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DEVELOPMENT OF ANALYTICAL METHODS FOR SIMULTANEOUS IDENTIFICATION AND DETERMINATION OF PHENOLIC COMPOUNDS

Doctoral dissertation

Maribor, March 2018

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Doctoral dissertation

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„Try not to become a man of success, but rather try to become a man of value.“

Albert Einstein

Abstract

The objective of this doctoral dissertation was to develop different analytical approaches for the extraction, separation, identification and quantitative determination of various phenolic compounds from different plants and their products. This dissertation is divided into the following four major segments, which, to some extent, can stand alone, but when it comes to the research, they are mutually very related:

- Segment 1: Short-term (up to 24 h) and long-term (up to 1 month) stability studies of *trans*-caffeic acid (*trans*-CA) and *trans*-ferulic acid (*trans*-FA) dissolved in two organic solvents (methanol and tetrahydrofuran) and exposed to a range of storage conditions (temperature, organic solvents used, influence of daylight and UV irradiation) were performed for the first time. Gas chromatography with mass spectrometry (GC-MS) was used to study the degradation of the samples and structural identification of the degradation products.

- Segment 2: The research within this segment focused on the optimization of a simple, fast and quantitative extraction method for the isolation of phenolic acids (PAs) from Slovenian red wine samples. Different extraction techniques were tested, and solid phase extraction (SPE) using HLB cartridges was selected as the optimal technique. For the identification and quantification of extracted analytes, the GC-MS method was optimized and validated. Different statistical and chemometrical tools were applied, and the wines were classified according to the Slovenian wine-growing regions and vine varieties.

- Segment 3: The main goal within this research segment was the development of an ultrasound-assisted extraction (UAE) method for the isolation of different polyphenol classes from coriander fruits. Additionally, for the isolation of total PAs (free and bound), two analytical steps were applied: UAE alkaline hydrolysis and clean-up using SPE HLB cartridges. The response surface methodology (RSM) combined with a Box-Behnken experimental design (BBD) were used for the optimization of the alkaline hydrolysis and for increasing the extraction yields of the PAs. In this way, most influencing factors (temperature, sonication time and NaOH concentration) were studied as independent variables. Extracted PAs were determined using the previously optimized GC-MS method.

- Segment 4: The main goal of this segment of the dissertation was to show the application of deep eutectic solvents (DESs) as a ‘green’ alternative to the conventional organic solvents for the isolation of phenolic compounds from plants such as *Aronia melanocarpa* (dried chokeberry) and *Olea europaea* (olive leaves). Different extraction

techniques and instrumental methods were applied for the determination of phenolic profiles. Phenols from chokeberries were obtained through UAE. Furthermore, for the simultaneous identification and quantitative determination of 21 different phenolic compounds from *Aronia melanocarpa*, the HPLC-UV method was optimized and validated. On the other hand, microwave-assisted extraction (MAE) was used to improve the extraction yields of phenolic compounds from olive leave samples, which were subsequently determined by using validated HPLC-DAD-ESI-TOF-MS method.

Keywords: phenolic compounds, phenolic acids, extraction, GC-MS, HPLC, method optimization, deep eutectic solvents, plant material.

UDK: **664.1.033:665.662.3(043.3)**

Povzetek

Cilj doktorske disertacije je bil razvoj različnih analitskih pristopov za ekstrakcijo, ločevanje, identifikacijo in kvantitativno določanje različnih fenolnih spojin iz različnih rastlin in njihovih produktov. Raziskovalna vsebina disertacije je razdeljena na štiri glavne segmente, ki so v določeni meri samostojni, vendar so raziskave medsebojno tesno povezane.

- Segment 1: ta segment je zajemal kratkoročne (do 24 h) in dolgoročne (do 1 meseca) študije stabilnosti *trans*-kavne kisline in *trans*-ferulne kisline, ki so bile izvedene pod različnimi pogoji hranjenja: temperatura, uporabljena organska topila, vpliv dnevne svetlobe in UV-sevanja. Prvič je bila uporabljena plinska kromatografija v povezavi z masno spektrometrijo (GC-MS) za študij degradacije vzorcev in strukturne identifikacije razgradnih produktov.

- Segment 2: raziskave v tem segmentu je bil poudarek na optimizaciji enostavne, hitre in kvantitativne metode za ekstrakcijo in izolacijo fenolnih kislin iz vzorcev slovenskih rdečih vin. Testirane so bile različne tehnike ekstrakcije, kot optimalna pa je bila izbrana ekstrakcija na trdno fazo (SPE) ob uporabi HLB kolonic. Za identifikacijo in kvantifikacijo ekstrahiranih analitov je bila optimizirana in validira GC-MS metoda. Uporabili smo različna statistična in kemometrična orodja, da smo lahko vina razvrstili glede na slovenska vinorodna območja in sorte vinske trte.

- Segment 3: glavni cilj tega raziskovalnega segmenta je bil razvoj ultrazvočne metode (UAE) za izolacijo različnih polifenolov iz koriandra. Poleg tega sta bila za izolacijo skupnih fenolnih kislin (prostih in vezanih) uporabljena dva analitska pristopa: alkalna hidroliza z UAE in čiščenje ob uporabi SPE in HLB kolonic. Optimizacijo alkalne hidrolize in povečanje izkoristkov ekstrakcije fenolnih kislin iz koriandra smo dosegli z metodologijo odzivne površine (RSM) z zasnovo Box-Behnken-ovega (BBD) eksperimentalnega načrta. Na ta način so bili kot neodvisne spremenljivke preučevani najbolj vplivni parametri (temperatura, ultrazvok in koncentracija NaOH). Ekstrahirane fenolne kisline so bile določene z uporabo predhodno optimizirane GC-MS metode.

- Segment 4: v tem delu disertacije je predstavljena uporaba globokih eutektičnih topil (DES), kot "zelena" alternativa običajnim organskim topilom, za izolacijo fenolnih spojin iz rastlin, kot so *Aronia melanocarpa* (posušena aronija) in *Olea Europaea* (oljčni listi). Za določitev fenolnih profilov smo uporabili različne ekstrakcijske tehnike in instrumentalne metode. Fenoli iz posušene aronije so bili ekstrahirani s pomočjo UAE. Za sočasno

identifikacijo in kvantitativno določitev enaindvajsetih različnih fenolnih spojin iz aronije je bila optimizirana in validirana HPLC-UV metoda. Za ekstrakcijo fenolnih spojin iz oljčnih listov pa je bila uporabljena ekstrakcija s pomočjo mikrovalov (MAE), spojine pa so določene z uporabo validirane HPLC-DAD-ESI-TOF-MS metode.

Ključne besede: fenolne spojin, fenolne kislin, ekstrakcija, GC-MS, HPLC, optimizacija metod, globok eutektična topil, rastlinski material.

UDK: **664.1.033:665.662.3(043.3)**

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Symbols and Abbreviations

Symbols

T	temperature (°C)
c	molar concentration (M, mol L^{-1})
m	mass (g)
V	volume (mL, µL)
MW	molecular weight (g mol^{-1})
L	pathlength (cm)
A	absorbance (-)
t	time (min)
R^2	coefficient of determination (-)
X_i	independent variable (°C, min, mol L^{-1} or %)
Y_i	response variable (different units)
pKa	logarithmic acid dissociation constant (-)
m/z	mass to charge ratio (-)
t_R	retention time (min)
v/v	volume to volume ratio (mL mL^{-1})
k	number of tested variables (-)
w/w	mass to mass ratio (g g^{-1})
ppm	parts per million

Greek symbols

β	regression coefficient (-)
γ	mass concentration (mg L^{-1})
$\mathcal{E}$	molar extinction coefficient (-)
λ	wavelength (nm)
Σ	sum (mg g^{-1} DW)

Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ACN	acetonitrile
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
BBD	Box-Behnken design
BD	between days
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene

BSA	N,O-bis(trimethylsilyl)acetamide
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CA	caffeic acid
CAT	catechin
CB	carbon block
CCBut	choline chloride-1,4-butanediol
CCEtg	choline chloride-ethylene glicole
CCFruc	choline chloride-fructose
CCGluc	choline chloride-glucose
CCLac	choline chloride-lactic acid (molar ratio 1:2)
CCLac1	choline chloride-lactic acid (molar ratio 1:1)
CCMalt	choline chloride-maltose
CCOx	choline chloride-oxalic acid
CCProp	choline chloride-1,2-propanediol
CCTart	choline chloride-tartaric acid
CCU	choline chloride-urea
CCXy	choline chloride-xylitol
CE	conventional extraction
CE-UV	electrophoresis ultraviolet detection
CLU	cluster analysis
CoA	coenzym A
Cya-3-Glu	cyanidin-3-glucoside
DAD	diode array detector
DCBA	dichlorobenzoic acid
DCM	dichlormethane
DESs	deep eutectic solvents
DF	dilution factor
DLLME	dispersive liquid-liquid micro-extraction
DMDCS	dimethyldichlorosilane
DMSO	dimethyl sulfoxide
DoE	Design of Experiments
DPPH	1,1-diphenyl-2-pircyl-hydrazyl
DSAE	dynamic sonication-assisted extraction
DW	dry weight
EASFE	enzyme assisted supercritical fluid extraction
ECD	electrochemical detector

EDTA	ethylenediamine-tetraacetic acid
ESI	electrospray ionization
EtOH	ethanol
EU	European Union
FA	ferulic acid
FCR	Folin-Ciocalteu reagent
Fe(III)TPTZ	ferric tripyridyltriazine
FF	fresh fruit
FIA	flow injection analysis
FIR	far-infrared radiation
FLD	fluorescence detector
FRAP	ferric reducing antioxidant power
GAE	gallic acid
GC	gas chromatography
GMF	generate molecular formula
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
HESI	heated electrospray ionization
HLB	hydrophilic lipophilic balance
HPICE	high-performance ion chromatography exclusion
HPLC	high-performance liquid chromatography
ICP-OES	inductively coupled plasma emission spectroscopy
ILs	ionic liquids
IRMS	isotope ratio mass spectrometry
ISTD	internal standard
LAB	lactic acid bacteria
LC	liquid chromatography
LDA	linear discriminant analysis
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
ME	maceration extraction

MeOH	methanol
MLF	malolactic fermentation
MS	mass spectrometry
MS/MS	mass spectrometry/mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MTBSTFA	N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide
MUAE	microwave-ultrasound assisted extraction
NADESs	natural deep eutectic solvents
ND	not detected
NI	not identified
NMR	nuclear magnetic resonance
NQ	not quantified
ORAC	oxygen radical absorbance capacity
OVAT	one variable at a time
PAHs	polycyclic aromatic hydrocarbons
PAs	phenolic acids
PAL	phenylalanine ammonia lyase
PCA	principal component analysis
PCs	phenolic compounds
PDA	photodiode detector
PEF	pulsed electric field
PFE	pressurized fluid extraction
PLE	pressurized liquid extraction
PTFE	polytetrafluoroethylene
RP-LC	reverse-phase liquid chromatography
RSD	relative standard deviation
RSM	response surface methodology
RT	room temperature
RUT	rutin
SC-CO_2	subcritical CO_2
SF	stationary phase
SIM	selected ion monitoring
SLE	solid-liquid extraction
S/N	signal to noise ratio

SNIF	site-specific natural isotope fractionation
S-N-K	Student-Newman-Keuls test
SPE	solid phase extraction
SPEL	screen-printed electrode
SWE	subcritical water extraction
TAC	total anthocyanin content
TAL	tyrosine ammonia lyase
TBHQ	*tert*-butylhydroquinone
TBDMSCl	tert-butyldimethylsilyl chloride
TFC	total flavonoid content
THF	tetrahydrofuran
TLC	thin layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
TOF	time of flight
TPC	total phenolic content
TRAP	total peroxyl radical trapping potential
UAE	ultrasound-assisted extraction
UHPLC	ultra high-performance liquid chromatography
UPLC	ultra performance liquid chromatography
USAEME	ultrasound-assisted emulsification micro-extraction
USE	United States
UV-ViS	ultraviolet-visible
QWPSR	quality wines produced in specified regions

1 Introduction

Generally, phenolic compounds (PCs) are the class of chemical compounds consisting of one or more hydroxyl groups (-OH) directly bonded to an aromatic hydrocarbon part [1]. PCs, or polyphenols, represent a large group of chemicals, which can additionally be divided into several subgroups according to their structural similarities. The term PCs has been used since 1894, but the interest for this group of natural chemicals has increased significantly when the public realised their health benefits. Today, a wide range of biological activities and pharmacological properties of PCs, such as antioxidant, antibacterial, anti-inflammatory and antitumor effects, have been confirmed [2]. Consequently, the PCs from natural sources can be used as replacements for synthetic antioxidant compounds. All these properties put PCs as a focus of interest for different industries, including the medicine, pharmacy, cosmetic and food-processing industries.

