RT): 27.57 min), 2 (RT: 30.13 min) and 3 (RT: 33.13 min) the molecular masses of 4834, 4835 and 4857 Da were found and they could be assigned as Viscotoxin A2, A3 and B respectively. Fig. 3.5 B depicts the HPLC-DAD chromatogram of purothionins after enrichment from a wheat sample extract. Peak 1 (RT: 27.57 min) and 2 (RT: 33.57 min) were identified as α1-, and β-purothionins exhibiting a molecular masses of 4834 and 4925 Da, respectively. In Fig. 3.5 C the chromatogram of isolated hordothionins from barley are depicted. Peak 1 (RT: 27.57 min) and 2 (RT: 33.57 min) could be identified as α- and β-hordothions with the molecular masses of 4856 and 4926 Da. The similar RTs displayed by the different isoforms of thionins could be ascribed to the amino acid sequence similarities of the isoforms. Sequence similarities between viscotoxins and other related α- and β-thionins alongwith disulphide bridge arrangements were described comprehensively by Orru et al. [40]. All these proteins consist of 45 or 46 amino acids and share a high degree of sequence homology. The RT of viscotoxin A2, α1-purothionin and α-hordothion was observed to be 27.57 min. α1-Purothionin and α-hordothion in their respective polypeptide chains share the similar amino acid residues at 40 positions while for viscotoxin A2 (depicting similar amino acids residues at 22 positions to α1-purothionin and α-hordothion) the resemblance of RT cannot be explained so convincingly. Likewise, the RTs displayed by viscotoxin B, β-purothionin and β-hordothion were 33.13, 33.57 and 33.57 min respectively. Again β-purothionin and β-
hordothionin only differs in five amino acid residues in their polypeptide chain, hence a very similar retention time was observed. The similar RT shown by Viscotoxin B which shares 22 amino acids residues to β-purothionin and β-hordothionin cannot be explained so persuasively.

3.3.3. Proposed Binding mechanism

The involved binding mechanism for the strong interaction between thionins and poly(STY-co-DVB) embedded zirconium silicate nano-powder can be explained by the experiment explained in 3.2.9. As the presented monolithic sorbent combines the characteristics of both materials, the organic polymer and the inorganic particles, it was important to investigate the binding behavior of both materials individually in order to obtain better understanding of the actual retention mechanism. Fig. 3.6 displays the evaluation of poly(STY-co-DVB) powder for wheat extract including the washing fraction 1, washing fraction 2 (A, B) (two washing fractions are presented in Fig. 6 to ascertain a non-selective binding of hydrophobic stationary phase to thionins) and the eluate (C) from the hydrophobic sorbent. The washing solution (methanol/water (1/1, v/v) containing 30% FA) could remove all the purothionins from the hydrophobic sorbent and no signal could be seen in the eluate. When the same sample was subjected to SPE analysis on the zirconium silicate nano-powder (predominantly electrostatic) sorbent, an increased binding to purothionins was observed in contrast to the hydrophobic poly(STY-co-DVB) resin. Fig. 3.7 displays the MALDI/TOF MS spectra of the fractions of washing (A) and elution (B) for the zirconium silicate nano-powder material, respectively.
Fig. 3.6: MALDI/TOF MS analysis of wheat extract evaluated on poly(STY-co-DVB) polymer powder sorbent. A) and B) are the wash fraction 1 and wash fraction 2 from the sorbent showing no specific retention for thionins. C) eluate from the same sorbent showing nothing retained specifically.

Based on these results it can be concluded that the main binding interaction between thionins and the developed sorbent is determined by the co-ordination between zirconium and the thiol-groups of the cysteine rich proteins. In general, the presented resin offers strong hydrophobic and electrostatic interaction sites which synergistically accounts for a very strong binding of thionins by allowing stringent washing steps in order to remove unspecific constituents.
**Fig. 3.7**: MALDI/TOF MS analysis of wheat extract evaluated on zirconium silicate nanopowder sorbent. A) is the wash from the sorbent B) eluate from the same sorbent showing strongly retained purothionins.

Zirconium has a co-ordination number (CN) of eight in zirconium silicate. Four of the co-ordination sites are occupied by oxygen atoms [48] and the remaining four are assumed to be surrounded by water molecules (after equilibration of the sorbent). These co-ordination sites surrounded by water molecules are proposed to be exchanged by an electron pair from sulphur atoms of thiol-groups for each co-ordination site causing strong retention of thionins.
3.4. Conclusion

Zirconium silicate embedded in poly(STY-co-DVB) hollow-monoliths are suitable for the purification and enrichment of thionins in extracts obtained from mistletoe, wheat and barley. Strong co-ordination between zirconium and the sulphur atoms of thiol-groups causes a strong binding of thionins to the developed material. This approach reduces the complexity of protein extracts enormously and therefore it offers a simpler chromatographic separation of individual isoforms. The SPE method may be suitable for screening of novel isoforms of thionins from still uninvestigated samples.

3.5. Acknowledgement

Special thanks to the Higher Education Commission of Pakistan and OeAD for Author’s PhD scholarship. The authors also want to thank Eurasia-Pacific Uninet (EPU) (Salzburg, Austria), the Ministry for Science and Research and the Ministry for Health, Family and Youth (Vienna, Austria) (Novel analytical tools for quality control of immunomodulatory, anti-inflammatory and neuroprotective agents in Traditional Chinese medicine). The authors also would like to acknowledge Prof. Dr. Richard Tessadri from Faculty of Geo- and Atmospheric Sciences, Institute of Mineralogy and Petrography, University of Innsbruck, for SEM analysis.
3.6. References

Chapter 3

SPE of thionins employing PSDZ sorbent


CHAPTER 4

Solid-Phase Extraction of Plant Thionins Employing Aluminum Silicate Based Extraction Columns*

4. Solid-Phase Extraction of Plant Thionins Employing Aluminum Silicate Based Extraction Columns


4.1. Introduction

Thionins comprise an assembly of cysteine-rich, low molecular weight (~5 KDa) basic proteins with a polypeptide chain of 45 to 48 amino acids and 3 to 4 internal disulfide bridges. Thionins are customarily found in the endosperms of Gramineae, e.g. wheat and barley, as well as in diverse plant species, including leaves and stems (e.g. mistletoe, pyrularia and rosids). They can be classified into two groups, namely α/β-thionins and γ-thionins (plant defesins). α/β-Thionins can be sub-divided into the types I, II, III, IV and V [1, 2].

All five types of α/β-thionins seem to be exceedingly homologous at the amino acid level. Primary structure determinations revealed that about 12 to 17% of the amino acids are cysteine residues. These cysteine residues are extremely conserved and are involved in disulphide bond formation [3, 4]. The crystal structure of crambin (type IV thionin) was the first to be resolved among thionins and was determined directly from anomalous scattering of sulphur. Crambin has the shape of the Greek capital letter gamma Γ, the stem of gamma is an antiparallel pair of helices and the cross-arm comprises of two antiparallel β-strands, an irregular strand and a classic β-turn [5]. A number of X-ray structure determinations of different thionins, along with NMR studies clearly demonstrated a distinct architectural
feature for these small proteins. Regardless of small variations in length (45-48 amino acids) α/β-thionins share similar three dimensional structure with minute exceptions [1].

Type I thionins are found in the endosperm of grains e.g. wheat and barley [1, 6] (α-, β-purothionins and hordothionins, respectively). These highly basic proteins encompass 45 amino acids, 8 of which are cysteines. α/β-Purothionins are poisonous to certain yeast strains [7] and insect larvae [8], antibacterial [9] and display in vitro protein synthesis inhibition [10]. α/β-Hordothionins are described to be effective against plant pathogenic bacteria [11] and actively obstruct endogenous mRNA translation [12]. α-Hordothionins are antifungal agents through breaking the fungal membrane prompted by augmented calcium ion uptake and permeabilization of the fungal membrane [13]. Synergistic improvement of the antifungal activity of purothionins and hordothionins by 2S albumins and trypsin inhibitors is also reported [14]. A relationship between hordothionins in barley kernels and beer gushing (uncontrolled release of CO₂ occurring when a bottle is opened and beer gushes out) was examined by Hegrova et al. Although the gushing of beer is also associated with the grains being attacked by fungi, yet a correlation between the presence of antifungal hordothionins and beer gushing could not be observed [15]. Type II thionins have been isolated from leaves and nuts of the parasitic plant Pyrularia pubera [16] and from the leaves of barley (Hordeum vulgare) [17]. These are rather less basic than type I thionins and comprise of 46 to 47 amino acids. Both, type I and II thionins possess four disulfide bonds.

Type III thionins, which are commonly called viscostoxins, have been extracted from leaves and stems of mistletoe species such as Viscum album L. [18]. Viscotoxins are as basic as type II thionins and enclose 45 to 46 amino acids with three disulfide bonds. The European
mistletoe (*Viscum Album L.*) grows on coniferous and leafy trees and its extracts are used as complementary medicine for cancer therapy [19].

There are various viscotoxins reported in the literature, namely A1, A2, A3, B, B2, 1-PS and C1 [20-22]. In spite of their sequence similarities, there are substantial differences in toxicity among the diverse viscotoxins. Viscotoxin A3 is demonstrated to be the most, while B is the least cytotoxic [23]. Their biological activity includes plant defense against pathogens [24], enhanced natural killer cell-mediated killing of tumor cells [25], immunomodulatory effect on human granulocytes [26, 27], antifungal [28], and induced apoptosis in human lymphocytes [29]. Type IV thionins, which consist of 46 amino acids, with three disulfide bonds are found in seeds of Abyssinian cabbage [30, 31]. Type V thionins are neutral thionins present in some grains like wheat without toxic activities [32].