Generally, raw fruits, vegetables, seeds, beverages and high-quality seasonings are among the richest sources of PCs found in nature. *The European Journal of Clinical Nutrition* published a list of the 100 richest dietary sources of PCs based on milligrams (mg) per 100 grams (g) or 100 mg of food [3]. Nevertheless, there are wide variations between the total phenolic content (TPC) determined for different fruits or vegetables and even between the same fruits or vegetables. First, this fact can be explained by the structural differences of this group of secondary metabolites, as well as the different methods involved in their extraction and instrumental analysis. Furthermore, the content of PCs from different natural sources highly depends on a number of intrinsic (genus, species and cultivars) and extrinsic (agronomic, environmental, handling and storage) factors.

Generally, the free forms of PCs in nature are very rare. Most of them occur in their glycosylated forms, although modifications such as esterification or polymerisation are also commonly found [4].

Because of their recognized antioxidant properties, PCs have been the focus of interest for many researchers over the past few decades, both for academic and industrial purposes. Many studies have been done to optimize their isolation and separation from natural sources. However, due to their complex structures, there is no universal method suitable for the extraction of all plant PCs [5]. Liquid-liquid extraction (LLE) and solid-liquid extraction (SLE) with different organic solvents and solvent mixtures are still the most useful extraction techniques for the isolation of different PCs. Additionally, to increase the extraction yield of

bioactive compounds and overcome the main disadvantages of standard processes, different modifications have been done. An increase in mass transfer between solid plant materials and solvents could be agitated by the use of ultrasonic waves, microwave irradiation or with pulsed electric fields [6]. Further, solid-phase extraction (SPE) is a powerful method for sample preparation and is used by most chromatographers today. Some advantages of SPE compared to the classical LLE or SLE methods are the small use of harmful organic solvents, the simplicity of the procedure, the selectivity, the increase in extraction recoveries, the shorter extraction time and so forth.

New trends in chemistry and environmental protection legislations have set requirements for the use of so-called 'green' solvents. For example, the European Union's (EU) environmental policy and legislation for the 2010–2050 period required a decrease in the use of petrochemical solvents and volatile organic compounds because most of these solvents are flammable, volatile and toxic [7]. Following these requirements, a new kind of non-conventional, alternative solvents were developed. These newly formed systems consisted of hydrogen-bond donor (HBD) and hydrogen-bond acceptor (HBA) compounds and were termed 'deep eutectic solvents-DESs'. Several recent studies have reported the successful application of DESs in the extraction of PCs, indicating its great potential in plant-extract production for direct use in human consumption [8–11]. Nevertheless, these studies are still in the initial development stage, more research must be performed.

Each of the above-mentioned extraction techniques has some advantages and disadvantages. Consequently, to select the best one, all influencing factors (structure of target analyte, plant matrix, operation cost, applicability in large-scale industrial processes, etc.) should be considered.

The identification and quantification of PCs are usually performed using two extremely powerful instrumental techniques: liquid chromatography and high-performance liquid chromatography (LC/HPLC) and gas chromatography (GC). The ability to connect LC/HPLC instruments to various detectors and easier sample preparation makes this technique the preferred one for the analytics of PCs. However, it is hard to negate some of the advantages of GC, primarily from the aspect of compound separation, simultaneous analysis and detection limits.

The detector used in the analysis of PCs is of great significance. Mass spectrometry (MS) or MS/MS detectors are some of the most powerful detectors when it comes to this group of

compounds. With the appropriate databases, using MS or MS/MS offers the identification of a large number of compounds with accuracy of almost 100%.

Except for laboratory development and the optimization of different analytical methods for the identification and quantification of individual PCs, requirements also moves more toward statistical-mathematical modelling. Almost 20 years ago, it was emphasised that univariate data analysis would no longer be sufficient for fully exploring the complexity of the vast amounts of available information [12]. Since then, multivariate analysis methods have been used as efficient tools for the extraction of meaningful experimental information. Namely, the combination of laboratory results with chemometric approaches can lead to very fast and precise retrieval of information related to the natural materials. Also, to reduce the cost of analytical process and analysis time, the design of experiments (DoE) was created and has become one of the most useful tools in the scientific and engineering disciplines [13]. In most cases, application of DoE in process optimization results in the increased process yields, reduced process variability and reduced overall costs [13].

1.1 Aims of PhD thesis

Widespread of PCs in the nature, their usability in different industries and complexity of their structures daily set requirements for the development and optimization of analytical methods for its determination from different plant matrixes. Consequently, the general aim of this PhD thesis was the application of acquired knowledge and the development of new extraction, separation, detection and statistical-mathematical methods for the analysis of PCs. The specific research goals for each individual research segment are described in the following paragraphs.

In the first segment of this PhD thesis, systematic stability tests on pure *trans*-CA and *trans*-FA and the determination of their degradation products were performed. Namely, CA and FA, as a part of hydroxycinnamic acids class, have double-bound structures. Therefore, they can exist in both geometric molecular arrangements (geometric isomers): *trans*- and *cis*-forms, depending on the different storage and handling conditions. Although, most studies on stability have been performed on HPLC systems, in this research, the GC-MS system was used.

As a rich source of PCs, wine could be valorised for further use in the production of functional food ingredients or supplements with high nutritional values. Generally, PCs from

wine can be classified into flavonoids and non-flavonoids. The main non-flavonoids in red wine are PAs, but there are other phenolic derivatives, including stilbenes. Consequently, the second segment of this PhD thesis dealt with the development of simple and quantitative extraction and an instrumental method for the isolation and the quantitative determination of selected PAs from Slovenian red wines, one of the most important products of the Slovenian economy. For the extraction of target compounds from wine samples, SPE using hydrophilic modified styrene (HLB) cartridges was used. For the quantitative determination of the target compounds, the GC-MS method was optimized and validated. The contents of bound PAs in the samples were determined after basic hydrolysis using NaOH in the presence of L-ascorbic acid and ethylenediaminetetraacetic acid (EDTA) as stabilizers. Additionally, the results obtained were used for the construction of statistical-mathematical models for the classification of Slovenian red wines according to the vine varieties and the regions they came from.

In the third research segment of this PhD thesis, the applicability of previously developed analytical methods (extraction and chromatographic) for isolation, separation and quantification of selected PAs from coriander fruits were tested. First, the TPC and total flavonoid content (TFC) in coriander methanolic extracts were determined using standard spectrophotometric methods. In the next step, alkaline hydrolysis and UAE method were used for the isolation of total PAs from coriander fruits. The effects of sonication time, temperature and concentration of NaOH were studied, and the optimal combination of extraction parameters were established using a Box-Behnken design combined with a response surface methodology. For the quantification of the target PAs, the previously developed and validated GC-MS method was used. The extraction recoveries of active compounds, according to the hydrolysis conditions, were also determined.

The main goal of the last part of this doctoral dissertation was the development of DESs-based extraction methods and the determination of their potential usefulness and effectiveness in the extraction of PCs. First, the UAE method involving DESs for the extraction of selected PCs from dried chokeberry was optimized. The obtained results were compared with the results of the most commonly used UAE methanolic extraction method. Additionally, the HPLC-UV method for the identification and quantification of 21 different PCs was developed and validated in terms of linearity, precision as repeatability, limit of detection (LOD) and limit of quantitation (LOQ). Similar research on a DESs-based extraction method for the

isolation of PCs was carried out on olive leave samples. In the present study, considering that DESs can efficiently absorb microwave energy, a microwave extractor was used to carry out the extraction performance. Based on the preliminary results of TPC content and concentration of individual PCs in the obtained extracts, the most effective DES was selected for further work. Additionally, an experimental design was implemented to optimize the main variables (temperature, irradiation time and water content in DES) involved in the extraction of bioactive compounds from olive leaves. The extracts were characterised by HPLC-DAD-ESI-TOF-MS.

2 Theoretical part

2.1 Chemical properties of phenolic compounds

The primary metabolites of a plant (carbohydrates, proteins, lignin and lipids) are the compounds directly involved in its growth and development [14]. On the other hand, secondary metabolites are not of importance for plant growth and development, but they are required for the plant to survive in its environment. Secondary plant metabolites eliciting pharmacological or toxicological effects in humans and animals are the plant's bioactive compounds [15]. Some of the most important classes of plant bioactive compounds are the following: terpenoids, alkaloids, sulphur-containing compounds, nitrogen-containing compounds and PCs [16].

PCs (or polyphenols) encompass approximately 8000 different naturally occurring bioactive compounds and are divided into subgroups [17]. Their classification can be made according to different criteria, but the most applicable classification is based on the number of carbon atoms (**Table 2-1**), a definition devised by Jeffrey Harborne and Simmonds in 1964 and published in 1980 [18].

Table 2-1. Classification of PCs according the number of C-atoms in their structures.

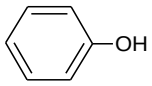
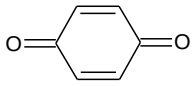
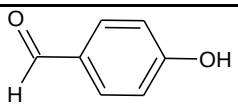
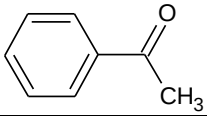
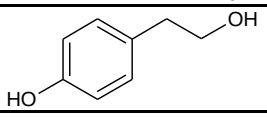
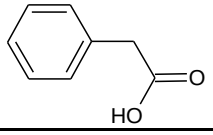
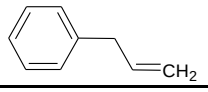
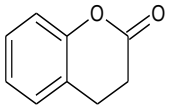
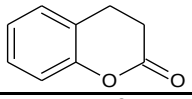
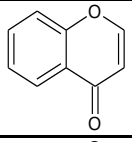
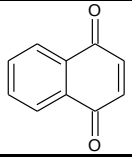
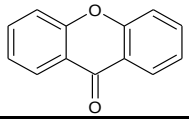
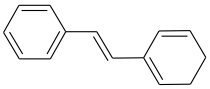
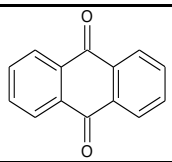
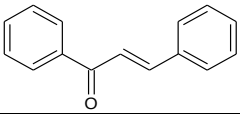
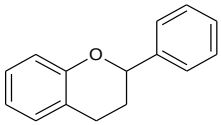
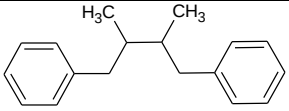
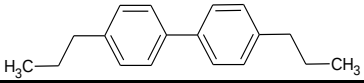
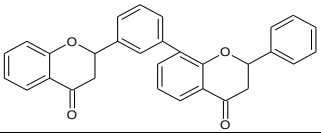
Basic skeleton	Structure	Class
C_6		Simple phenols
		Benzoquinones
C_6-C_1	See **Figure 2-2**	Hydroxybenzoic acids
		Phenolic aldehydes
C_6-C_2		Acetophenones
		Tyrosine derivatives
		Phenylacetic acids

Table 2-1. Continued.

C_6-C_3	See **Figure 2-2**	Hydroxycinnamic acids
		Phenylpropenes
		Coumarins
		Isocoumarins
		Chromones
C_6-C_4		Naphthoquinones
C_6-C_1-C_6		Xanthonoids
C_6-C_2-C_6		Stilbenoids
		Anthraquinones
C_6-C_3-C_6		Chalconoids
		Flavonoids
$(C_6$-$C_3)_2$		Lignans
		Neolignans
$(C_6$-C_3-$C_6)_2$		Biflavonoids
$(C_6$-$C_3)_n$, $(C_6)_n$, $(C_6$-C_3-$C_6)_n$	n>12	Lignins, Catechol melanins, Condensed tannins, Polyphenolic proteins

The starting material for the synthesis of most PCs found in nature is the shikimate. **Figure 2-1** represents the biosynthesis of PCs following the pentose phosphate, shikimate and phenylpropanoid pathways.

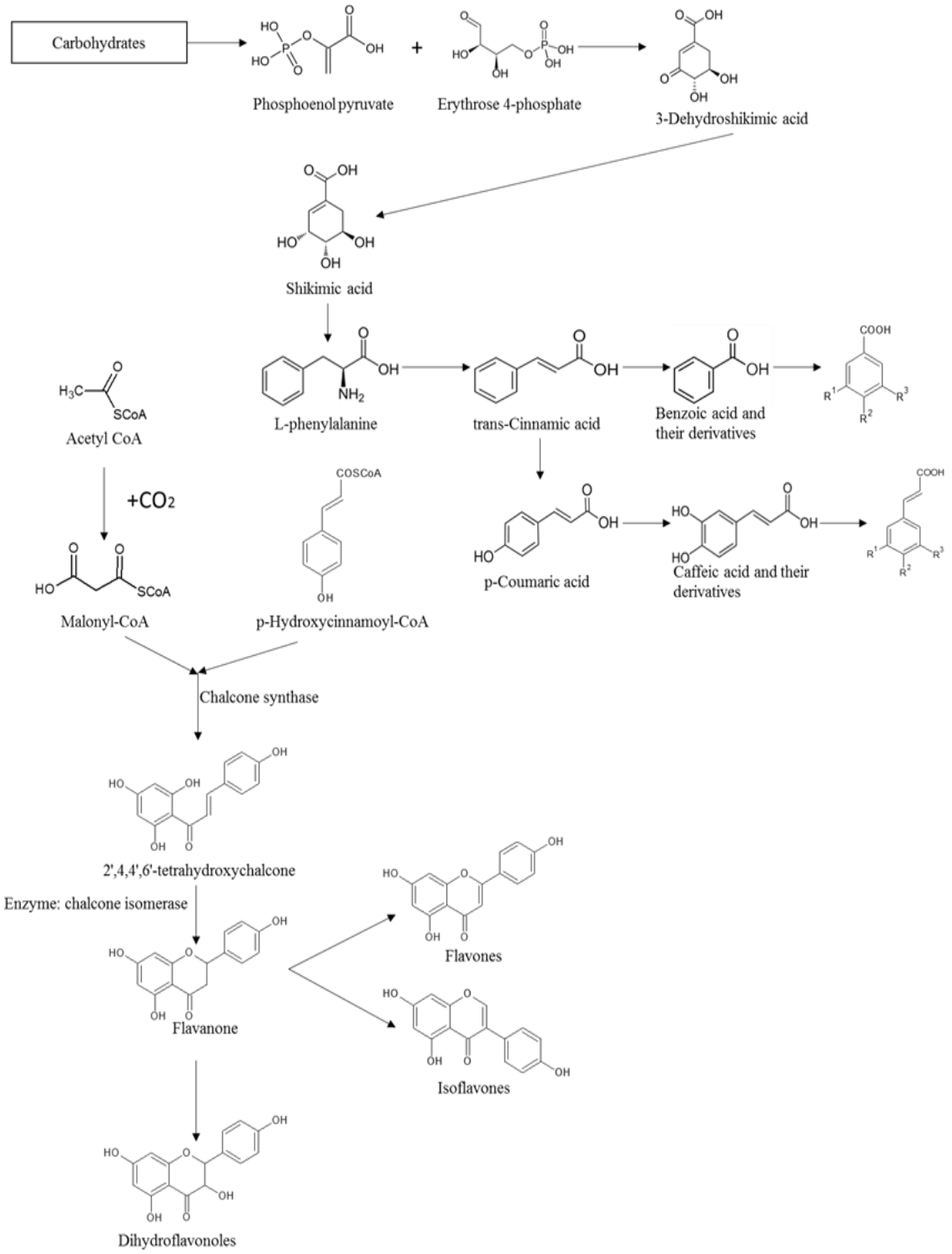


Figure 2-1. Biosynthetic pathways of PCs [19].

2.1.1 Phenolic acids

PAs are the simplest compounds from the polyphenol class; their general structure is represented in **Figure 2-2**.

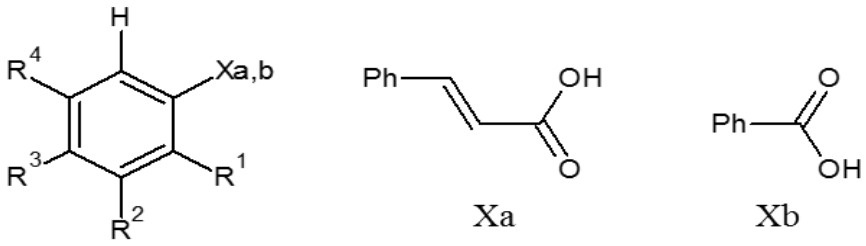


Figure 2-2. General structure of PAs (Ph-phenyl ring).

PAs with a functional group Xa are classified as derivatives of hydroxycinnamic acid, and PAs that contain Xb as the functional group are classified as derivatives of hydroxybenzoic acid. The most commonly found, natural structures of PAs are summarised in **Table 2-2**.

Table 2-2. The most common PAs found in nature.

R^1	R^2	R^3	R^4	Common name
Derivatives of hydroxycinnamic acid (X=Xa)				
H	H	H	H	Cinnamic acid
-OH	H	H	H	o-Coumaric acid
H	H	-OH	H	p-Coumaric acid
H	-OH	H	H	m-Coumaric acid
H	$-OCH_3$	-OH	H	Ferullic acid
H	$-OCH_3$	-OH	$-OCH_3$	Sinapic acid
H	-OH	-OH	H	Caffeic acid
Derivatives of xydroxybenzoic acid (X=Xb)				
H	H	H	H	Benzoic acid
-OH	H	H	H	Salicylic acid
H	H	-OH	H	p-Hydroxybenzoic acid
H	$-OCH_3$	-OH	H	Vanillic acid
H	$-OCH_3$	-OH	$-OCH_3$	Syringic acid
H	-OH	-OH	H	Protocatechuic acid
-OH	H	H	-OH	Gentisic acid
-OH	-OH	-OH	-OH	Gallic acid
H	$-OCH_3$	$-OCH_3$	H	Veratric acid

Chlorogenic acid is also a natural chemical compound, an ester of caffeic acid and (−)-quinic acid.

PAs may occur in plants in their free forms, but usually, they are esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyfatty acids and sterols [20]. PAs and their derivatives are widely distributed in plants, and some plants with the highest content of PAs are listed in **Table 2-3**.

Table 2-3. The list of plants with the highest content of PAs [21].

Phenolic acid	Plant	The Latin name	Content (*ppm*)
Cinnamic acid	Sweetgum	*Liquidambar styraciflua*	230 000
	Oriental Styrax	*Liquidambar orientalis*	150 000
o-Coumaric acid	Peanut-seed	*Arachis hypogaea*	10
	Wheat-seed	*Triticum aestivum*	10
p-Coumaric acid	Indian bean-leaf	*Catalpa bignonioides*	0-15 000
	Sunflower-seed	*Helianthus annuus*	1 100
Sinapic acid	Sunflower-seed	*Helianthus annuus L*	5 700
	Brussels-Sprouts-leaf	*Brassica oleracea var. gemmifera var. gemmifera*	107
Caffeic acid	Jalap-tuber	*Ipomoea purga*	40 000
	Pear-fruit	*Pyrus communis*	19 700
Benzoic acid	White Peony-root	*Paeonia lactiflora*	11 000
	Cowberry-fruit	*Vaccinium vitis-idaea var. minus*	1 360
Salicylic acid	Commom Licorice	*Glycyrrhiza glabra*	567
	Peanut-seed	*Arachis hypogaea*	18
p-Hydroxybenzoic acid	Juniper-plant	*Juniperus communis*	2 500
	Coriander-fruit	*Coriandrum sativum L.*	960
Vanillic acid	Picrorhiza	*Picrorhiza kurrooa*	1 000
	Coriander-fruit	*Coriandrum sativum L.*	960
Syringic acid	Peanut-seed	*Arachis hypogaea L.*	51
	Chia-seed	*Salvia sp.*	0-25
Protocatechuic acid	Onion-bulb	*Allium cepa L.*	17 540
	Coriander-fruit	*Coriandrum sativum L.*	760
Gentisic acid	Tarragon-leaf	*Artemisia dracunculus L.*	150
	Horseradish-root	*Armoracia rusticana*	15
Gallic acid	Mango-flower	*Mangifera indica L.*	90 000
	Witch Hazel-leaf	*Hamamelis virginiana L.*	54 400

The most dominant PA found in nature is CA. It is found in almost all plants because it is a key intermediate in the biosynthesis of lignin, one of the principal components of plant biomass and its residues. Many studies have shown that CA can be linked to strong antioxidant properties [22,23].

PAs are synthesised following the shikimate pathway from l-phenylalanine or l-tyrosine, as shown in **Figure 2-3**. The enzymes involved in the reactions are listed in **Table 2-4**.

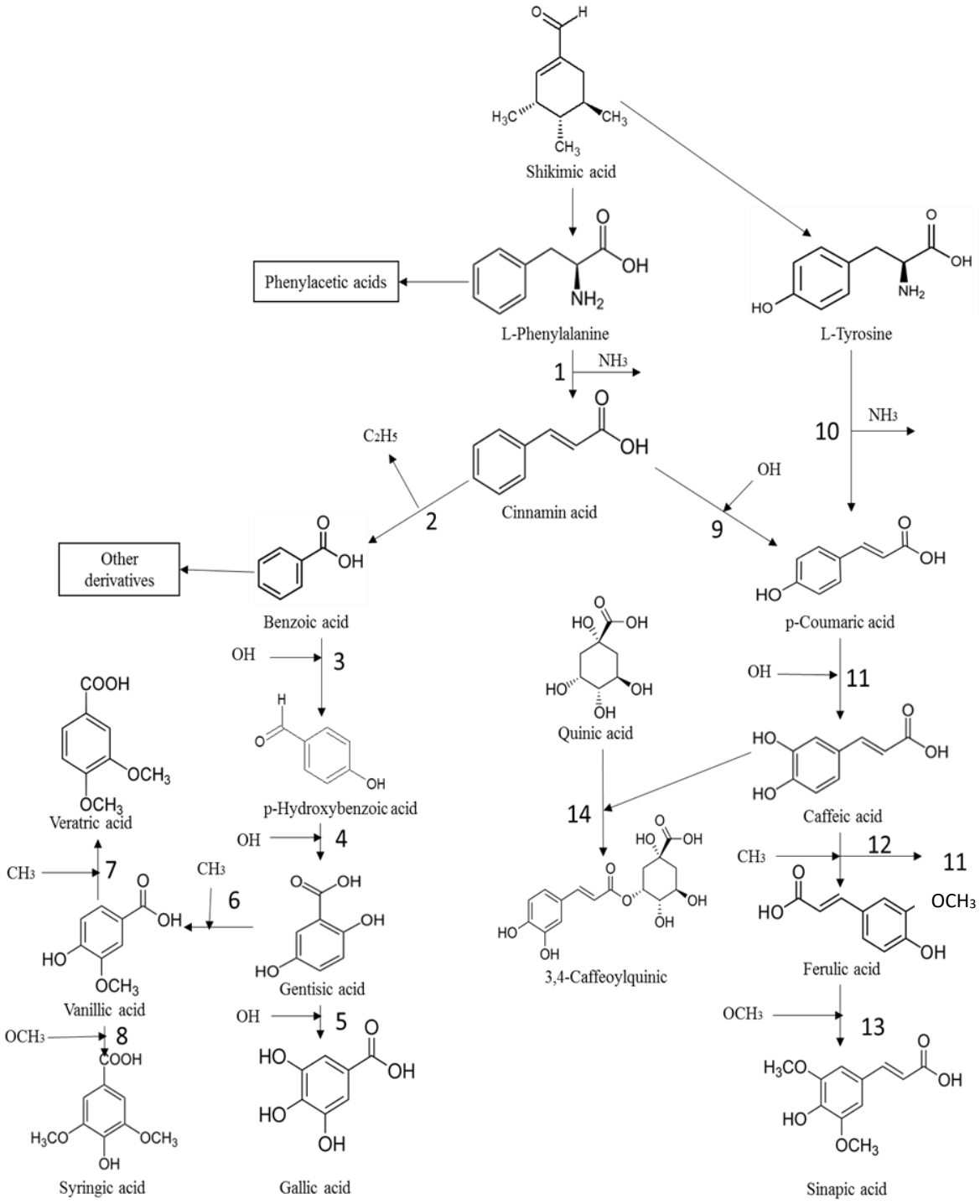


Figure 2-3. Biosynthetic pathway of PAs [24].

Table 2-4. The list of enzymes involved in the biosynthetic pathway of PAs.

Reaction	Enzyme
1	Phenylalanine ammonia lyase (PAL)
2	Oxidaze (presumed β-oxidation)
3	Benzoic acid 4-hydroxylase
4	p-Hydroxybenzoic acid 3-hydroxylase
5	Protocatechuic acid 5-hydroxylase
6	Protocatechuic acid 3-O-methyltransferase
7	Vanillic acid 4-O-methyltransferase
8	Vanillic acid 5 hydroxylase and vanillic acid 5-O-methyltransferase
9	Cinnamic acid 4-hydroxylase
10	Tyrosine ammonia lyase (TAL)
11	p-Coumaric acid 3-hydroxylase
12	Caffeic acid 3-O-methyltransferase
13	Ferulic acid 5-hydroxylase and caffeic/5-hydroxyferulic acid O-mehyltransferase
14	4-Caffeate coenzym A (CoA) ligase and quinate O-hydroxycinnamoyltransferase

2.1.2 **Flavonoids**

Flavonoids are the most common and widely distributed group of plant PCs. Their basic structure is a skeleton of two benzene rings (ring A and B) linked by a three-carbon chain that forms a closed pyran ring (**Figure 2-4**) [25]. Additionally, flavonoids are divided into several subclasses. The structures and the sources with the highest content of different flavonoid subclasses found in nature are represented in **Table 2-5**.

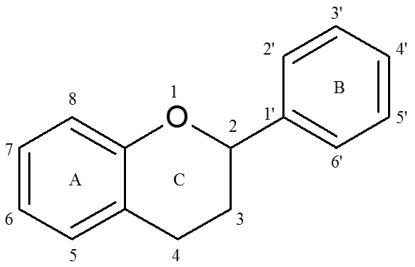
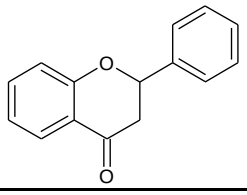
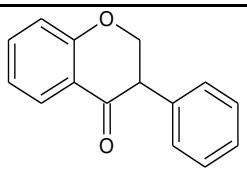
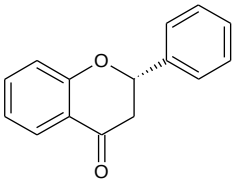
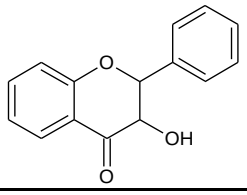
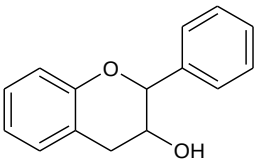
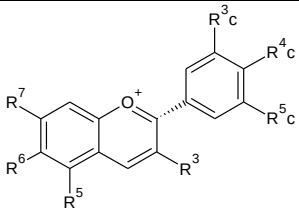


Figure 2-4. Basic flavonoid structure.