Solid-phase extraction (SPE) is an operative practice to clean up and pre-concentrate the targeted analytes for succeeding instrumental analysis. Specific polypeptides can be isolated from complex biological tissues [33] and from food samples [34] employing different SPE methods. SPE sorbents encompassing ethyl, cyclohexyl, octyl, cyanopropyl, phenyl, or octadecyl functional groups can be employed to provide an extensive range of options for pre-concentration of peptides. The sorbent must be selected with respect to the polarity, hydrophobicity and length of the peptide [35].

The present study describes the potential of mullite as an exceedingly selective sorbent for the isolation of plant thionins. It is an excellent ceramic material having high thermal and chemical stability, creep resistance and corrosion stability together with suitable strength [36]. To the author’s knowledge mullite is used for the very first time as a sorbent for the selective isolation of plant thionins.
4.2. Materials and methods

4.2.1. Chemicals and reagents

Acetonitrile (ACN, for HPLC, ≥99.9%), Methanol (LC-MS grade) and water (HPLC grade) were purchased from Carl Roth GmbH+Co. KG (Karlsruhe, Germany). Aluminum silicate powder (Al₆Si₂O₁₃), iodoacetamide (IAA, ≥98.0%), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, matrix substance for MALDI-MS, ≥99.0%), n-octyl β-D-glucopyranoside (nOGP, 98%), α-cyano-4-hydroxycinnamic acid (HCCA, matrix substance for MALDI-MS, ≥99.0%), were purchased from Sigma Aldrich (St. Louis, MO, USA). Formic acid (FA) was received from Merck KGaA (Darmstadt, Germany). Trifluoroacetic acid (TFA, for protein sequence analysis), dithiothreitol (DTT, ≥99.0%), ammonium hydroxide (25% aqueous solution) and ammonium bicarbonate (ultra, ≥99.5%) were purchased from Fluka (Buchs, Switzerland). Trypsin (sequencing grade modified) was obtained from Promega Biosciences (San Luis Obispo, CA, USA). The protein and peptide standards were bought from Bruker Daltonics Care (Bremen, Germany). Columns (11 µm frit size) and phosphate buffer saline (PBS, 0.01 M Na₂HPO₄/NaH₂PO₄ and 0.15 M NaCl, pH=7.4) were provided from PhyNexus, Inc. (San Jose, CA, USA). BCA™ protein assay kit was from Pierce–Thermo Fisher Scientific (Rockford, Illinois, USA).

4.2.2. Instrumentation

For qualitative MS analysis, a Bruker Daltonics Ultraflex I MALDI-TOF/TOF instrument (Bremen, Germany) was used. 1 µl of the sample was spotted on a stainless steel target followed by 1 µl of saturated SA solution (SA in ACN/water (1/1, v/v) containing 0.1% TFA) for protein analysis. 1 µl of the saturated HCCA solution was used as a matrix substance in case of digested thionins. All measurements were recorded in linear (for proteins) and
reflectron mode (for peptide analysis). An external calibration was achieved by spotting 0.5 µl of protein or peptide calibration standard (Bruker Daltonics, Bremen, Germany). Mass spectra were documented by summing up 500 laser shots. Laser power was set at 60-70% of its maximal intensity, using a 337 nm nitrogen laser at 50 Hz. The Flex Analysis version 2.4 software provided by the manufacturer was used for data processing. Database searching analysis was executed with Mascot software (http://matrixscience.com) and SwissProt as database. For peptide mass-fingerprint (PMF) database searching analysis, the parameters were set as following: C-carbamidomethyl (fixed modification), M-oxidation, mass value (monoisotopic), peptide mass tolerance (200 ppm), mass tolerance (0.6 Da), missed cleavage (1 to 3) and taxonomy other green plants). Centrifugation was done with an Eppendorf Centrifuge 5415 D (Hamburg, Germany). An Eppendorf Concentrator 5301 was used for evaporating solvents. Infrared spectra of mullite were recorded on a Perkin-Elmer Spectrum 100 ATR-IR spectrometer (Perkin-Elmer, Waltham, USA) and a Spectrum software version 6.3.1 (Perkin-Elmer, Waltham, USA). The measured wavenumber range was 4000–520 cm\(^{-1}\) with 16 scans. The spectral resolution was 1.00 cm\(^{-1}\) and temperature was set to 22 °C. UV–Vis spectrometer (Janwey Genova Plus Bibbly Scientific Ltd, UK) was used for quantification of thionins in SPE eluates at 562 nm.

4.2.3. Preparation of extracts

Barley seeds and mistletoe were milled with a Retsch ZM 200 mill (Retsch, Hann, Germany) to a particle size less than 0.5 mm. In parallel, 200 mg of mistletoe powder, wheat flour and barley flour were separately extracted in 5 ml PBS for 60 min in ultra-sound. After extraction the samples were centrifuged and supernatants were stored at -20°C.
4.2.4. SPE Method

30 mg of ultra-sonicated mullite powder was filled into column having a frit size of 11 µm. Activation was done with 100 µl of methanol/water (1/1, v/v) by aspirating and expelling slowly several times. Additionally, 100 µl of sample (mistletoe/wheat/barley extract) was loaded onto the sorbent by aspirating and dispensing several times. Washing was achieved with 100 µl of methanol/water (1/1, v/v) thrice. Finally, the elution was brought to 100 µl using 5% FA in methanol/water (1/1, v/v).

4.2.5. Scanning electron microscopic analysis

Scanning electron microscopy (SEM) of the sorbent was executed by Electron Micro Probe (JEOL 8100, Tokyo, Japan) for determination of particle size distribution. Before inspection under the scanning electron microscope, the mullite powder was sputtered with gold. SEM pictures were taken with an accelerating voltage of 15 kV and currents of 5-10 nA.

4.2.6. Recovery Study

Protein quantification of extracted thionins through mullite (30 mg) was performed with the BCA™ protein assay kit (Pierce– Thermo Fisher Scientific, Rockford, Illinois, USA). The assay is based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total proteins. 1 mg/ml stock solution of thionins was prepared by extracting viscotoxins. For the extraction of viscotoxins the above mentioned SPE protocol was employed with the surplus amount of mullite (500 mg sorbent per ml of mistletoe extract), in 2 ml vials. The eluate was dried down in Eppendorf Concentrator and reconstituted at a concentration of 1 mg/ml in 5% FA in methanol/water (1/1 v/v). Reference standards (50, 100, 150, 200, 250, 300 and 350 µg/ml) were prepared from appropriate dilutions of stock solutions in 5% FA in
methanol/water for calibration. Similarly, for the experiments describing the binding capacity and recovery from mullite the thionins standards (50-350 µg/ml) were prepared from the dilution of stock solution in PBS and the pH (7.4) was adjusted with 25% NH₄OH solution in water. 2 ml of the BCA™ working solution were added to 100 µl of the reference standards or the SPE eluates of thionins standards and each sample (mistletoe, wheat and barley) fraction. Subsequently, incubation was carried out on a thermomixer (Eppendorf AG, Hamburg, Germany) for 30 min at 60 °C at 800 rpm. After incubation, the supernatants were taken for UV-VIS measurements at 562 nm. The protein concentration corresponds to the amount of extracted thionins in the eluate.

4.2.7. Protein digestion

For thionins digestion, a method according to Güzel et al. was employed with some adjustments [37]. Shortly, each SPE-eluate (100 µl) was added with 40 µl of 0.5 M ammonium bicarbonate (pH < 8.0), 8 µL 40 mM nOGP and 8 µl 45 mM DTT. The samples were positioned on a Eppendorf thermomixer at 37 °C for 30 min and 900 rpm to attain denaturation. After cooling down to room temperature, alkylation of denatured proteins was achieved by adding 8 µl 100 mM IAA to each fraction and 30 min incubation in the dark. Subsequently, 8 µl of 0.1 µg/µl trypsin solution (diluted in 50 mM acetic-acid buffer pH 3.0) were supplemented with the samples and placed on a thermomixer for digestion at 37 °C for 16 h. Digestion progression was stopped by adding 5 µl of 5% TFA solution (pH < 3.0). Afterwards, the digested protein solutions were spotted on a stainless steel target followed by the addition of saturated HCCA matrix (HCCA in 50% ACN /0.1% TFA solution) for MALDI/TOF MS analysis.
4.2.8. Selectivity comparison

A selectivity comparison of mullite with PSDZ hollow-monolithic sorbent [38] was performed. Barley and wheat extracts were loaded onto the sorbents and SPE was carried out according to the protocols described for both sorbents. The respective eluates were subjected to MALDI/TOF MS analysis for investigating the selectivity of both sorbents.

4.3. Results and discussions

Isolation of individual isoforms of thionins from complex samples is often intricate and requires several purification steps. This unwieldy procedure can be significantly simplified by a selective SPE method prior to the instrumental analysis. In our earlier study, we described the SPE procedure for plant thionins employing PSDZ sorbent. The study also presented the HPLC-DAD separation of currently investigated isoforms after SPE [38]. In continuation to this principle, the mullite has shown a remarkable improvement to the SPE of thionins. Mullite further abolished the unspecific proteins, which were not possible to exclude from the complex extracts employing PSDZ sorbent. Mistletoe, wheat and barley extracts were loaded onto the sorbent and selectively isolated in mullite containing extraction column. A schematic outlook of the entire SPE method is depicted in Supporting Information Fig. S1 along with the proposed binding mechanism for cysteine rich thionins.