Table 2-5. Classification of naturally occurred flavonoids [26].

Flavonoid subclass	Structure	Examples	Sources	Ref.
Flavone		Apigenin Luteolin Chrysin Diosmin Rutin	Chamomile teas Parsley leaves	[27]
Isoflavone		Genistein Daidzin	Soybean	[28]
Flavanone		Naringin Naringenin Taxifolin Hesperidin	Citrus fruits	[29]
Flavonol		Kaempferol Quercetin Myricetin Tamarixetin	Onion Red wine Berries	[30]
Flavanol		(+)-Catechin (−)-Epicatechin Epigallocatechin	Grape Apple Pear Green tea	[31]
Anthocyanidin		Apigenidin Cyanidin	Berries Plums Red onion Radish	[32]

Chalcones and dihydrochalcones are flavonoids with open structures (**Figures 2-5a** and **2-5b**). They are classified as flavonoids due to having similar synthetic pathways.

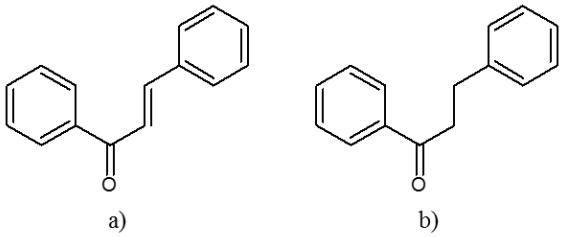


Figure 2-5. Structure of: a) chalcone and b) dihydrochalcones.

2.2 Natural sources of phenolic compounds

The content of PCs varies significantly from plant to plant. *The European Journal of Clinical Nutrition* published a list of the 100 richest dietary sources of PCs based on milligrams per 100 g or 100 mg of plant material [3]. One the other hand, the United States Department of Agriculture recently published its Database for the Flavonoid Content of Selected Foods [33]. This database includes 506 plants with their determined flavonoid subclasses. Likewise, the distribution of PCs in a different part of the same plant (root, leaves, flower, seeds, etc.) is not uniform. Soluble flavonoids are concentrated in the cell vacuoles, whereas the insoluble flavonoids are found in cell walls [34]. Generally, numerous factors, such as degree of ripeness at the time of harvest, environmental factors (soil type, sun exposure, rainfall, etc.) and processing and storage conditions, affect the content of PCs in plants. **Table 2-6** provides a brief review of recent studies related to the content of PCs (expressed as TPC) in different plants.

Table 2-6. A brief review of PCs content identified in different plant materials.

Natural source	PCs sub-class	Amounts (gallic acid aquvalent)	Ref.
		Fruits	
Apple	Flavonols Flavanols Phenolic acids Chalcones	from 1.21 up to 6.46 mg g^{-1} DW[a] (depending on the apple variety)	[35]
Blueberry	Anthocyanins Flavonols Flavanols Phenolic acids	between 103.40 and 131.30 mg g^{-1} DW (depending on the extraction procedure applied)	[36]
Grape	Anthocyanins Condesed tannins	between 9.46 and 12.87 mg g^{-1} DW (depending on the harvesting date and extraction procedure applied)	[37]
Citrus fruits	Phenolic acids	from 0.22 up to 0.53 mg g^{-1} FF[b] (depending on the citrus fruit type)	[38]
		Vegetables	
Artichoke	Phenolic acids Flavones	between 200 and 300 mg g^{-1} DW (depending on extraction solvent used)	[39]
Broccoli	Phenolic acids Flavanols	4.94±0.06 mg g^{-1} DW	[40]
Red cabbage	Anthocyanins Flavonols Phenolic acids	28.54±2.73 mg g^{-1} of raw material	[41]

Table 2-6. Continued

Tomatoe	Flavonols Phenolic acids Flavanols	between 489.30 and 997.45 mg 100 g^{-1} DW (depending on the cultivars)	[42]
Grains			
Rice	Phenolic acids Flavones Anthocyanins Proanthocyanins	from 7.57 up to 878.07 mg 100 g^{-1} DW (depending on the rice type and extraction solvent used)	[43]
Wheat	Phenolic acids Flavonoids Coumarins Stilbens Proanthocyanins	between 1.05 and 4.66 mg g^{-1} DW (depending on the cultivars and extraction procedure applied)	[44,45]
Corn	Flavonoids Anthocyanins Proanthocyanins	between 2.09 and 10.16 mg g^{-1} DW (depending on the cultivares)	[46]
Buckwheat	Flavonoids Phenolic acids	4.46 ± 0.51 mg g^{-1} DW	[47]
Aromatic plants			
Oregano	Phenolic acids Flavones Flavonones	between 79.50 and 147.30 mg g^{-1} DW (depending on the cultivares)	[48]
Basil	Rosmarinic acid Phenolic acids Flavonols	from 12.89 up to 26.04 mg 100 g^{-1} DW (depending on cultivares)	[49]
Sage	Phenolic acids Rosmarinic acids Flavones Flavanones	between 4.25 and 5.95 mg g^{-1} DW (depending on the extraction solvent used)	[50]
Thyme	Phenolic acids Rosmarinic acids Flavones Flavanones	between 4.75 and 8.10 mg g^{-1} DW (depending on the extraction solvent used)	[50]

[a]DW- dry weight
[b]FF- fresh fruits

2.3 Antioxidant activity of phenolic compounds

Oxidative stress caused by free radicals leads to many human diseases, such as atherosclerosis, cancer, neurodegenerative diseases, diabetes, ageing and more [51]. Antioxidants are the molecules that repair damage caused by free radicals and that help protect healthy cells [51,52]. The use of the synthetic antioxidants such as BHT-butylated

hydroxytoluene, BHA-butylated hydroxyanisole and TBHQ-*tert*-butylhydroquinone has been related to health risks, resulting in strict regulation of their use in foods [53]. Consequently, research on plants as potential sources of natural antioxidants that can be used to enhance the properties of foods, for both nutritional purposes and preservation, is currently of major interest. Some studies have confirmed the antioxidant properties of PCs. Namely, the hydroxyl group from PC molecules can accept one electron and form relatively stable phenoxyl radicals, consequently disrupting chain oxidation reactions in cellular components [54].

The antioxidant potential (activity) of plant extracts that contain PCs can be measured *in vitro* by different methods. Oxygen radical absorbency capacity (ORAC) [55] and Total Peroxyl Radical Trapping (TRAP) [56] are based on the inhibition of peroxy radical induced by oxidation, which involves hydrogen donation from antioxidant molecules.

Likewise, the antioxidant activity of different samples can be determined using a 1,1-diphenyl-2-picryl-hydrazyl (DPPH) reagent, which is a stable free radical with an unpaired valence electron at one atom of the nitrogen bridge [57]. Scavenging of the DPPH radical is the basis of a popular DPPH antioxidant assay. Many research groups have used different protocols for the concentration of DPPH, incubation time, reaction solvent and pH of the reaction mixture to determine the antioxidant activity of target samples [38,58].

One more method that can be used for the determination of total antioxidant activity is a ferric-reducing antioxidant power (FRAP) assay. A FRAP assay depends on the reduction of the ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ) complex by a reductant at low pH. Martins et al. determined antioxidants in teas using the FRAP method in combination with flow injection analysis (FIA) systems [59]. Some of the advantages of the FRAP method over other methods are that it FRAP has an easy and fast determination of antioxidant activity, there is a reduction of the total analysis costs and there is a total waste reduction [60,61].

However, it is difficult to compare the described methods because of the complexity and different principles of the reactions, which often results in contradictory values. Generally, different PCs classes have different biological activities and mechanisms by which they could contribute to disease prevention, and these are still unknown [62].

2.4 Sampling and sample pretreatment

The basic steps in the analytical determination of PCs from plant materials are: sampling, sample pretreatment, extraction of target compounds, separation (clean-up-isolation), qualitative identification and quantification using different instrumental techniques.

Sampling procedure strongly depend on the aims and scopes of the investigation, but there are several basic rules applicable to the all analysis. Before a plant analysis result could be used effectively, it is necessary to determine what part or portion of the selected plant, vine or tree should be sampled and the time for sampling [63]. Namely, the content of a target analytes in plant is not a fixed entity, but varies from month to month, day to day, and even from hour to hour, as well as differing between the various parts of the plant itself [63]. Once it has been determined which plant part should be sampled to represent the plant's constituent status, the representative number of plant to samples and sampling procedure (random or selected) must be decided [64]. It has also been defined list with the samples which should be avoided: plants covered with soil, dust, or residue chemicals; plants damaged by insects, mechanically injured, or diseased; plants under moisture or temperature and nutritional stress; border-row plants or end-row plants [63]. Collected plant tissue is very perishable, requiring special handling during the transport and storage processes in order to reduce the influence of external factors on the analysis results.

Furthemore, sample pretreatment is one of the initial steps of the qualitative and quantitative analysis of PCs, and pretreatment depends on the matrix of the particular sample. Namely, before the extraction, plant samples are usually treated by milling, grinding and homogenization, which may be preceded by air-drying or freeze-drying [65]. Some liquid samples, such as urine, plasma and some beverages, can be injected directly into the chromatographic system after filtration.

As mentioned above, the PCs, especially PAs, occur in the form of esters, glycosides or amides, but rarely in a free form within plant material. Consequently, for the quantitative extraction and determination of the target analytes, a hydrolysis step is usually necessary. Acidic, alkaline and enzymatic hydrolysis are the most commonly used methods. An alkaline hydrolysis is used to break ester complexes while acidic hydrolysis is used for breaking glycosidic links.

Acidic hydrolysis is often performed using from 2-6 M HCl at higher temperatures (100°C) for a couple of hours [66]. Alkaline hydrolysis uses 1-4 M NaOH as a reagent. The reaction time of the breaking of ester complexes is tested in time intervals ranging from 1 hour up to the 24 h under different conditions. To reduce the loss of analytes during alkaline hydrolysis, EDTA and L-ascorbic acid or BHT are widely used as stabilizers [67]. In both cases (acidic or alkaline hydrolysis), after the reaction has been completed, the pH of the sample is adjusted to pH=1 or pH=2; after this, the PCs are extracted using the usual techniques. Enzymatic hydrolysis not used as often compared to the two previously described methods [68].

2.5 Extraction techniques

Sample extraction is often considered one of the most important steps in a whole analytical procedure. This step helps not only to clean up the interferences from the complex matrices, but also to isolate and concentrate target analytes. In this way, lower detection limits can be achieved. The extraction efficiency in an analytical process depends on various factors, such as the chemical and physical characteristics of the target compounds, extraction solvents used, extraction time, temperature, pH value, the matrix nature and so forth. However, there is no universal, optimal method for the extraction of all PCs or even one subclass of PCs from plant materials.

Generally, all extraction techniques involved in the separation of PCs may be classified into two main groups: conventional and modern extraction techniques.

2.5.1 Conventional extraction techniques

LLE and SLE are the oldest types of extraction techniques and use organic solvents such as methanol (MeOH), ethanol (EtOH), propanol, acetone, ethyl acetate and their mixtures, usually with different proportions of water [69–72]. The extraction solvent, solvent-to-solid ratio and process temperature are the most important parameters when it comes to extraction efficiency. The selection of an appropriate solvent primarily depends on the chemical properties of the target compounds. This extraction method is usually performed at room temperature (RT) to avoid the degradation of PCs, but temperatures from 30–50ºC can also be applied.

Conventional extraction techniques can be improved by the influence of external factors such as ultrasound or microwave irradiation.

The UAE technique is based on the formation of high-frequency ultrasonic waves that cause cavitation due to the expansion and contraction cycles experienced by the material [73]. During the UAE, temperatures usually range from RT up to the 45°C. Higher temperatures are not recommended because of the degradation or polymerisation of PCs, which leads to a decrease in the extraction efficiency [73,74]. The optimization for an UAE method should include the following factors: sonication time, temperature, ultrasound amplitude, cycle and type and volume of solvent used. Corbin et al. optimized UAE for the determination of PCs from flax seeds [75]. They tested different extraction conditions: different solvents (MeOH, EtOH, butanol and water), different extraction times from 0-60 min and different operating temperatures, ranging from 25–60 °C. The following conditions were determined as optimal: water as a solvent supplemented with 0.2 N of sodium hydroxide for the alkaline hydrolysis, an extraction time of 60 min at a temperature of 25 °C and an ultrasound frequency of 30 kHz.

Microwave-assisted extraction, which uses frequencies from 0.3–300 GHz, is also considered a novel method for extracting PCs. The MAE system increases the temperature rapidly, and this results in shorter extraction times and higher extraction recovery of the target analytes. MeOH, EtOH and water are the conventionally used solvents for MAE extraction methods. MAE extraction efficiency is also highly dependent on many factors, such as the particle size of the sample, applied microwave power, irradiation time and so forth. Due to the previously mentioned reasons, the optimization of MAE is a very important step for the quantitative determination of target PCs. Bouras et al. optimized MAE for the extraction of PCs from Quercus bark [76]; they concluded that the best conditions were an irradiation time of 60 min, power of 45 W, pH of 10.75, EtOH content of 33%, MeOH content of 0.38% and particle size of 0.5 cm. The MAE method for the extraction of PCs from different types of samples has also been described by other authors [42,77,78].

Microwave-ultrasound assisted extraction (MUAE) combines ultrasonic and microwave extraction.

A brief review of recent studies based on the LLE and SLE extractions of PCs published by different authors is summarised in **Table 2-7**.

Table 2-7. An overview of LLE and SLE methods for the extraction of PCs from different plant materials.

Natural source	Extraction solvent	Extraction conditions	Instrumental analysis	Identified compounds	Ref.
Asteraceae plants	Distilled water	Heated up to the 80 °C	Spectrophotometry	TPC, antioxidant activity	[79]
Apple tree wood residues	EtOH:water (50:50)	Heated at 55 °C for 2 h	HPLC-PDA	Flavnonols Flavanones	[69]
Nuts	Acetone	50 °C-30 min	UHPLC-MS/MS	Phenolic acids Flavanols Rutin	[70]
Tea infusions	Distilled water	94 °C-15 min	HR-CS GFAAS and HPLC-MS/MS	Rutin Quercetin Phenolic acids	[71]
Serbian unifloral honeys	Ethyl acetate	Not-defined	UHPLC-HESI-MS/MS	Phenolic acids Flavonoids	[72]
Brassica oleracea	70% MeOH	Heated-5 min	Capillary zone electrophoresis	Phenolic acids	[80]
Wheat flours produced	EtOH:water (80:20)	Chilled	HPLC-UV	Phenolic acids	[81]
Fresh olive	MeOH: water (80:20)	UAE, 47 °C-30 min (optimized by RSM)	HPLC-DAD	Oleuropein Hydroxytirosol	[82]
Chokeberry	EtOH	UAE, 90 °C -30 and 60 min	HPLC-DAD	Anthocyanins Flavonoids	[83]
Soy isoflavone concentrate	MeOH:water (4:1, v:v)	UAE, 40 °C -10 min	HPLC–DAD–ESI-MS	Isoflavones Flavones Phenolic acids	[84]
Cereals	70% MeOH	UAE, RT-15 min	HPLC–DAD	Free and bound phenolic acids	[85]
Mango seed kernel	EtOH with different percentages of water	MAE, 75 °C-2 extraction cycle (optimized by RSM)	HPLC-ESI-MS	Flavonoids	[86]
Rice grains	MeOH with different percentages of water	MAE, 185 °C-20 min (optimized by RSM)	HPLC-FD	Phenolic acids	[87]
Apple tree wood residues	EtOH:water (60:40)	MAE, 100 °C-20 min	HPLC-PDA	Flavnonols Flavanones	[69]
Satureja macrostema	Different solvents were tested	MUAE, extraction parameters were optimized by RSM	Spectrophotometry	TPC TFC Antioxidant activity	[88]

SPE is a powerful method for sample preparation, and it is used by most chromatographers today. SPE can be used in a broad range of applications, such as environmental analysis, pharmaceutical and biochemical analysis and organic chemistry and food analysis [89]. Some of the advantages of SPE compared to the classical LLE and SLE techniques are the low solvent consumption, shorter extraction time, better reproducibility, cleaner extracts and the potential for automation. SPE procedure usually used for the extraction of PCs is shown in **Figure 2-6**.

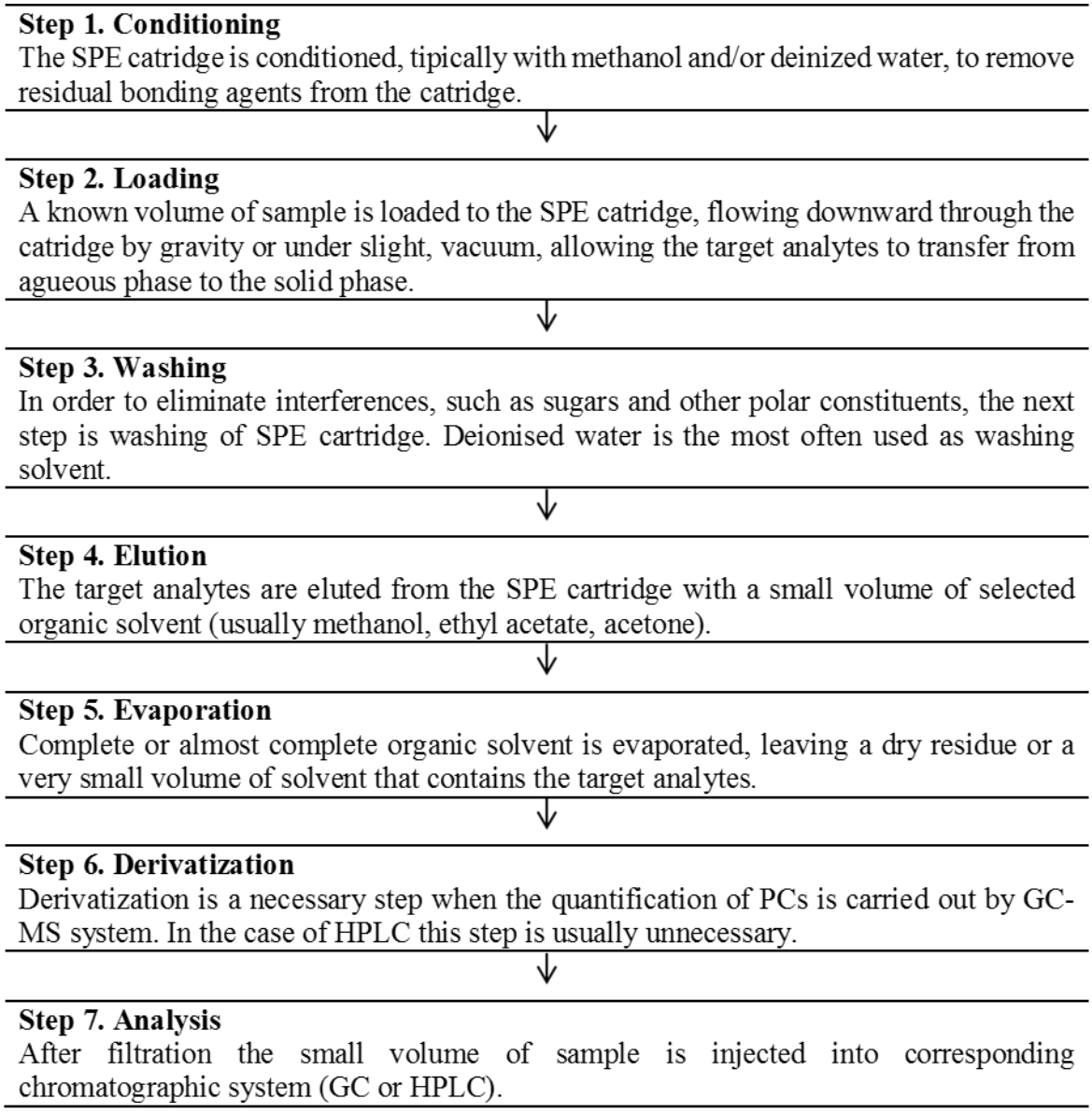


Figure 2-6. Steps involved in typical SPE procedure [90].

SPE cartridges with a diol-bond, C8 and C18 stationary phase, are the most commonly used for the isolation of PCs from different materials. For example, Alarcon-Flores et al. proposed diol stationary phase for the extraction of PAs, as well as of oleuropein, tyrosol, hydroxytyrosol and luteolin from olive oil [91]. They obtained extraction recoveries over 90% for of the investigated compounds when using MeOH as an extraction solvent, acetonitrile for residue solubilisation and two washing steps with hexane. Barnaba et al. [92] tested C18 and C8 SPE cartridges for the isolation of 56 simple PCs from wine, spirit and vinegar samples. Recent studies have shown several disadvantages of these cartridges for the extraction of PCs; therefore, SPE methods with stationary phases based on different copolymers were developed. Irakli et al. [85] optimized the SPE method with Oasis HLB water cartridges for the isolation of free and bound PAs from cereals, in this case using aqueous MeOH as the eluent. Sadiki and Martin [93] used the cartridges with the same stationary phase (HLB) for the extraction of several PCs from maple syrup.

2.5.2 Modern extraction techniques

Pressurised fluid extraction (PFE) or pressurised liquid extraction (PLE) are two new extraction techniques that use liquid solvents at increased temperatures and pressures to extract bioactive compounds. These methods are principally similar to classical Soxhlet extraction, with one important modification. Namely, during the process, the solvent condition inside the PLE cell approaches the supercritical region, which results in more efficient extractions. The main advantages of PLE over the conventional extraction techniques are higher extraction efficiencies, the use of solvents generally classified as green and safe (e.g., EtOH, water and their mixtures) and reduced extraction time [94]. Some recently published articles have described the application of this extraction technique in the analysis of PCs [94–96].

Campone at al. [97] developed a new, rapid and simple dispersive liquid-liquid microextraction (DLLME) for the determination of PAs and flavonoids in honey samples. The first use of this extraction method was reported in 2006, when Rezaee et al. [98] developed DLLME for the pre-concentration of polycyclic aromatic hydrocarbons (PAHs) in water. Using this technique, a mixture of organic solvents as the extractant and a disperser solvent were rapidly injected into an aqueous sample so that the turbulence caused the formation of fine droplets, which then dispersed through the aqueous sample [99].

Reboredo-Rodríguez et al. applied ultrasound-assisted emulsification–microextraction (USAEME) for the isolation of PCs from olive oils [100]. USAEME is a variant of DLLME, but it uses ultrasonic radiation as an alternative to the disperser solvent to assist in emulsification [101].

Subcritical water extraction (SWE) was compared to the conventional SLE for the determination of PCs from potato peels [102]. The results of this study showed that SWE could be maximized at 180 °C and 60 min. Also, when compared to SLE, SWE required about 50% less solvents.

A green and environmental friendly supercritical CO_2 (SC-CO_2) extraction is also a relatively new technique for the isolation of PCs prior to instrumental analysis [103–105]. However, CO_2 has a very limited capacity regarding its ability to dissolve polar and high molecular weight compounds.

Mushtaq et al. developed a new, effective, fast and environmentally friendly enzyme assisted supercritical fluid extraction (EASFE) for the isolation and determination of PCs from pomegranate peel samples [106]. Five different PAs were identified in the samples using the HPLC-DAD-MS technique, and the extraction process was twice as effective compared to the currently applied conventional methods.

2.5.3 Comparison of different extraction techinques

Each of the previously described extraction techniques has its advantages and disadvantages. To correctly choose the most suitable extraction technique, many different factors affecting the extraction process (matrix from which target compounds should be extracted, the chemical and physical properties of the target analytes, economic feasibility, influence on the environment, etc.) should be considered. **Table 2-8** compares different extraction techniques used in the analysis of PCs from the aspects of usability, time consumption, energy consumption (in the form of heat) and extraction solvent type.

Table 2-8. Comparison of different extraction techniques used for the isolation of PCs from different plant materials.

Matrix	Extraction technique	Extraction solvent	Time consumption	Temperature	Ref.
Grape pomace	SLE	EtOH:water	48 h	RT	[107]
Walnut leaves	ME	EtOH	90 min	112-150 °C	[108]
Spent coffee grounds	UAE	EtOH	34 min	40 °C	[74]
Walnut leaves	MAE	67.9% EtOH	3 min	107.5 °C	[108]
Wheat bran	SC-CO_2	SC-CO_2	6 h	70 °C	[109]
D. kotschyi	PLE	MeOH	11.3 min	74 °C	[110]
Pomegranate peel	EASFE	SC-CO_2 EtOH (80%)	30-120 min	55 °C	[106]
Bitter melon	SWE	Water	Fractionated extraction (2 h)	130-200 °C	[111]
Olive oils	USAEME	MeOH:H_2O (80:20 v:v)	15 min (40 kHz)	25±3 °C	[100]
Honey	DLLME	$CHCl_3$ (DLLME extractant) Me_2CO (DLLME disperser)	10 s + 5 min centrifuge	RT	[97]

2.6 Detection of phenolic compounds

2.6.1 Spectrophotometric methods

Spectrophotometry is the main instrumental technique used for the quantification of the different subclasses of PCs (TPC, TFC, total anthocyanin content-TAC, etc.) due to its simplicity and low cost. Some spectrophotometric methods that are used later in this doctoral dissertation are shortly described in the following subsections. However, the main disadvantage of all spectrophotometric assays is the non-selectivity over the individual PCs.