The SEM micrographs of the mullite sorbent (Supporting Information Fig. S2 A-D) depicts the micrographs recorded at different (50 μm (A), 10 μm (B), 5 μm (C) and 1 μm (D)) resolutions. The SEM pictures reveal the particle size of the sorbent particles to be around ~15-55 μm. Extract of mistletoe displays the least number of interfering components and serves as a decent sample extract for the SPE method development for thionins. Fig. 4.1 demonstrates the selective isolation of viscotoxins from mistletoe sample, the mass spectra of the
mistletoe extract (prior SPE) (A) and the eluted viscotoxins (B) is depicted. Inset C represents an expanded mass region around the molecular masses of viscotoxins (5 KDa) for clarity. The viscotoxins which could be detected in the eluted fraction are listed in Table 4.1 [39-44]. The reported molecular masses are based upon their known amino acid sequences.

Supporting Information Fig. S4.1: Schematic view of the SPE procedure along with the proposed binding mechanism between thionins and mullite.

Supporting Information Fig. S4.2: SEM micrographs of mullite powder recorded at 50 µm (A), 10 µm (B), 5 µm (C) and 1 µm (D) resolutions.
Protein Information Resource (PIR) software was used for the determination of the exact mass [http://pir.georgetown.edu/pirwww/search/comp_mw.shtml].

![MALDI/TOF MS analysis](image)

**Fig. 4.1:** MALDI/TOF MS analysis of the European mistletoe sample. A) Mass spectra of mistletoe extract prior to SPE, B) eluate-retained viscotoxins inset C is the expanded of mass spectra around 5 kDa.

Fig. 4.2 and 4.3 display the MALDI/TOF MS spectra of wheat and barley sample, respectively. Owing to the presence of a diverse range of proteins in these samples, a
selective SPE of thionins is extremely important for succeeding instrumental analysis. The presented SPE sorbent offers stronger interaction for purothionins and hordothionins (thionins from wheat and barley, respectively) which allows the removal of weaker bound non-specific proteins by methanol/water (1/1, v/v) washings.

**Fig. 4.2:** MALDI/TOF MS exploration of wheat flour extract. A) Mass spectra of wheat extract prior to SPE, B) methanol/water (1/1, v/v) wash of unspecific components C) 5% FA in methanol/water (1/1, v/v) elution of retained purothionins.

Mass spectrum A in Fig. 4.2 and 4.3 reveals the entire range of proteins up to 14 kDa in wheat and barley extract, before SPE. Spectrum B represents proteins removed by the
washing step and spectrum C of Fig. 4.2 and 4.3 displays the eluted fractions of purothionins and hordothions. The isolated and detected purothionins and hordothions are listed in Table 4.1 [45-48].

**Fig. 4.3:** MALDI/TOF MS analysis of barley. A) Mass spectra of barley extract before subjecting to SPE, B) methanol/water (1/1, v/v) wash of unspecific components from mullite C) 5% FA in methanol/water (1/1, v/v) elution of retained hordothions.
Protein quantification of extracted thionins from mullite was accomplished as described in “Recovery study”. The recovery rates of the thionins standards were found to be ~100% up to 100 µg/ml, which demonstrate the binding capacity for 30 mg of mullite powder employing the stated SPE protocol. Standards with a concentration higher than the binding capacity of the sorbent revealed a gradual decrease in recovery. The extraction efficiencies for the different standard solutions from mullite are listed in Supporting Information Table S1.

**Supporting Information Table S4.1: Recovery study of thionins standards from 30 mg mullite powder.**

<table>
<thead>
<tr>
<th>Loading Conc. µg/ml</th>
<th>Measured value µg/ml</th>
<th>Recovery %</th>
<th>S.D (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>50.21</td>
<td>100.42</td>
<td>0.49</td>
</tr>
<tr>
<td>100</td>
<td>99.90</td>
<td>99.90</td>
<td>1.68</td>
</tr>
<tr>
<td>150</td>
<td>102.45</td>
<td>68.30</td>
<td>1.14</td>
</tr>
<tr>
<td>200</td>
<td>103.30</td>
<td>51.65</td>
<td>7.24</td>
</tr>
<tr>
<td>250</td>
<td>104.67</td>
<td>41.86</td>
<td>6.57</td>
</tr>
<tr>
<td>300</td>
<td>104.35</td>
<td>34.78</td>
<td>5.72</td>
</tr>
<tr>
<td>350</td>
<td>105.35</td>
<td>30.10</td>
<td>8.28</td>
</tr>
</tbody>
</table>

Table 4.2 is based upon the BCA assay for the colorimetric quantitation of total thionins in eluted SPE fractions from mistletoe, wheat and barley. The recoveries of investigated thionins (from 30 mg of mullite and 100 µl of respective plant extract) were found to be, on average, 61.5 µg/ml (S.D 2.39) for viscotoxins, 71.2 µg/ml (S.D 4.03) for purothionins and
67.5 µg/ml (S.D 6.02) for hordothionins. Moreover, tryptic digest of the eluted SPE fractions were performed as described in “Protein digestion”. Thionins with the most intensive signals were confirmed through their peptide mass-fingerprint while the minor constituents could not be confirmed. Peptide mass-fingerprint examinations of the isolated and digested mistletoe, wheat and barley proteins employing Mascot as a search engine are listed in Supporting Information Table S2. Viscotoxin A2 unveiled sequence coverage of 65%, whereas B and A3 revealed the sequence coverage of 65 and 100%, respectively. α1-, α2-, and β-purothionins showed a sequence coverage of 34, 50 and 51%, respectively. α-hordothionin disclosed a sequence coverage of 47%, while 21% could be achieved for β-hordothionin from barley.

**Table 4.1: Detectable thionins from the SPE eluates of European mistletoe, wheat and barley extracts. The theoretical mass of an isoform is stated based on its amino acid sequence as reported in the literature.**

<table>
<thead>
<tr>
<th>Mistletoe</th>
<th>Observed mass</th>
<th>Theoretical mass</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscotoxin A1</td>
<td>4,890.6</td>
<td>4,889.53</td>
<td>[40]</td>
</tr>
<tr>
<td>Viscotoxin A2</td>
<td>4,835.7</td>
<td>4,834.42</td>
<td>[39]</td>
</tr>
<tr>
<td>Viscotoxin A3</td>
<td>4,835.7</td>
<td>4,835.53</td>
<td>[38]</td>
</tr>
<tr>
<td>Viscotoxin B</td>
<td>4,857.8</td>
<td>4,857.45</td>
<td>[41]</td>
</tr>
<tr>
<td>Viscotoxin B2</td>
<td>4,967.3</td>
<td>4,977.65/4,967*</td>
<td>[42]</td>
</tr>
<tr>
<td>Viscotoxin 1-PS</td>
<td>4,904.8</td>
<td>4904.02</td>
<td>[43]</td>
</tr>
<tr>
<td><strong>Wheat</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-1-purothionin</td>
<td>4,825.6</td>
<td>4,825.70</td>
<td>[44]</td>
</tr>
<tr>
<td>α-2-purothionin</td>
<td>4,925.3</td>
<td>4,929.82</td>
<td>[44]</td>
</tr>
<tr>
<td>β-purothionin</td>
<td>4,925.3</td>
<td>4,926.81</td>
<td>[45]</td>
</tr>
<tr>
<td><strong>Barley</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-hordothionin</td>
<td>4,856.8</td>
<td>4,855.75</td>
<td>[46]</td>
</tr>
<tr>
<td>β-hordothionin</td>
<td>4,926.4</td>
<td>4,926.81</td>
<td>[47]</td>
</tr>
</tbody>
</table>

* Kong et al. documented viscotoxin B2 to display the molecular mass at 4967 Da in MALDI/TOF MS [43]
**Table 4.2:** Quantification of eluted thionins, retained on 30 mg of mullite, from their respective samples.

<table>
<thead>
<tr>
<th>Al-silicate (mg)</th>
<th>Sample (100 µl)</th>
<th>Calibration range (µg/ml)</th>
<th>Regression equation</th>
<th>R-value</th>
<th>Absolute recovery (µg/ml)</th>
<th>S.D. n=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Mistletoe</td>
<td>50-350</td>
<td>y = 0.0017x + 0.0205</td>
<td>0.9994</td>
<td>61.5/viscotoxins</td>
<td>2.39</td>
</tr>
<tr>
<td>30</td>
<td>Wheat</td>
<td>50-350</td>
<td>y = 0.0017x + 0.0205</td>
<td>0.9994</td>
<td>71.2/purothions</td>
<td>4.03</td>
</tr>
<tr>
<td>30</td>
<td>barley</td>
<td>50-350</td>
<td>y = 0.0017x + 0.0205</td>
<td>0.9994</td>
<td>67.5/hordothions</td>
<td>6.02</td>
</tr>
</tbody>
</table>

**Supporting Information Table S4.2:** Identification of thionins from their peptide mass-fingerprints analysis.

<table>
<thead>
<tr>
<th>seq. coverage [%]</th>
<th>mascot search score</th>
<th>average error [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>European Mistletoe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viscotoxin-A2</td>
<td>65</td>
<td>52</td>
</tr>
<tr>
<td>Viscotoxin-B</td>
<td>65</td>
<td>59</td>
</tr>
<tr>
<td>Viscotoxin-A3</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>Wheat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-Purothionin</td>
<td>34</td>
<td>130</td>
</tr>
<tr>
<td>α2-Purothionin</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>β-Purothionin</td>
<td>41</td>
<td>36</td>
</tr>
<tr>
<td>Barley</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Hordothionin</td>
<td>69</td>
<td>51</td>
</tr>
<tr>
<td>β-Hordothionin</td>
<td>32</td>
<td>125</td>
</tr>
</tbody>
</table>

A selectivity comparison between previously reported results on PSDZ [38] with mullite was accomplished and is depicted in Fig. 4.4. Mullite offers a significant increase in selectivity for thionins compared to the PSDZ sorbent. PSDZ provides a sturdier interaction for the
encountered unspecific proteins (wheat and barley proteins, particularly) in comparison to mullite.

![Selectivity comparison between PSDZ sorbent and mullite.](image)

**Fig. 4.4:** Selectivity comparison between PSDZ sorbent and mullite. A and A1 are the mass spectra of eluates from PSDZ and mullite, respectively. B and B1 depict the mass spectra of barley eluate from PSDZ and mullite, respectively.

Therefore, more stringent washing steps are required in order to reach an acceptable level of selectivity for the PSDZ sorbent. Furthermore, a considerable non-specific retention pattern was observed in wheat and barley sample around 7 kDa and 10 kDa. On the contrary, mullite offers less affinity towards unspecific proteins that enables convenient washings of non-specific components by considerably increasing selectivity. No definite pattern of unspecific retention was observed using mullite for any of the applied samples except for barley where a minute unspecific binding pattern was observed around 10 kDa.

The composition of mullite ranges from 3Al₂O₃.2SiO₂ (3:2) to 2Al₂O₃.1SiO₂ (2:1). The ratio Al₂O₃/SiO₂ may be used to characterize the chemical composition of mullite whose
complete formula looks like $\text{Al}_{4+2x}\text{Si}_{2-2x}\text{O}_{10-x}$, where $x = 0$ corresponds to sillimanite (commonly occurring as an aluminosilicate mineral with the chemical formula $\text{Al}_4\text{Si}_2\text{O}_{10}$ (1:1 alumina/silica), $x = 0.25$ corresponds to 3:2 mullite ($\sim 72\% \text{Al}_2\text{O}_3$) and $x = 0.40$ corresponds to 2:1 ($\sim 78\% \text{Al}_2\text{O}_3$) mullite. The average structure of mullite is similar to closely related, but structurally simple sillimanite. Mullite structures consist of distorted edge-sharing octahedral ($\text{AlO}_6$) chains running parallel to the crystallographic $c$ axis, the chains are cross linked by Si-O and Al-O corner sharing tetrahedra. With increasing alumina content the Si$^{+4}$ is replaced by Al$^{+3}$, oxygen vacancies are created to maintain the charge neutrality that are randomly distributed throughout the structure [36, 49-51]. The characteristic IR absorption frequencies observed for the sorbent at 720 cm$^{-1}$, 880 cm$^{-1}$, 1108 cm$^{-1}$, 1125 cm$^{-1}$ and 1165 cm$^{-1}$ (IR spectra not shown) were in agreement with MacKenzie’s IR frequency calculations for 3:2 mullite [52] and Rüsher et al. [53]. The IR spectrum of mullite gives a clear indication about the composition of mullite i.e. 3:2 mullite can be distinguished from 2:1 mullite on the basis of IR spectral comparisons especially from 1100 cm$^{-1}$- 1200 cm$^{-1}$ [53].

The higher affinity of cysteine containing peptides to the different metal atoms has been reported in the literature. Xu et al. found increased retention of cysteine containing peptides by using gold nano-particles in the stationary phase [54]. A similar behavior of cysteine rich thionins on zirconium based PSDZ sorbent was demonstrated in our prior work [38]. In this regard, mullite has demonstrated the outstanding selectivity towards thionins. The interaction of aluminum with cysteine and the stability constant of their co-ordination complex has been well documented [55, 56]. The affinity of aluminum towards thiol group of cysteine provides a foundation for selective immobilization of sulphur rich thionins onto mullite. The increased selectivity of aluminum(III) compared to the zirconium(IV) based
sorbents can be explained by the stronger electrostatic character of zirconium. The stronger interactions provided by zirconium(IV) silicate based sorbent retains protein too strongly and consequently difficult to wash away, henceforth, makes it less selective as compared to mullite.

4.4. Conclusions

Mullite is an excellent sorbent for the selective isolation of thionins in plant extracts obtained from mistletoe, wheat and barley. Co-ordination between aluminum and the thiol group of cysteine results in a stronger affinity of thionins to the material compared to unspecific proteins. Mullite has shown to remarkably improve the selective SPE of thionins compared to previously described PSDZ phases. This approach diminishes the complexity of protein extracts enormously and therefore offers a simpler chromatographic separation of individual isoforms. The SPE method is suitable for screening of novel isoforms of thionins from still investigated samples.

4.5. Acknowledgement

Special thanks to the Higher Education Commission of Pakistan and OeAD for Author’s PhD scholarship. The authors also want to thank Eurasia-Pacific Uninet (EPU) (Salzburg, Austria), the Ministry for Science and Research and the Ministry for Health, Family and Youth (Vienna, Austria) (Novel analytical tools for quality control of immunomodulatory, anti-inflammatory and neuroprotective agents in Traditional Chinese medicine). The authors also would like to acknowledge Prof. Dr. Richard Tessadri and Martina Trebus from Faculty of Geo- and Atmospheric Sciences, Institute of Mineralogy and Petrography, University of Innsbruck, for SEM analysis.
Lastly, we would like to acknowledge Dr. Douglas T. Gjerde from PhyNexus, Inc., San Jose, CA, USA for providing PhyTip® columns and the valuable scientific discussions.

4.6. References

Chapter 4  SPE of thionins employing aluminium silicate


CHAPTER 5

Zirconium Silicate Assisted Removal of Residual Proteins after Organic Solvent Deproteinization of Human Plasma, Enhancing the Stability of the LC-ESI-MS Response for the Bioanalysis of Small Molecules*


5.1. Introduction

Blood plasma contains about 10,000 different proteins with the total concentration of 6 to 8 g dL\(^{-1}\) [1]. The generally used sample preparation techniques for biological sample cleaning include protein precipitation, liquid-liquid extraction (LLE), SPE and ultrafiltration [2, 3]. Each of these approaches is associated with certain advantages and disadvantages.

Protein precipitation is one of the earliest and the least time-consuming sample preparation technique for purifying biological fluids. It involves denaturation of proteins by external stress (such as strong acid/base, heat or organic solvents) and consequently disrupts protein-drug binding [4, 5]. However, not all protein precipitation methods are suitable for removing the proteins for the analysis of small molecules. The protein precipitation techniques based upon salts, acids and heat usually trap the analytes in the protein aggregates leaving a small quantity in the supernatant. Therefore, nearly all bioanalytical
protein precipitation methods use water miscible organic solvents for protein denaturation [2, 6]. Most of the organic solvent protein precipitation methods achieve only about 90-96% protein precipitation from human plasma [7]. The remaining proteins still can interfere with the chromatographic procedures by generating matrix effects. Many approaches have been employed to overcome this problem by additional cleaning of the sample. In this regard, salting-out assisted liquid/liquid extraction technique (SALLE) has recently been introduced to further clean-up the biological samples for the subsequent liquid chromatography (LC). SALLE involves double protein precipitation i.e. conventional organic solvent deproteinization followed by the addition of salts into the supernatant, which results in the removal of more than 99% plasma proteins [8, 9].

LLE provides cleaner samples and is applied in many studies, but it is quite time consuming due to slow drying of extraction solvents. Moreover, LLE is unsuitable for hydrophilic compounds [3]. SPE is easy to use and efficient, but rather expensive compared to protein precipitation. It further needs the selection of an appropriate affinity sorbent, method development, and optimization for isolating different classes of compounds from the biological samples [2]. Ultrafiltration is the least common sample preparation technique for the biological samples. It has the potential to deplete more than 99% of proteins from human plasma [10]; however, the ultrafiltrate only provides a measure of drug not bounded to proteins as opposed to total drug. Therefore, it is highly inappropriate for strongly protein-bound drugs. In addition, separate experiments must be executed to determine, and correct for the drug-membrane binding. The choice of membrane is certainly critical for the analysis as membrane contaminants and structure can affect the quantitation and hence results in the loss of recovery [11, 12].
In this study, we combined methanol-protein precipitation and a follow-up cleaning using zirconium silicate powder in spin-columns. Artichoke leaves extract (ALE) tablets 400 mg were administrated orally for three days to a healthy volunteer for setting-up a practical application of the clean-up method for the isolation of CQAs and their metabolites from human plasma.

ALE is traditionally used for the treatment of hepatic and dyspeptic orders. Efficacy has been established in numerous clinical studies [13]. ALE is used for the treatment of the irritable-bowel syndrome [14] and reduces the risk of coronary heart disease by lowering plasma cholesterol levels [15]. Antioxidant activities [16] and the inhibition of hepatocellular cholesterol biosynthesis [17] were also observed. CQAs are one of the key active substances of ALE. CQAs are the esters of quinic acids with caffeic, ferulic or p-coumaric acids. Humans routinely ingest CQAs from various plants [18], fruits [19], coffee [20] and green tea [21]. There have been numerous reports on the pharmacological activities of CQAs [22-29]. CQAs (chlorogenic acid (CGA) and cynarin (CYN)) and three of their potential metabolites [30] (caffeic acid (CA), ferulic acid (FLA) and 3,4-dihydroxyhydrocinnamic acid (DHCA)) were selected as model compounds for the study.