2.6.1.1 Folin Ciocalteu reagent assay for TPC

One of the most useful methods for the determination of TPC is a Folin-Ciocalteu method, which uses gallic acid (GAE) or catechin (CAT) as standards. The Folin-Ciocalteu reagent (FCR) is a mixture of phosphomolybdate and phosphotungstate. The reduction of this reagent to a blue-coloured complex in an alkaline solution occurs in the presence of PCs [112]. The disadvantages of the method are its non-selectivity toward different PCs subclasses and the fact that the results can also be affected by other nonphenolic-reducing molecules such as ascorbic acid [112]. Despite the disadvantages, many authors have described this method as the most suitable for the determination of TPC in different matrices [113–115]. The final

concentrations of TPC are usually expressed as mg of gallic acid per gram of dry weight (mg GAE g^{-1} DW).

2.6.1.2 Total flavonoid content

One of the most commonly used spectrophotometric methods for the determination of TFC is the aluminium chloride colorimetric method. It is based on the fact that $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols (**Figure 2-4**) [116]. $AlCl_3$ also forms acid-labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (**Figure 2-4**). Absorbance measurements are performed on 415 nm against the blank. Studies have proved rutin as a suitable standard for the determination of TFC in different samples, and the final results are expressed as mg of rutin per gram of dry weight (mg RUT g^{-1} DW) [117,118].

2.6.1.3 Total anthocyanin content

The pH differential method is often used for the determination of TAC. Here, monomeric anthocyanin pigments reversibly change colour with a change of pH value. Namely, at the pH value of 1.0, anthocyanins exist as coloured oxonium ions while the colourless hemiketal form is predominant at a pH value of 4.5 [119]. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. The results are usually expressed as mg of cyanidin-3-glucoside equivalents per gram of dry weight (mg Cya-3-Glu g^{-1} DW).

2.6.2 Chromatographic analysis

Beside studies related to the development of new extraction techniques for the isolation of PCs, many researchers have focused on the optimization of suitable chromatographic methods for their identification and quantification.

2.6.2.1 Thin layer chromatography (TLC)

TLC was previously used for the qualitative analysis of PCs. TLC is performed on a sheet of glass, plastic or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide or cellulose [120]. This layer is called the stationary phase (SF). Maleš et al. optimized the TLC method for the determination of flavonoids and PAs from *Helleborus atrorubens* [121]. The identification of the extracted compounds was done by

comparing retention times with the standard compounds. The TLC method for the qualitative determination of the PCs has also been published in other articles [122], [123]. TLC has its advantages and disadvantages. The method is very simple, fast, cheap and sensitive. On the other hand, the main disadvantages of TLC are its application to only non-volatile compounds, limited separation, inability to be typically automated and limited reproducibility.

2.6.2.2 Gas chromatography

GC is the most commonly used technique for the determination of volatile components. Coupled with MS as a detector, GC allows the simultaneous identification and quantification of a large number of compounds. The compounds intended for determination by a GC-MS chromatographic system should be in gas phases or must be transformed into gas phase without decomposition (thermostable compounds).

The main components of a typical GC-MS system are gas supply, injector, thermostatic oven, GC column, ion source, mass analyser and detector (**Figure 2-7**).

The separation of target compounds is performed in the GC column, and it is highly dependent on the following factors: choice of the suitable column (regarding the used SF, column length, diameter and film thickness), selection of the appropriate mobile phase (helium or argon as carrier gas) and chromatographic conditions (oven temperature programme, transfer-line temperature, etc). The selection of the GC column depends on the target analytes, but for the analysis of PCs, the mostly used is a capillary column with 5% phenylmethylpolysiloxane SF.

The most common types of MS detectors associated with a GC are ion trap and quadrupole mass spectrometers. MS detectors require a vacuum to operate in a predictable and efficient way. When the sample exits at end of the GC column, it is fragmented by ionization, and the fragments are sorted by mass to form a fragmentation pattern. Most MS detectors work in the electron impact ionization mode, with the ionization voltage set to the standard 70 eV. The spectra are collected tipically up to *m/z* 650 in the SCAN or SIM (selected ion monitoring) mode. SIM mode allows the MS detection of specific compounds with very high sensitivity because the instrument is set to gather data at masses of interest instead of stepping the mass filter over a wide range of masses [124].

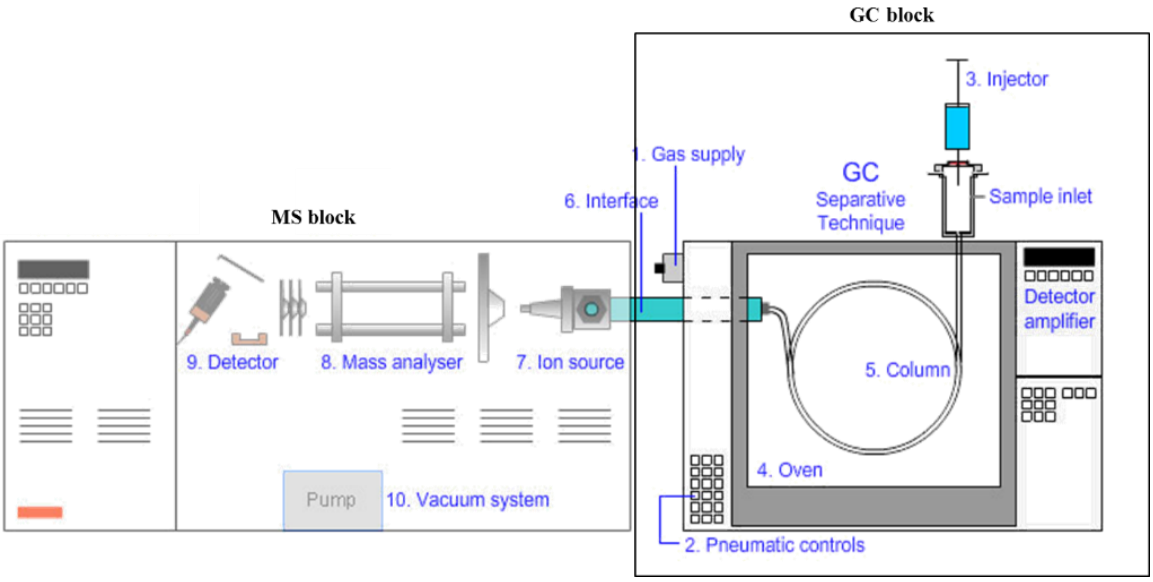


Figure 2-7. Shematic representation of GC-MS system [125].

A chemical characteristic of the -OH groups in PCs is the hydrogen-bonding capability, which increases PCs melting point. Consequently, the low volatility of PCs represents the main limitation for the identification and quantification of these compounds by GC-MS [18]. To overcome this disadvantage, during the sample preparation for a GC analysis, one additional step is needed, namely derivatization of the compounds [126]. During the derivatization, PCs are transformed into volatile and thermostable compounds suitable for GC separation.

The most useful derivatization reaction is silylation using different reagents such as BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide), MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide), BSA (N,O-Bis(trimethylsilyl)acetamide) and their mixtures, usually in pyridine [126,127]. Derivatization reaction can be performed at RT or an elevated temperature and for various time periods. For the successful silylation of PCs, all protic organic solvents and traces of water should be previously removed from the samples. The final products are the trimethylsilyl (TMS) derivatives of PCs.

Some interesting, recent articles based on the development of GC-MS methods for the determination of PCs from different matrices are summarised in **Table 2-9**.

Table 2-9. An overview of the GC-MS methods for the quantification of PCs from the different matrices.

Sample	Extraction procedure	Derivatization	Temperature programme	Reference
Virgin olive oil	SPE extraction with Diol-SF	BSTFA + 1% TMCS- at RT for 30 min	160 °C for 5 min 160-188 °C, 3 °C min^{-1} 188 °C for 1 min 188-241 °C, 15 °C min^{-1} 241 °C for 1 min 241-282 °C, 2 °C min^{-1} 282-310 °C, 5 °C min^{-1} 310 °C for 5 min	[128]
Human plasma after coffee consumption	Without extraction step	BSTFA + 1% TMCS or MTBSTFA + 1% TBDMSCl at 60 °C for 2h	150 °C for 1 min 150-210 °C, 15 °C min^{-1} 210-310 °C, 5 °C min^{-1} 310 °C for 1 min	[129]
Aromatic plants	62.5% MeOH	BSTFA with 5% DMDCS	70-135 °C, 2 °C min^{-1} 135 °C for 10 min 135-220 °C, 4 °C min^{-1} 220 °C for 10 min 220-270 °C, 3.5 °C min^{-1} 270 °C for 10 min	[130]
Aromatic plants	Dynamic sonication-assisted extraction (DSAE)	BSTFA- at 25 °C for 60 min	100 °C for 5 min 100-280 °C, 5 °C min^{-1} 280 °C for 5 min	[131]
Balsamic vinegar from Modena	Chem-Elut and SPE extraction. Elution solvent: ethyl acetate	BSTFA- at 70 °C for 30 min	90 °C for 1 min 90-240 °C, 20 °C min^{-1} 240 °C for 10min 240-280 °C, 20 °C min^{-1} 280 °C for 1 min	[132]
Sicilian olive oils	Extraction with MeOH:water (80:20)	BSTFA:TMCS (99:1)- at RT for 30 min	60-275 °C, 15 °C min^{-1} Isothermal for 15 min at 275 °C	[133]
Triticum aestivum L (wheat)	Extraction with diethyl ether	MSTFA- at 60 °C for 30 min	80 °C for 1 min 80-160 °C, 10 °C min^{-1} 160-235 °C, 5 °C min^{-1} 235- 280 °C, 50 °C min^{-1}	[134]

2.6.2.3 Liquid chromatography

One of the most useful instrumental methods for the analysis of PCs is absolute LC. The main advantage of LC compared to GC is that it allows the identification of components that cannot be determined by the GC system, such as low-volatility and therm unstable compounds. The most important parts of the LC system are: pumps, injector, chromatographic column, thermostatically controlled oven, detector and data-acquisition system (**Figure 2-8**).

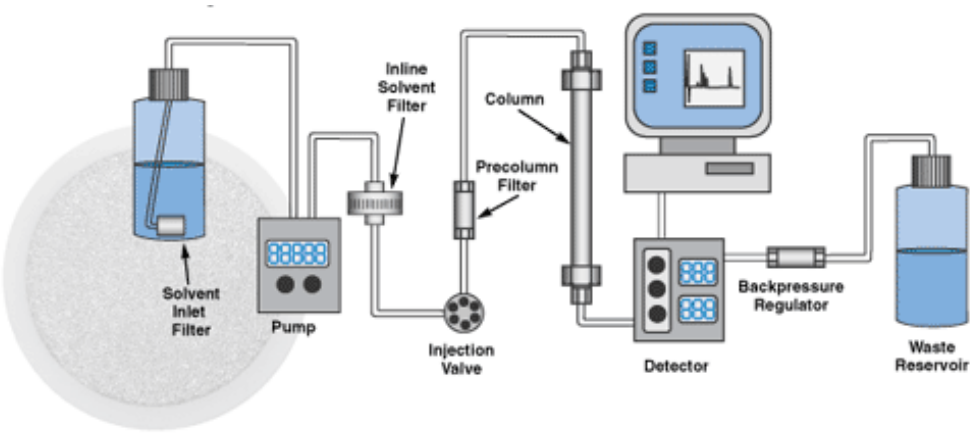


Figure 2-8. Scheme of a typical LC system [135].

The types of LC techniques based on different interactions between target compounds and stationary phases of the column are as follows [136]:

1. Partition Chromatography
2. Ion Exchange or Ion Chromatography
3. Size Exclusion Chromatography
4. Affinity Chromatography
5. Chiral Chromatography

Regardless of the interactions being exploited, LC is carried out by the following six steps:

1. Column equilibration
2. Sample loading
3. Washing
4. Elution
5. Final column washing
6. Column regeneration

The compounds of the sample, distributed between the mobile phase (which is in the liquid state) and SF (typically consisting of small porous particles with high surface areas), travel through the chromatographic column. Depending on the physical and chemical properties, each component in the sample interacts slightly differently with the SF. This phenomenon causes different flow rates for the different components and leads to the separation of the components as they flow out of the column. Reverse-phase liquid chromatography (RP-LC), where the SF of the column is less polar compared to the mobile phase, is the most commonly used type for the separation of PCs. In this chromatographic type, less polar compounds leave the column, and it has the longest retention times.

HPLC, a type of LC, relies on pumps to pass a pressurised liquid solvent containing the sample mixture through a column filled with a solid adsorbent material.

Sophisticated versions of HPLC are: ultra-performance liquid chromatography (UPLC) and ultra-high performance liquid chromatography (UHPLC).

The LC/HPLC system can be coupled with different detectors, such as ultraviolet-visible (UV-ViS), MS, DAD, electrochemical detector (ECD) and rarely used detectors: PDA and fluorescence detector (FLD). Additionally, for the identification of compounds when the standards are not available, nuclear magnetic resonance (NMR) can also give all the necessary information. The choice of the appropriate detector depends on several factors, such as nature and properties of the investigated analytes, required sensitivity and information that needs to be collected (structural, quantitative, etc.). However, the most useful detectors in the analytics of PCs are UV-ViS and MS.