The aim of this study was to design a rapid, convenient and an efficient clean-up method for human plasma samples. The presented analytical workflow should assist the popular organic solvent deproteinization as a powerful sample preparation tool. Furthermore, this novel method shows great potential to be established as an analytical platform for the isolation of small molecules from human plasma.
5.2. Materials and methods

5.2.1. Reagents

Acetonitrile (ACN, HPLC-grade), methanol (ultra LC-MS grade) and water (HPLC-grade) were purchased from Carl Roth Gmbh + Co. KG (Karlsruhe, Germany). Formic acid (FA) was obtained from Merck KGaA (Darmstadt, Germany). Sinapinic acid (SA), zirconium(IV) silicate (325 mesh), Supel\textsuperscript{TM} QuE Z-Sep/C18 (sorbent amount 60 mg, centrifuge tube volume 2 mL), SigmaPrep\textsuperscript{TM} spin columns (7-20 microns polyethylene frit, 800 µL sample capacity), bovine serum albumin (BSA) and human plasma (blank plasma), chlorogenic acid (CGA, 95%), ferulic acid (FLA, 99%), 3,4-dihydroxyhydrocinnamic acid (DHCA, 98%), were purchased from Sigma Aldrich (St. Louis, USA).

![Fig. 5.1: The chemical structures of investigated CQAs and their metabolites. A) chlorogenic acid (CGA), B) cynarin (CYN), C) caffeic acid (CA), D) ferulic acid (FLA), E) 3,4-dihydroxyhydrocinnamic acid (DHCA).](image-url)
Cynarin (CYN) was from Extrasynthese (Genay, France), caffeic acid (CA, 97%), trifluoroacetic acid (TFA, for protein sequence analysis), gallic acid, were from Fluka (Buchs, Switzerland). The chemical structures of CGA, CYN, CA, FLA and DHCA are shown in Fig. 1 A-E, respectively. BCA™ protein assay kit was from Pierce–Thermo Fisher Scientific (Rockford, Illinois, USA).

5.2.2. Instrumentation

5.2.2.1. MALDI-TOF-MS

For qualitative analysis of proteins, a Bruker Daltonics Ultraflex I MALDI-TOF/TOF instrument (Bremen, Germany) was used. 1 µL of the sample was spotted on a stainless steel target followed by 1 µL of saturated SA solution (SA in ACN/water (1/1, v/v) containing 0.1% TFA). All measurements were recorded in linear mode. Mass spectra were recorded by summing up 500 laser shots. Laser power was attenuated at 70% of its maximal intensity, using a 337 nm nitrogen laser at 50 Hz. The Flex Analysis version 2.4 software provided by the manufacturer was used for data processing.

Centrifugation was carried out with an Eppendorf Centrifuge 5415 D (Hamburg, Germany). An Eppendorf Concentrator 5301 was used for evaporating solvents. UV-Vis spectrometer (Janwey Genova Plus, Bibbly Scientific Ltd, Staffordshire, UK) was used for quantification of proteins at 562 nm using the BCA assay.

5.2.2.2. HPLC-DAD/ESI-MS

A Shimadzu (Tokyo, Japan) HPLC-DAD/ESI-MS was used for the analysis comprising an online degasser unit (DGU-20A5R), two solvent delivery pumps (LC-30AD), an auto-injector (SIL-30AC), column oven (CTO-20AC), a DAD (SPD-M20A) and an electrospray ionization (ESI)
Chapter 5  Bioanalysis of phenolic acids

single quadrupole liquid chromatograph mass spectrometer LC/MS (LCMS 2020). System control and data analysis were performed by using the manufacturer’s software packages (LabSolutions LCMS version 5.42SP6).

A (BDS) Hypersil C18 column (250 × 4 mm, 5 µm particle size, 130 Å pore size), with a guard column (50 × 4 mm, 5 µm particle size, 130 Å pore size) from Thermo Scientific, Berlin, Germany was used for chromatographic separations. The mobile phase was consisted of acetonitrile (B) and 0.5% FA in water (A).

5.2.3. HPLC-DAD/ESI-MS conditions

HPLC separation of the target compounds was executed employing a linear gradient as: (min/% B) 0/5, 20/25, 21/95, 27/95, 28/5, 34/5. The flow rate was 0.8 mL/min and the injection volume was 20 µL. The column-oven was set at 30 °C. The diode-array detector was scanning between 190-400 nm. The flow cell temperature was set at 40 °C and the slit width was adjusted to 8 nm.

MS was carried out using ESI in positive-ion mode for all the analytes except DHCA. DHCA was analyzed in a separate injection using negative-ion mode. The injection volume was 20 µL, scan speed was 790 u/sec, interface temperature and voltage was 350 °C and 4.5 kV, respectively. Desolvation line (DL) temperature was 250 °C, drying gas flow was set at 15 L/min while the nebulizing gas flow was adjusted at 1.5 L/min. The heat block temperature was fixed at 200 °C. Scanned mass range was m/z 120-900.

5.2.4. Clean-up protocol

100 µL of plasma were supplemented with 350 µL of methanol containing (0.1 mg mL⁻¹) gallic acid. The samples were mixed for two min and centrifuged at 16.1 × 1000 g for ten min.
Afterwards, the supernatant was separated into 1.5 mL vials. The protein pellet was washed with 50 µL 2% FA solution in methanol by gentle pipetting. The washing solution was combined with the supernatant.

In the second step, 400 mg of zirconium silicate (325 mesh) were filled in spin column and activated with 500 µL methanol twice, followed by an equilibration with 500 µL water twice at $0.4 \times 1000 \times g$. Subsequently, the supernatant from the first step (protein precipitation) was loaded onto the spin column and allowed to flow through the zirconium silicate bed once at $0.4 \times 1000 \times g$. Following this, the elution was achieved (fraction of target compounds attached to zirconium silicate eluted while still holding the proteins) by recycling 100 µL 2% FA in methanol three times and combined with the flow-through sample.

In the comparative studies of zirconium silicate based clean-up with Supel™ QuE Z-Sep/C18, the same protocol in spin columns was employed by using 100 mg of the sorbent and subsequently reducing the volume of sample and solvents to one-fourth.

5.2.5. Clean-up efficiency of the method

Proteins were quantified by the BCA™ protein assay kit. The assay is based on BCA for the colorimetric detection and quantitation of total proteins. 2 mg mL$^{-1}$ stock solution of bovine serum albumin (BSA) was prepared in aqueous solution. Reference standards (25, 50, 100, 200, 400, 800 and 1600 and 2000 µg mL$^{-1}$) were prepared from appropriate dilutions of stock solutions in water. 2 mL of BCA™ working solution were added to 100 µL of the reference standards or the samples (human plasma, supernatant from methanol deproteinization, zirconium silicate assisted clean-up, Supel™ QuE Z-Sep/C18 assisted clean-up). The protein quantification of the human plasma sample was achieved by a 100 times dilution in water.
Subsequently, they were placed at room temperature for two hours. Finally, samples were taken for UV-VIS measurements at 562 nm.

The protein removal efficacy was calculated using the formula:

\[
\% \text{ efficiency} = \frac{A - B}{A} \times 100
\]

where, A is the total proteins in plasma and B the proteins present after methanol deproteinization/clean-up procedure using zirconium silicate or Supel\textsuperscript{TM} QuE Z-Sep/C18. The above mentioned formula was also used for calculating the efficiency of zirconium silicate and Supel\textsuperscript{TM} QuE Z-Sep/C18 over methanol deproteinization.

5.2.6. Preparation of stock and working solutions

Stock solutions of CQAs (CGA and CYN), three potential metabolites of CQAs (CA, FLA, and DHCA) were prepared by dissolving 1 mg of each compound in 1 mL methanol/water (1/1, v/v), separately. From these stock solutions two sets (A: CGA, CYN, B: CA, FLA, DHCA) of working solutions were prepared. CGA and CYN were pooled together at concentrations of 250, 100 and 10 µg mL\textsuperscript{-1} by a suitable dilution in methanol/water (1/1, v/v). Similarly, CA, FA and DHCA were pooled together at the same concentrations. All working solutions were stored at -20 °C.

5.2.7. Calibration standards, quality control standards and neat standards

The working solutions were further diluted in cleaned-up blank plasma (zirconium silicate assisted cleaned-up plasma after methanol deproteinization, see Sec 5.2.4) for the preparation of reference standards. Reference standards for both of the pools (A and B) were prepared at concentration levels (µg mL\textsuperscript{-1}): 0.02, 0.05, 0.10, 0.50, 0.80, 1.5, 3.0, 5.0, 8.0,
11.0 and 15.0. Four quality control (QC) standards (µg mL⁻¹): low (0.1), medium (0.6, 3) and high (10) concentrations were also prepared in the similar manner in cleaned-up blank plasma for accuracy, precision and recovery studies. Neat standards (for analyte loss and matrix effect experiments) were prepared at concentration levels: 3 µg mL⁻¹ and 0.01 µg mL⁻¹ by dilution of working solutions in methanol/water (1/1, v/v). The calibration curve was constructed for each analyte by plotting the peak area (HPLC-DAD chromatogram) vs concentration of each target compound.

5.2.8. Validation: accuracy, precision and stability

Accuracy and precision of the method were determined by HPLC-DAD analysis of QC standards. Inter-day accuracy and precision were calculated by analyzing the QC standards over three consecutive days. Four sequential assays were performed with five replicates (n=5) of QC standards. Precision was expressed as a co-efficient of variance percent (% CV) and accuracy was calculated as percent relative error (% RE). Stability was monitored by HPLC-DAD analysis of the QC standards at room temperature for four hours (short term stability). Stability was also documented for three freeze and thaw cycles (freeze and thaw stability) and for two weeks at -80 °C (long term stability). The obtained data were in accordance with the FDA-Industry Guidelines for Bioanalytical Method Validation (2001) [31].