UV-ViS detection

UV-ViS is a non-destructive detection method but has limited applications that can be applied only on samples that have absorbed radiation at the wavelength of the source light. Another disadvantage of a UV-ViS detector is that it gives no structural information about the analyte. In the best-case scenario, it can be used for the determination of the subclasses of PCs because every family has specific bands of maximum absorption (**Table 2-10**) [137]. If the subject of the analysis is a well-known matrix or if the standard compounds are available for the structure confirmation of target analytes, UV-ViS detection gives satisfactory results.

Table 2-10. Absorption bands for the different PCs subclasses [137].

PCs subclasses	Absorption maximum (nm)	
Phenolic acids		
Hydroxybenzoic acids	270-280	
Hydroxycinnamic acids	305-325	
Flavonoids		
Isoflavonoids	245-270	300-340
Flavones	250-270	330-350
Flavonols	250-270	350-380
Anthocyanidin	240-280	450-560
Flavanones	270-295	
Flavanols	270-280	
Other PCs classes		
Coumarins	220-230	310-350

MS detection

The main difference between MS detectors coupled to the LC system and these coupled to the GC is the ionization. The ionization types for MS coupled to the LC are as follows:

1. **Electrospray ionization (ESI)**: Liquid samples are pumped through a metal capillary maintained at 3 to 5 kV and nebulised at the tip of the capillary to form a fine spray of charged droplets. The droplets are rapidly evaporated by the application of heat and dry nitrogen, and the residual electrical charge on the droplets is transferred to the analytes. The ionised analytes are then transferred into the high vacuum of the mass spectrometer via a series of small apertures and focusing voltages. The ion source and subsequent ion optics can be operated to detect positive or negative ions, and switching between these two modes within an analytical run can be performed [138].

2. **Atmospheric pressure chemical ionization (APCI)**: Like the ESI, liquid is pumped through a capillary and nebulised at the tip. A corona discharge takes place near the tip of the capillary, initially ionising the gas and solvent molecules present in the ion source. These ions then react with the analyte and ionise it via charge transfer [138].

The use of the buffers (as the mobile phase) containing inorganic ions such as phosphate and sodium acetate should be avoided. These buffers cause significant ion suppression, which can lead to the creation of MS sodium and potassium adducts and can quickly contaminate the ion source. The buffers that are acceptable for the LC-MS systems are based on ammonium acetate, ammonium formiate or ammonium bicarbonate. **Table 2-11** summarises some of the recent papers on the determination of PCs based on LC/HPLC analysis.

Table 2-11. An short overview of the recent published LC/HPLC methods for the quantification of PCs from the different matrices.

Sample source	Sample preparation	Stationary phase/Mobile phase	Detector	Ref.
Crude red grape skin extracts	Analytes were extracted with 15 mL of 70% alcohol for 24 h in the dark. Samples were then ultrasonicated (60 kHz) for 25 min.	Agilent Poroshell 120 EC-C18 (100 mm × 3.0 mm, 2.7 μm)/ A (0.2% formic acid in Milli-Q water) and B (acetonitrile). The gradient was as follows: 13% B (9 min), 13% to 27% B (5 min), 27% to 40% B (5 min), and 40% to 50% B (7 min).	DAD-MS	[139]
Raw propolis	a) Maceration extraction with EtOH–H_2O (80:20, v:v) at RT for 24 h under stirring. b) Heat reflux extraction (HRE) with EtOH-H_2O (80:20, v:v) at 70 °C for 1 h under stirring using a water bath. c) The UAE with EtOH–H_2O (80:20, v/v) at 70 °C for 1 h using an ultrasonic bath.	Ascentis Express C_{18} column (150 mm × 3.0 mm I.D., 2.7 μm)/ (A) 0.1% formic acid in H_2O and (B) ACN. The gradient elution was modified as follows: 0–3 min 20% B, 3–10 min from 20 to 30% B, 10–40 min from 30 to 40% B, 40–50 min from 40 to 60% B, 50–60 min from 60 to 80% B, 60–65 min from 80 to 50% B.	ESI-MS/MS	[140]
Herba Lysimachiae and Herba Desmodii Styracifolii	The crude powder was soaked in EtOH for 1h and extracted in an ultrasonic bath for 45 min, where the bath temperature was 50 °C.	Diamonsil C_{18} column (150 × 4.6 mm, 5 μm)/ (A) MeOH and (B) 0.1% acetic acid aqueous solution. The gradient condition was as follows: 0–34 min, 20–34% A; 34–38 min, 34–95% A; maintained 95% A for the next 4 min.	MS/MS	[141]
Red wines	a)LLE- acidified a wines to pH 2.0 and extracted with ethylacetat. b)SPE- cartridges were conditioned with 2.5 acetonitrile, followed by MeOH, followed by water. Compounds were eluted with acetonitrile: MeOH (1:1, v:v).	Eclipse XDB C18 column (250 mm × 4.6 mm, 5 μm)/ (A) formic acid, MeOH (B) and water (C). Start, A:B:C (0.2:35:64.8) at a flow rate of 1 mL min^{-1}. After 6 min, the pumps were adjusted to A:B:C (0.2:60:39.8) at a flow rate 1 mL min^{-1}. At 9 min the conditions were A:B:C (0.2:80:19.8) at a flow rate 1 mL min^{-1} until termination of the run at 14 min.	UV-254 UV-280 UV-323	[142]
Cereals	Extraction of free PAs: 70% MeOH, and after that cleannig using SPE. Elution with MeOH:acidified water (9:1). Extraction of bound PAs: Basic hydrolisis using 4 M NaOH for 90 min at the 40 °C. The liberated PAs were extracted with ethyl acetate.	Nucleosil 100 C_{18} column (250 mm × 4.6 mm i.d., 5 μm)/ (A) MeOH and (B) water:acetic acid (99:1 v:v). The gradient was linear at a flow rate of 1.3 mL min^{-1} from 90% to 80% solvent B for 10 min, from 80% to 75% B for the next 10 min, from 75% to 65% B for 10 min and finally from 65% to 35% B for another 10 min.	DAD	[85]

Table 2-11. Continued.

Citrus honey	Honey was dissolved with water, and exposed to UAE for 10 min. The sample was homogenized and filtered through 0.45 μm membrane filter.	Zorbax SB-C_{18} column (150 × 4.6 mm, 5 μm) connected with a Zorbax SB-C_{18} guard column (20 × 4.0 mm, 5 μm)/ (A) 4% aqueous acetic acid and (B) MeOH. The gradient programme was: 5–20% B at 0–10 min, 20–40% B at 10–15 min,40–60% B at 15–20 min, 60% B at 20–25 min. The flow rate was 1.0 mL min^{-1}.	ECD	[143]
Portuguese wild mushrooms species	Samples were extracted with acetone:water (80:20) mixture at -20 °C for 6 h. After that, the extract was exposed to UAE for 15 min, and filtered. The residue was then extracted with acetone:H_2O mixture.The aqueous phase was washed with n-hexane, extracted with diethyl ether (3x) and ethyl acetate (3x).	Spherisorb reverse phase C_{18} column (150 mm × 4.6 mm, 3 μm)/ (A) 2.5% acetic acid in water, (B) acetic acid 2.5%/acetonitrile (90:10) and (C) acetonitrile. The gradient was: isocratic 100% A for 10 min, 50% A and 50% B for 10 min, isocratic 100% B for 15 min, 90% B and 10% C for 10 min, 70% B and 30% C for 10 min, 50% B and 50% C for 5 min, 20% B and 80% C for 5 min, 100% A for 5 min, at a flow rate of 0.5 mL min^{-1}.	DAD-ESI/MS	[144]
Fruit juices	The sample was cleaned up using the Sep-Pak Plus t C_{18} and Bond Elut PSA cartridges. The PAs were elueted with 10 mL of MeOH.	L-column ODS (250 × 4.6 mm, 5 μm)/ Mobile phase was: 5 mmol L^{-1} potassium dihydrogenphospate solution (pH 2.5)-acetonitrile (41:9) at a flow rate of 1.0 mL min^{-1}.	UV-280 nm	[145]
Human plasma	The target compounds were extracted with EtOH.	C_{18}-column Acquity UPLC BEH (2.1 mm × 150 mm, 1.7 μm)/ (A) water with 1% acetic acid and (B) acetonitrile with 1% acetic acid. The gradient condition was as follows: 0–2 min 97% A; 2–10 min 80% A; 10-20 min 70% A, 20-30 min 10% A and 30-40 min 7% A.	MS/MS	[146]
White wine	Without extraction step.	Luna C_{18} (150 × 2 mm, 3 μm)/ (A) water with 1% formic acid and (B) acetonitrile. 0 min 2% B; 0-10 min 13% B; 10-25 min 15% B; 25-30 min 22% B; 30-40 min 22% B.	DAD-MS/MS	[147]

2.6.3 Other analytical methods

Research in the last couple of years has resulted in the development of various electrochemical and electrophoresis methods for the determination of PCs in the different matrices. Oliveira-Neto et al. used the electrochemical behaviour of phenolic antioxidants to determine their concentration in selected coffee samples. The measurements were performed in a 5.0 mL one-compartment electrochemical cell with a three-electrode system consisting of a carbon paste electrode (prepared as a piston-driven holder containing 75% graphite powder and 25% Nujol), a Pt wire and the Ag/AgCl/KCl salt [148].

Talarico et al. constructed a new electrochemical sensor for the determination of PCs. The sensor was constructed by modifying the working electrode surface of a screen-printed electrode (SPEL) with carbon block (CB) dispersion. In their studies, they also detected caffeic acid and gallic acid and demonstrated that CB-SPEL exhibited better electrochemical properties than bare SPE in terms of reducing peak-to-peak separation and the intensity of the peak for selected analytes [149].

Chlorogenic acid was determined in the coffee samples using a tetranuclear copper (II) complex that mimics the active site of catechol oxidase. The recovery of this method was between 93% and 106% [150]. Other authors have described procedures for the determination of PCs using different standard and modified electrodes, as well as other electroanalytical methods such as voltammetry [151–155].

Electrophoresis was first used in 2003 by Bendini et al. for the determination of PAs. The method was developed to monitor PAs in olive oil [156]. As a reference method, HPLC was used. The authors concluded that electrophoresis can be a viable alternative when it comes to the determination of PAs, with high sensitivity, speed of the analysis and reduced usage of organic solvents.

Hurtado-Fernández et al. developed new, sensitive electrophoresis techniques based on capillary electrophoresis-ultraviolet detection (CE-UV) for the determination of PAs and their subsequent characterisation in avocado [157].

Likewise, some other authors have described the use of this instrumental method for the determination of PCs in different samples [80,158,159].

3 Experimental part and results

3.1 Stability studies of *trans*-Caffeic and *trans*-Ferulic acid

PAs are precursors for the biosynthesis of many other PCs, such as flavonoids, lignin, chalconoids etc. PAs represent a significant part of the human diet, owing to their antioxidant and prooxidant properties and effects on human health [160,161].

3-(3,4-dihydroxyphenyl)-2-propenoic acid (Caffeic acid) and (E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid (Ferulic acid) belong to the group of hydroxycinnamic acids with prominent bioactivity and have been widely used in the medicine, food and cosmetic industries [162,163]. Both compounds can exist as *trans*- and *cis*-geometric isomers. *Trans*-forms are naturally predominant isomers, but studies have shown that *cis*- isomers can also be isolated [164].

In nature, *trans*-CA is formed from 4-hydroxycinnamic acid and transformed to *trans*-FA, which is the precursor for the synthesis of many other PCs. CA was found in high concentrations in plants from the *Lamiaceae* family, often in combination with rosmarinic acid, which is an ester of CA and 3, 4-dihydroxyphenyllactic acid [165]. Several studies have confirmed that *trans*-CA is not stable under the influence of light, and isomerization from *trans*- to *cis*-form can occur as well as photo-conversion to esculetin (6,7-dihydroxy-2H-1-benzopyran-2-one) [166–169]. The proposed mechanism of the photo-degradation of *trans*-CA is shown in **Figure 3-1**.

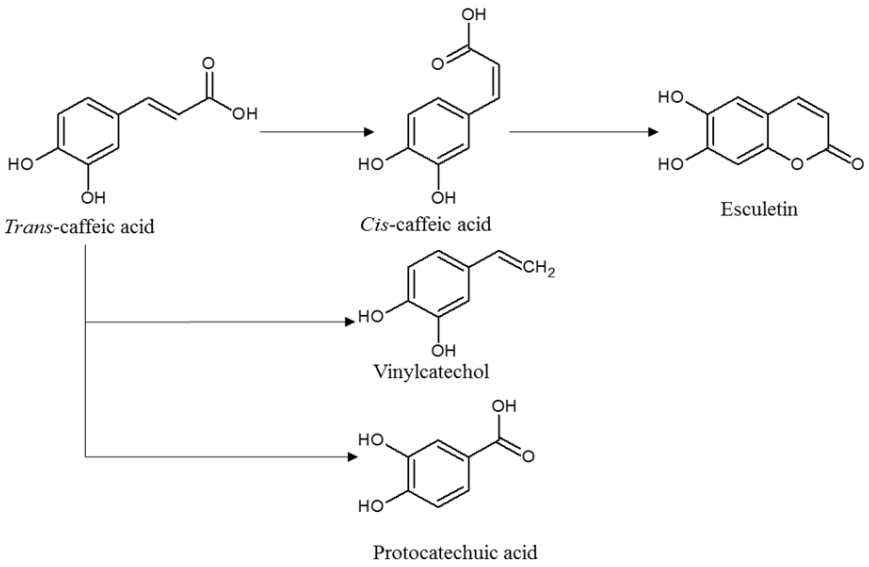


Figure 3-1. Proposed mechanism of the photo-degradation of *trans*-CA [167].