5.2.9. Extraction efficiency

The extraction efficiencies for each analyte were determined at four concentration levels of QC standards by a comparison of peak areas of analytes extracted from spiked blank plasma samples, employing the stated protocol (Sec 5.2.4), to the peak area obtained from the QC
standards without extraction protocol. Three replicates (n=3) of extracted and unextracted samples were measured at each concentration level.

5.2.9.1. Analyte loss during clean-up

The potential analyte loss during the zirconium silicate assisted clean-up was estimated by comparing the peak areas of neat standards (0.1 µg mL\(^{-1}\) and 3 µg mL\(^{-1}\)) to the peak areas of the standards (spiked into blank plasma at same concentration levels) after different stages of extraction procedures, namely protein precipitation, protein precipitation along with pellet wash and after clean-up with zirconium silicate.

5.2.10. Matrix Effect

The matrix effect (matrix suppression or enhancement of ionization) of CQAs and their metabolites was investigated by comparing the peak areas of neat standards (0.1 µg mL\(^{-1}\) and 3 µg mL\(^{-1}\)) to the same standards spiked in four different lots of human plasma each cleaned-up with zirconium silicate, cleaned-up with Supel\(^{TM}\) QuE Z-Sep/C18 and methanol deproteinized supernatant without further clean-up.

The matrix effect was measured using the following equation:

\[
\% \text{ matrix effect} = \frac{B - C}{C} \times 100
\]

where, C is the peak area of neat standard and B is the peak area of the same standard spiked in blank plasma cleaned-up with zirconium silicate/cleaned-up with Supel\(^{TM}\) QuE Z-Sep/C18 or methanol deproteinized supernatant without further clean up.
5.2.11. Example application to Human Plasma

A plasma sample was collected from a healthy, non-smoking individual with normal blood pressure (aged 29 years) after the oral administration of ALE tablets (400 mg) for three days (one tablet three times a day). The volunteer was not taking coffee and coffee-related items for five days prior to the collection of the blood sample. Blood was taken one hour after the ingestion of the last dose of ALE tablets using heparin as an anticoagulant. The samples were immediately centrifuged at 6.0 × 1000 g for 10 minutes at 5 °C and stored at -80 °C. HPLC-DAD/ESI-MS analysis of plasma samples was executed after clean-up with zirconium silicate (sec 5.2.4), either with or without β-glucuronoside treatment. β-glucuronoside treatment was performed according to the method of Wittemer et al. with slight adjustments [32]. 500 µL of plasma were adjusted to pH 5 using concentrated FA. Afterwards 30 µL of β-glucuronoside solution were added. The mixture was incubated at 37 °C for one hour.

5.3. Results and discussion

In this study, an efficient blood plasma clean-up method is reported, which combines simple methanol protein precipitation and a rapid SPE procedure employing zirconium silicate powder to remove the interfering plasma proteins which were still existent after the solvent precipitation. Zirconium silicate was reported to have a very strong binding for proteins [33] due to the strong electrostatic interactions provided by zirconium(IV). Therefore, zirconium silicate has been used to make human plasma samples free of interfering proteins for subsequent LC. A schematic illustration of the entire SPE workflow is depicted in Fig. 5.2. By running the deproteinized plasma sample over a bed of zirconium silicate (once) in spin columns, most of the CQAs and its metabolites passed through whereas proteins were retained. In our enrichment study on CQAs from Arnicae flos samples it was demonstrated
that approximately ten times recycling through the zirconium silicate powder [34] were required to have a significant retention of CQAs onto the sorbent. Nevertheless, a minute quantity that might have been retained over the sorbent was eluted using a 2% FA in methanol. Due to the presence of a large number of coordination sites (O, S and N) proteins have a much higher tendency to bind to the zirconium silicate compared to the investigated small molecules. The difference in the binding efficiencies of proteins and the analytes towards the sorbent enables an effective immobilization of proteins onto zirconium silicate while eluting less strongly retained CQAs. Analyte loss during the different stages of clean-up procedure employing zirconium silicate was calculated (see Supporting Information Table S5.1) for the standards (spiked into blank plasma at 0.1 µg mL⁻¹ and 3 µg mL⁻¹). CGA and CYN experienced the maximum recovery loss (14-20%) during the protein deproteinization step (presumably due to the presence of carboxylic group and large number of hydroxyl- and carbonyl groups they encounter strong interaction with plasma proteins). CA, FLA and DHCA demonstrated no recovery loss at the protein precipitation.
**Supporting Information Table S5.1:** Analyte loss during different stages of extraction procedure. Mean values of three replicates measurements (n=3) are reported, RSD < 5 %.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Conc. (µg mL(^{-1}))</th>
<th>Recovery % after methanol precipitation</th>
<th>Recovery % after methanol precipitation besides pellet wash</th>
<th>Recovery % after clean-up with zirconium silicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHCA</td>
<td>3</td>
<td>101</td>
<td>101</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>99</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>CGA</td>
<td>3</td>
<td>82</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>86</td>
<td>89</td>
<td>83</td>
</tr>
<tr>
<td>CA</td>
<td>3</td>
<td>101</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>99</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td>CYN</td>
<td>3</td>
<td>80</td>
<td>87</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>81</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>FLA</td>
<td>3</td>
<td>99</td>
<td>99</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>98</td>
<td>99</td>
<td>90</td>
</tr>
</tbody>
</table>

**Fig. 5.3:** MALDI/TOF MS investigation of clean-up method. A) Mass spectrum of supernatant after methanol deproteinization, B) Mass spectrum after zirconium silicate assisted clean-up of supernatant from methanol deproteinization.
The pellet wash minimized (3-6%) the recovery loss of CGA and CYN from 14-20% to 11-14%.

Zirconium silicate assisted clean-up resulted in 3-9% recovery loss for all the tested analytes. Fig. 5.3 displays the efficacy of zirconium silicate in cleaning up the methanol deproteinized human plasma. There were considerable proteins detected in MALDI/TOF MS spectrum recorded after methanol deproteinization (A), spectrum B was recorded post zirconium silicate supported follow-up cleaning and depicts a substantial removal of residual proteins.

Table 5.1 represents the efficiency comparison of protein exclusion by zirconium silicate and Supel™ QuE Z-Sep/C18 for three replicate measurements (n=3). The protein removal

Table 5.1: Protein depletion efficacy of the different methods employed (n=3).

<table>
<thead>
<tr>
<th>Plasma/supernatant volume (µL)</th>
<th>Sorbent/amount mg</th>
<th>Total plasma proteins/residual proteins after methanol deproteinization (µg mL⁻¹) mean ± % RSD</th>
<th>Proteins in cleaned-up plasma (µg mL⁻¹) mean ± % RSD</th>
<th>Protein depletion efficacy %</th>
<th>Sorbent efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50/200</td>
<td>ZrSiO₄/400</td>
<td>621.3 × 100 ± 1.95/1870.7 ± 1.68</td>
<td>191.6 ± 1.62</td>
<td>99.69</td>
<td>89.75</td>
</tr>
<tr>
<td>100/400</td>
<td>ZrSiO₄/400</td>
<td>1870.7 ± 1.68</td>
<td>192.7 ± 2.18</td>
<td>99.68</td>
<td>89.73</td>
</tr>
<tr>
<td>150/600</td>
<td>ZrSiO₄/400</td>
<td>211.2 ± 2.40</td>
<td>192.7 ± 2.18</td>
<td>99.66</td>
<td>88.70</td>
</tr>
<tr>
<td>200/800</td>
<td>ZrSiO₄/400</td>
<td>263.2 ± 4.68</td>
<td>192.7 ± 2.18</td>
<td>99.57</td>
<td>85.90</td>
</tr>
<tr>
<td>25/100</td>
<td>Z-Sep-C18/100</td>
<td>183.2 ± 0.93</td>
<td>192.7 ± 2.18</td>
<td>99.70</td>
<td>90.10</td>
</tr>
</tbody>
</table>

capacity of zirconium silicate was calculated by monitoring the clean-up efficiency for different volumes (50-200 µL) of human plasma. Zirconium silicate demonstrated a constant protein removal up to 100 µL of plasma (clean-up capacity), plasma volumes more than 100 µL depicted a gradual decrease in sorbent efficiency. Therefore, 100 µL of plasma volume was used for the comparative studies between zirconium silicate and Supel™ QuE Z-Sep/C18. Both the sorbents demonstrated > 99.6% removal of the human plasma proteins, whereas methanol deproteinization achieved 96.9% removal of plasma proteins. The
efficacy of zirconium silicate and Supel™ QuE Z-Sep/C18 was found to be 89.7% and 90.1%, respectively over conventional methanol deproteinization. The clean-up procedure abolishes the use of organic- or inorganic salts to further deplete the residual proteins after organic solvent deproteinization. The consumption of inorganic salts in the most recent SALLE technique, can particularly raise concerns for subsequent LC-MS analysis [35].

5.3.1. Linearity

The calibration curve from reference standards was found to be linear over the tested range (0.02 – 15 µg mL⁻¹ for CGA, CYN, FLA, CA and 0.05 – 15 µg mL⁻¹ for DHCA). A least square regression equation was found to have the best fit over the whole concentration range and was used for accuracy, precision and stability evaluations of QC standards. The regression equations of these curves and their co-relation coefficients are as:

CGA, \( y = 7504.314 + 183602.227x \) (R = 0.9996); CYN, \( y = 9809.413 + 241518.817x \) (R = 0.9992); CA, \( y = 5867.943 + 363642.284x \) (R=0.9993); FLA, \( y = 1447.996 + 406492.765x \) (R = 0.9991); DHCA, \( y = -4394.057 + 59554.779x \) (R = 0.9994). The lower limit of quantification (LLOQ) for the tested compounds (signal to noise ratio, S/N ~ 10) was observed to be 0.02 µg mL⁻¹ for CGA, CYN, FLA, and CA while DHCA resulted in a LLOQ of 0.05 µg mL⁻¹.

5.3.2. Extraction Efficiency

Table 5.2 reveals the extraction efficiency of the applied method. The scheme demonstrates high recoveries for standard compounds spiked into blank human plasma. The recoveries for CYN was found to be in the range of 81-86%, CGA demonstrated the recoveries between 78-83%, CA was extracted in the excess of 89-92%, while FA and DHCA were recovered to an extent of 87-91% and 92-95%, respectively. Relative standard deviation (RSD) for the data was found to be below 5% for n=3.
Table 5.2: Extraction efficiencies (mean recovery %) of CGA, CYN, CA, FLA and DHCA from human plasma samples spiked at different concentration levels (n=3).

<table>
<thead>
<tr>
<th>Conc. µg mL⁻¹</th>
<th>Mean recovery % (n=3) RSD &lt; 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYN</td>
</tr>
<tr>
<td>0.1</td>
<td>86</td>
</tr>
<tr>
<td>0.6</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>81</td>
</tr>
<tr>
<td>10</td>
<td>83</td>
</tr>
</tbody>
</table>

5.3.3. Precision and Accuracy

Table 5.3 demonstrates the precision and accuracy of the data for the investigated compounds. The % CV for each of the QC standard was found below 5%. The % RE values were also turned out according to the FDA guidelines, i.e. below 15% for higher concentrations and below 20% for lower concentrations (concentrations around LLOQ).

5.3.4. Stability

The stability of CGA, CYN, FLA, CA and DHCA which were spiked into cleaned-up human plasma was investigated under altered storage and handling conditions at 0.1 µg mL⁻¹ and 3 µg mL⁻¹ concentration levels. Supporting Information Table S2 reveals the stability data for short term stability (four hours at room temperature), Freeze and thaw stability (three recycles) and long term stability (-80 °C two weeks). Relative standard deviation (RSD) for the data was found to be below 5% for n=3. No stability related issues were observed during storage and handling i.e., the recoveries were below 15% for higher concentrations and below 20% for lower concentrations according to the FDA guidelines.
**Table 5.3:** Intra- and inter-assay precision and accuracy data for the investigated compounds (n=5).

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Conc. (µg mL⁻¹)</th>
<th>Intra-day % CV</th>
<th>Inter-day % CV</th>
<th>Measured value (µg mL⁻¹)</th>
<th>% RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGA</td>
<td>10</td>
<td>1.96</td>
<td>1.84</td>
<td>9.92</td>
<td>-0.80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.51</td>
<td>2.51</td>
<td>3.25</td>
<td>8.33</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>0.70</td>
<td>0.90</td>
<td>0.67</td>
<td>11.67</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.19</td>
<td>0.27</td>
<td>0.80</td>
<td>-20</td>
</tr>
<tr>
<td>CYN</td>
<td>10</td>
<td>1.70</td>
<td>1.08</td>
<td>10.28</td>
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<tr>
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<td>1.91</td>
<td>3.40</td>
<td>13.33</td>
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<tr>
<td></td>
<td>0.6</td>
<td>2.12</td>
<td>2.40</td>
<td>0.69</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.90</td>
<td>2.31</td>
<td>0.83</td>
<td>-17</td>
</tr>
<tr>
<td>CA</td>
<td>10</td>
<td>0.30</td>
<td>0.30</td>
<td>10.38</td>
<td>3.83</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.41</td>
<td>0.80</td>
<td>3.25</td>
<td>8.33</td>
</tr>
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<td>0.6</td>
<td>0.25</td>
<td>0.63</td>
<td>0.68</td>
<td>13.88</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.16</td>
<td>1.43</td>
<td>0.12</td>
<td>12</td>
</tr>
<tr>
<td>FLA</td>
<td>10</td>
<td>0.15</td>
<td>4.46</td>
<td>9.50</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.45</td>
<td>2.81</td>
<td>3.17</td>
<td>5.66</td>
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<tr>
<td></td>
<td>0.6</td>
<td>0.57</td>
<td>1.52</td>
<td>0.63</td>
<td>5</td>
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<tr>
<td></td>
<td>0.1</td>
<td>2.16</td>
<td>3.34</td>
<td>0.11</td>
<td>10</td>
</tr>
<tr>
<td>DHCA</td>
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<td>1.28</td>
<td>0.95</td>
<td>9.17</td>
<td>-8.33</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<tr>
<td></td>
<td>0.6</td>
<td>0.38</td>
<td>2.62</td>
<td>0.67</td>
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</tr>
<tr>
<td></td>
<td>0.1</td>
<td>1.09</td>
<td>3.02</td>
<td>0.11</td>
<td>16.66</td>
</tr>
</tbody>
</table>

**Supporting Information Table S5.2:** Stability data from QC samples of CQAs and their metabolites in cleaned-up blank plasma under different storage and handling conditions, mean values of three replicates measurements (n=3) are reported, RSD < 5 %.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Conc. (µg mL⁻¹)</th>
<th>Short-term stability Recovery %</th>
<th>Freeze and thaw stability Recovery %</th>
<th>Long-term stability Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGA</td>
<td>3</td>
<td>97.4</td>
<td>103.6</td>
<td>101.8</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>87.2</td>
<td>91.3</td>
<td>105.1</td>
</tr>
<tr>
<td>CYN</td>
<td>3</td>
<td>104.3</td>
<td>106.6</td>
<td>109.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>116.1</td>
<td>118.2</td>
<td>95.1</td>
</tr>
<tr>
<td>CA</td>
<td>3</td>
<td>108.6</td>
<td>111.6</td>
<td>112.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>118.2</td>
<td>115.3</td>
<td>116.4</td>
</tr>
<tr>
<td>FLA</td>
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<td>96.0</td>
<td>97.3</td>
<td>101.6</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>90.2</td>
<td>90.7</td>
<td>88.4</td>
</tr>
<tr>
<td>DHCA</td>
<td>3</td>
<td>96.4</td>
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<td>90.6</td>
</tr>
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<td></td>
<td>0.1</td>
<td>118.3</td>
<td>119.1</td>
<td>116.5</td>
</tr>
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</table>
5.3.5. Matrix effect

The matrix effects were measured by comparing the peak areas of the spiked standards in cleaned-up plasma (zirconium silicate assisted clean-up/Supel™ QuE Z-Sep/C18 assisted clean-up or the methanol deproteinized supernatant without further clean-up) to the neat standards. Fig. 5.4 depicts the HPLC/ESI MS chromatograms recorded in positive ion mode for the spiked standards (at 3 μg mL⁻¹) in different cleaned-up plasma samples. The positive ion mode was preferred to the negative ion mode because it was found more sensitive and less noisy except for DHCA analysis. DHCA was analyzed in a separate injection using negative ion mode (negative ion mode chromatograms not shown). Chromatograms A and B are the extracted ion chromatograms (XIC) (at m/z 183, 355, 181, 517 and 195 corresponding to [M+H]⁺ of DHCA, CGA, CA, CYN and FLA, respectively) which demonstrate the matrix effects observed for the spiked (3 μg mL⁻¹ each) standards into Supel™ QuE Z-Sep/C18 and zirconium silicate assisted cleaned-up plasma, respectively. XIC C represents the matrix effects witnessed for the same standards spiked in methanol deproteinized supernatant. XIC D is the representative chromatogram for the neat standards. The retention times of the standard compounds were found to be CGA: 9.99 ± 0.09 min, CYN: 13.07 ± 0.11, FLA 15.08 ± 0.17, CA: 10.64 ± 0.13, DHCA 9.08 ± 0.17. The results for the matrix effects, as summarized in Table 5.4, clearly indicates that the matrix effects were evident at both the concentration levels (-12.11% to -26.23% for 3 μg mL⁻¹ and -18.28% to -40.12% for 0.1 μg mL⁻¹) of all the standards spiked into methanol deproteinized supernatants. The matrix effects for the standards spiked at 3 μg mL⁻¹ into Supel™ QuE Z-Sep/C18 extracted plasma were acceptable (-3.37% to -9.32%) whereas, for the standards spiked at 0.1 μg mL⁻¹ the matrix effects (-14.91% to -18.76 %) were found higher than (±15%) the acceptable values for matrix effects as established by FDA guidelines [31].
Table 5.4: Calculated % matrix effect of the CQAs and their metabolites in zirconium silicate assisted clean-up, Supel™ QuE Z-Sep/C18 assisted clean-up and methanol deproteinized supernatant.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Conc. (µg mL⁻¹)</th>
<th>ZrSiO₄ Mean ± SD</th>
<th>Z-Sep/C18 Mean ± SD</th>
<th>Methanol deproteinization Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHCA</td>
<td>3</td>
<td>-2.05 ± 1.29</td>
<td>-3.37 ± 1.30</td>
<td>-12.11 ± 3.43</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-4.49 ± 2.25</td>
<td>-17.34 ± 4.48</td>
<td>-21.42 ± 5.47</td>
</tr>
<tr>
<td>CGA</td>
<td>3</td>
<td>-1.05 ± 3.98</td>
<td>-6.73 ± 4.59</td>
<td>-26.23 ± 5.60</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-5.08 ± 2.34</td>
<td>-18.2 ± 6.02</td>
<td>-29.14 ± 6.39</td>
</tr>
<tr>
<td>CA</td>
<td>3</td>
<td>-1.16 ± 2.83</td>
<td>-6.36 ± 4.78</td>
<td>-21.21 ± 7.14</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-5.10 ± 2.34</td>
<td>-14.91 ± 5.98</td>
<td>-28.75 ± 5.81</td>
</tr>
<tr>
<td>CYN</td>
<td>3</td>
<td>1.42 ± 2.47</td>
<td>-8.28 ± 4.16</td>
<td>-22.27 ± 8.49</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-4.51 ± 2.14</td>
<td>-17.5 ± 4.04</td>
<td>-40.12 ± 6.90</td>
</tr>
<tr>
<td>FLA</td>
<td>3</td>
<td>0.82 ± 0.52</td>
<td>-9.32 ± 3.83</td>
<td>-18.28 ± 7.02</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>-3.30 ± 2.66</td>
<td>-18.76 ± 6.23</td>
<td>-21.34 ± 7.02</td>
</tr>
</tbody>
</table>

Fig. 5.4: HPLC/ESI MS (XIC) of examined CQAs and their metabolites in different cleaned-up samples. A) XIC of standards (3 µg mL⁻¹ each) spiked into Supel™ QuE Z-Sep/C18 assisted cleaned-up plasma, B) XIC of standards (3 µg mL⁻¹ each) spiked into zirconium silicate assisted cleaned-up plasma, C) XIC of standards (3 µg mL⁻¹ each) spiked into methanol deproteinized supernatant without further clean-up, D) neat standards (3 µg mL⁻¹ each).
On the contrary, zirconium silicate demonstrated no matrix related problems for all of the tested standards at both 3 µg mL\(^{-1}\) (0.82% – 2.05%) and 0.1 µg mL\(^{-1}\) (-3.30% – 5.10%) concentration levels.

5.3.6. Application to pharmacokinetic study

In a further approach the present technique was applied to extract CQAs and their metabolites from human plasma (Sec 5.2.11). Fig. 5.5 depicts the HPLC/ESI MS selected ion monitoring (SIM) chromatograms (in positive ion mode, at m/z 181 and 195 corresponding to \([M+H]^+\) of CA and FLA, respectively) of the β-glucuronoside treated human plasma samples, which were extracted after ALE tablets intake for three days, respectively. β-glucuronoside treatment is imperative to carry out as the metabolites of CQAs have the tendency to exist in their conjugated (glucuronides or sulfates) forms [30]. The extracted sample displayed no peak (chromatogram not shown) from any of the target analytes before β-glucuronoside treatment (conjugates probably co-eluted with matrix compounds). After hydrolyzing with β-glucuronoside, the extraction procedure (with zirconium silicate) revealed quite a few compounds from plasma samples. Neither CGA nor CYN was observed in the treated sample, according to the findings reported in the literature for the pharmacokinetics of CQAs in human plasma [30].

Among the metabolites of CQAs, FLA and CA could be confirmed on the basis of retention times of standards and MS. All the other potential metabolites could not be confirmed due to the sensitivity limit of the conventional HPLC system used.
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Fig. 5.5: HPLC/ESI MS (SIM) chromatograms of β-glucuronoside hydrolyzed plasma after zirconium silicate assisted clean-up. A) SIM chromatogram of CA, B) SIM chromatogram of FLA.

The detected compounds (CA and FLA) were below the LLOQ of the instrument so a 6 times concentration of the extracted sample was achieved by evaporating the extracted sample in an Eppendorf concentrator, and reconstituting it in 100 µL of methanol/water (1/1, v/v). The amounts of the FLA and CA were found to be ~10 ng and ~17 ng mL⁻¹, respectively.

5.4. Conclusions

The presented method enhances the efficiency of the organic solvent protein precipitation by a follow-up cleaning using zirconium silicate which was found to be a convenient biological sample preparation technique. Zirconium silicate assisted clean up effectively
removes > 99.6% of the human plasma proteins and significantly removes the matrix effects. The matrix effects were below -2.05% for higher concentration levels of CQAs and their metabolites, whereas lower concentrations witnessed the matrix effects below -5.10%. The method offers a rapid and effective sample preparation in bioanalytical LC of pharmaceutical compounds. The scheme also demonstrated high recoveries for the spiked standards in plasma. This novel method provides a great analytical tool for the rapid depletion of proteins from human plasma for the subsequent isolation of small molecules.

5.5. Acknowledgements

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5.6. References


CHAPTER 6

Summary and future prospects
6. Summary and future prospects

The dissertation reports the SPE methods for the selective and efficient isolation of natural bioactive substances of plant origin. Four novel sorbents are described, namely bismuth citrate, zirconium silicate, PSDZ and aluminum silicate. The selective isolation of phenolic acids from their plant sources was achieved employing bismuth citrate and zirconium silicate. The SPE method was optimized and compared to commercial products. Sorbents with electrostatic interactions (zirconium silicate and bismuth citrate) were found to be more selective and presented better recovery rates than the sorbents with pure hydrophilic or hydrophobic interaction sites. It was demonstrated that zirconium silicate exhibited best selectivity among all the tested sorbents. The SPE method for phenolic acids described in this dissertation is unique due to the ease it can be operated and tuned. The amount of sorbent particles can be adjusted depending on concentration of the samples. Zirconium silicate, in particular, is stable under the whole pH range from 1 to 14, on the contrary to bismuth citrate, which dissolves in basic pH. The developed SPE method also excludes chlorophyll and waxes from complex mixtures to protect the analytical columns. Furthermore, these particles can turn out to be helpful for screening phenolic acids from uninvestigated plants in a very short time. The offered SPE technique may help to find new sources of these important bioactive substances and the search for low abundant novel isomers can be optimized. Moreover, by using the introduced conditions, other metal particles can bring further improvement of the reported SPE method and the present work can be a valuable base for future investigations of medicinal plants.

The synthesized PSDZ sorbent was found suitable for the purification and enrichment of thionins in extracts obtained from mistletoe, wheat and barley. Strong co-ordination
between zirconium and the sulphur atoms of thiol-groups resulted in strong binding of thionins to the developed material. Moreover, mullite was also proved to be excellent material for the isolation of thionins from mistletoe, wheat and barley. In comparison to PSDZ sorbent, aluminum (III) silicate showed reduced interactions towards proteins which resulted in superior washings of unspecific compounds while still retaining cysteine rich thionins. This approach diminishes the complexity of protein extracts enormously and therefore offers a simpler chromatographic separation of individual isoforms. The SPE method is suitable for screening of the novel isoforms of thionins from still investigated samples.

Lastly, the dissertation reports the SPE clean-up method for the isolation of small molecules from human plasma. The presented method enhanced the efficiency of the organic solvent protein precipitation by a follow-up cleaning using zirconium silicate which was found to be a convenient biological sample preparation technique. Zirconium silicate assisted clean up effectively depleted >99.6% of the human plasma proteins and significantly diminished the matrix effects. The matrix effects were below -2.05% for higher concentration levels of CQAs and their metabolites, whereas lower concentrations witnessed the matrix effects below -5.10%. The method offers a rapid and effective sample preparation in bioanalytical LC of pharmaceutical compounds. The scheme also demonstrates high recoveries for the spiked standards in plasma. This novel method provides a great analytical tool for the rapid depletion of proteins from human plasma for the subsequent isolation of small molecules.

The presented SPE strategies provide great analytical platform for the isolation of natural bioactive substances from the complex samples. The techniques can assist to discover the
new natural sources of these pharmacologically active compounds. Moreover, the described clean-up method can be used to study the pharmacokinetics of bioactive substances.
Curriculum Vitae

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2. **Solid-phase extraction method for the isolation of plant thionins from European mistletoe, wheat and barley using zirconium silicate embedded in poly (styrene-co-divinylbenzene) hollow-monoliths**


3. **Solid-phase extraction of plant thionins employing aluminum silicate based extraction columns**


4. **Zirconium silicate assisted removal of residual proteins after organic solvent deproteination of human plasma, enhancing the stability of LC-ESI-MS response**


5. **Comparison of NIR and ATR-IR spectroscopy for the determination of the antioxidant capacity of Primulae flos cum calycibus**
6. **Highly selective enrichment of phosphopeptides using aluminum silicate**


**Poster Presentations**

1. **Solid-phase extraction of galloyl- and caffeoylquinic acids from natural sources**

   * (Galphimia glauca and Arnicae flos) using pure zirconium silicate and bismuth citrate powders as sorbents inside micro spin columns

   AOAC Europe International Workshop, Quality Control of Botanicals, TCM, Herbal Food Supplements and Herbal Medicinal Products, Erlangen, 10-12 October 2011.

2. **Novel SPE Sorbents for Isolation of Phenolic Acids from Natural Sources**

   8th Meeting of Life Science Doctoral Students, Innsbruck, 5 October 2011.
3. **Solid-phase extraction method for the isolation of plant thionins from European mistletoe, wheat and barley using zirconium silicate embedded in poly (styrene-co-divinylbenzene) hollow-monoliths**


4. **Solid-Phase Extraction of Plant Thionins Employing Aluminium Silicate Based Extraction Columns**


5. **Solid-Phase Extraction of Plant Thionins Employing Aluminium Silicate Based Extraction Columns**


Oral Presentation

6. **Novel SPE Sorbents for Isolation of Phenolic Acids from Natural Sources**