FA, like many other natural PCs, is an antioxidant in vitro [170]. It may be effective in preventing cancer, but it also has a neuroprotective, radioprotective, pulmonary protective and anti-atherogenic effect [171]. It has also been proven that *trans*-FA is very unstable under varied conditions, and that its most important degradation (isomerization) product is *cis*-FA [172]. Antioxidant studies, carried out on the *trans*- and *cis*-FA isolated as the major components of bound phenolics from rice bran, revealed that *cis*-isomers possessed almost identical DPPH hydroxy radical and ABTS+ radical scavenging activities to the corresponding *trans*-isomers [173,174].

From the literature, it is obvious that the most common technique for the different stability studies is HPLC with a UV-ViS or DAD detector. In comparison with HPLC, a minor disadvantage of the GC-MS method is the necessity for a derivatization step, required to ensure the volatility of the compounds [132,175]. Nevertheless, GC-MS also offers some advantages such as simultaneous, complete and high-resolution separation of *trans*- and *cis*- isomers in one chromatographic run, their sensitive detection, as well as unambiguous identification and quantification [176].

3.1.1 Chemicals

Standard compounds: *trans*-FA (99.5%) and *trans*-CA (99.5%) were supplied by Merck (Germany).

Solvents: tetrahydrofuran (THF) and pyridine (99.9%), were supplied by Merck (Germany). HPLC-grade MeOH (99.8%) was purchased from Sigma (USA), while GC-grade toluene (99.5%) was purchased from Carlo Erba (Italy). The water used was obtained from a Milli-Q water purification system.

Other chemicals: MSTFA was purchased from Sigma (USA).

3.1.2 Preparation of calibration curves

The stock solutions were prepared by accurately weighing 10 mg of the *trans*-CA and *trans*-FA standard compounds, and dissolving them in 10 mL glass flasks with THF or MeOH, to obtain concentrations of 1 000 mg L^{-1}. Five working calibration solutions for both acids (concentration range from 1 to 100 mg L^{-1}), in the two different solvents, were prepared from standard stock solutions by further dilution with the appropriate solvent. As the internal

standard (ISTD) 2,5-dichlorobenzoic acid (2,5-DCBA) was used. The concentration of ISTD in all working solutions was 50 mg L^{-1}.

The derivatization was performed by evaporating the solvent to absolute dryness by rotary evaporation and adding 100 μL of MSTFA and 50 μL of pyridine, and further by heating at 80 °C for 1 h in a sand bath. After the derivatization was finished and the solutions were cooled to RT, quantitatively transferred to a 1 mL volumetric flask, and brought up to the mark with the toluene. The calibration solutions were injected into the GC-MS system in triplicates. Calibration curves were constructed by linear regression of the peak area ratio of individual *trans*-isomers to the ISTD (y) versus the concentration in mg L^{-1} (x). The concentrations of the photoproduced *cis*-CA and *cis*-FA, which standards are not commercially available, were calculated as the difference between the initial and the residual concentrations of corresponding *trans*-isomers. The calibration curve solutions were prepared fresh daily.

3.1.3 Instrumentation and chromatographic conditions

Analyses were carried out on a Varian 3900 GC, coupled to an Agilent 2100T MS. GC separation was performed using a Varian capillary column VF-5ms CP8944 (30 m x 0.25 mm, with 5% phenylmethylpolysiloxane stationary phase 0.25 μm). 1 μL of the sample was injected in the split injection mode (split ratio 1:10). The carrier gas was He (5.0 UHP) at 1.0 mL min^{-1} flow rate. The initial oven temperature was 40 °C, held for 1 min, and then the temperature was raised to 320 °C at a rate of 10 °C min^{-1}, and finally, held for 3 min. The total run time was 32 min. The injection-port was set to 250 °C and the transfer-line to 170 °C. A mass spectrometer recorded the entire spectrum (SCAN mode) in a range from *m/z* 50 to *m/z* 650, using electronic ionization energy at 70 eV. The peak identification was done by comparison to standard compounds or by library matching from the NIST MS library, containing the mass spectra of TMS derivatives of studied compounds.

The method was validated for linearity, precision as repeatability, LOD and LOQ. The repeatability of the analyses was evaluated by relative standard deviation (% RSD) of three replicate analyses of five calibration solutions within a day. The sample preparation repeatability (method precision) was established by carrying out the complete analysis three times. The LOD and LOQ were determined as the lowest concentration injected giving signal to noise ratio (S/N) $\geq$ 3 and 10, respectively.

3.1.4 **Stability tests with discussion**

The pure standards of *trans*-CA and *trans*-FA were dissolved in THF and MeOH to obtain concentrations of 1 000 mg L^{-1}. An aliquot of 50 μL from each solution was taken, spiked with 50 μL of ISTD (γ = 1 000 mg L^{-1}), evaporated, derivatized, diluted and measured by GC–MS immediately after preparation. To perform short- and long-term stability studies, each solution was divided into equal parts and transferred into graduated, glass-stoppered test tubes. Four parts of each solution were placed in conditions of RT and daylight; the second four parts were placed in conditions of RT and darkness, and the last four parts were stored in the freezer in the dark at -18 °C for varied periods of time (1, 10, 20 and 30 days).

In parallel, three parts of each solution were placed under the influence of UV at two different wavelengths, 254 nm and 366 nm, for varied periods of time (2, 4 and 6h). For this purpose, a UV cabinet (Camag 4) was used. After exposure to these conditions, an aliquot of 50 μL from each solution was combined with 50 μL of ISTD, evaporated, derivatized, diluted and analysed, according to the previously described procedure. The concentrations of *trans*-CA and *trans*-FA were determined from the corresponding calibration curves. The concentrations of the corresponding *cis*-forms were calculated from the difference between the total PAs contents and the concentrations of individual *trans*-isomers. *Cis*-isomers were the only degradation products confirmed.

Linear regression analysis proved that the responses for the investigated compounds were linear over tested concentration ranges from LOQ's−100 mg L^{-1}. The correlation coefficients (R^2) were above 0.9990 for both compounds in all calibration curves. The repeatability of chromatographic analyses confirmed by RSD, it was from 2.7% to 5.4% for FA, and from 1% to 5.7% for CA. The LOD and LOQ for *trans*-CA were 0.23 mg L^{-1} and 0.77 mg L^{-1}, respectively. The LOD and LOQ for *trans*-FA were 0.15 mg L^{-1} and 0.50 mg L^{-1}, respectively.

Although few studies have been reported about the *trans–cis* photoisomerization of PAs [58,167,177,178], this is the first work where the stabilities of *trans*-CA and *trans*-FA have been systematically tested under a range of storage conditions.

3.1.4.1 Stability of *trans*-CA

After being stored for 1 day at RT in darkness or in the refrigerator at -18 °C, the *trans*-CA dissolved in MeOH or THF was found to be quite stable, and isomerization occurred only in a small portion (up to 4.6%). This observation was previously confirmed and published by A. Le Person et al. (2013) [167], they proved that *trans*-*cis* isomerization is a reversible process. As expected, much more progressive isomerization of *trans*- to *cis*-CA was observed when the solutions were exposed for the same period of time to RT and daylight (up to 28%). The results showed that, with increasing storage time, the reduction of *trans*-CA dissolved in MeOH or THF, exposed to different storage conditions was in accordance with the increase in the *cis*-CA amount. The highest rate of isomerization was achieved after one month of aging of the MeOH-solutions at RT and in daylight (33%). In general, more protic solvent, higher temperature and longer time led to intensive isomerization. *Cis*-CA was the only degradation/isomerization product of *trans*-CA under all tested conditions, confirmed by GC-MS. Typical chromatogram of silylated compounds in a freshly prepared solution of *trans*-CA in MeOH is showen in **Figure 3-2**.

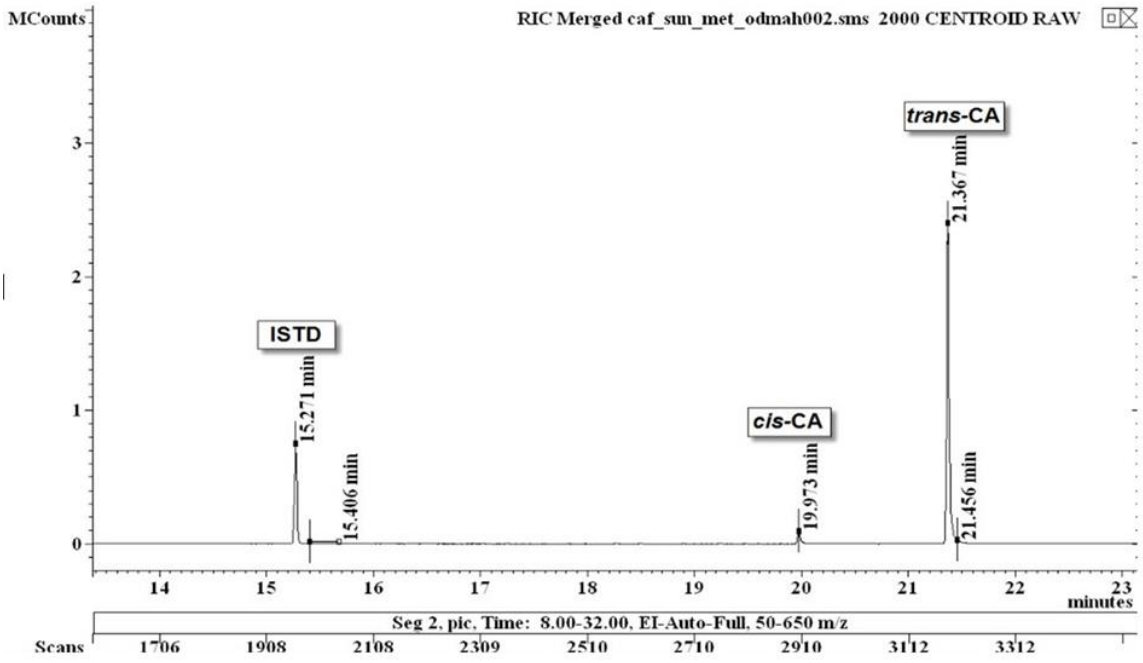


Figure 3-2. Typical chromatogram of silylated compounds in a freshly prepared solution of *trans*-CA in MeOH.

Further isomerization of *cis*-CA to esculetin and other products was not observed in our study. This result probably can be explained by the fact that photoisomerization of *trans*-caffeic acid followed by an intramolecular cyclization to esculetin is pH depending process

[58,167]. Complete results of the stability tests for pure *trans*-CA dissolved in two different organic solvents are presented in **Table 3-1**.

Table 3-1. Isomerization of *trans*-CA dissolved in THF or MeOH under the influence of various storage conditions.

Solvent	Storage conditions	RT				Freezing at -18 °C	
		Darkness		Daylight			
		Trans (%)	*Cis (%)*	*Trans (%)*	*Cis (%)*	*Trans (%)*	*Cis (%)*
THF	1 day	96.2±4.2	3.8±0.1	75.5±2.1	24.5±0.2	96.8±2.4	3.2±0.1
	10 days	85.1±3.8	14.9±0.7	70.9±0.5	29.1±0.5	85.7±5.3	14.3±0.2
	20 days	90.8±5.4	9.2±0.6	69.5±1.7	30.5±0.5	92.5±3.0	7.5±0.1
	30 days	91.8±5.6	8.2±0.1	67.4±1.4	32.6±0.1	92.1±0.7	7.9±0.9
MeOH	1 day	95.3±3.7	4.7±0.1	72.1±1.5	27.8±0.4	94.9±2.1	5.1±0.1
	10 days	79.8±2.2	20.2±0.3	68.4±1.4	31.6±0.5	86.1±2.6	13.9±0.1
	20 days	82.5±1.9	17.5±0.2	65.7±1.1	34.3±0.5	86.4±1.8	13.6±0.7
	30 days	82.6±2.0	17.5±0.2	65.4±2.0	34.6±0.7	87.1±1.2	12.9±0.4

The mass spectra of TMS-*trans*-CA and TMS-*cis*-CA (**Figure 3-3**) were almost identical, but when comparing both spectra in detail, it can be concluded that they differ slightly with respect to the intensities of certain fragment ions. The most intensive (100%) is a molecular ion and can be observed at *m/z* 396 for both compounds. Small differences appear in the intensity of the fragment ion signal at *m/z* 381, where it was more intensive for *cis*-CA. The loss of a methyl group from the molecular ion resulted in the fragment ion *m/z* 381, and further loss of the TMSO group produced the ion at *m/z* 293. Loss of the TMSO group from the main molecule led to the formation of the ion *m/z* 307. Simultaneous eliminations of TMS group and the methyl group led to the intensive *m/z* 219 ion.

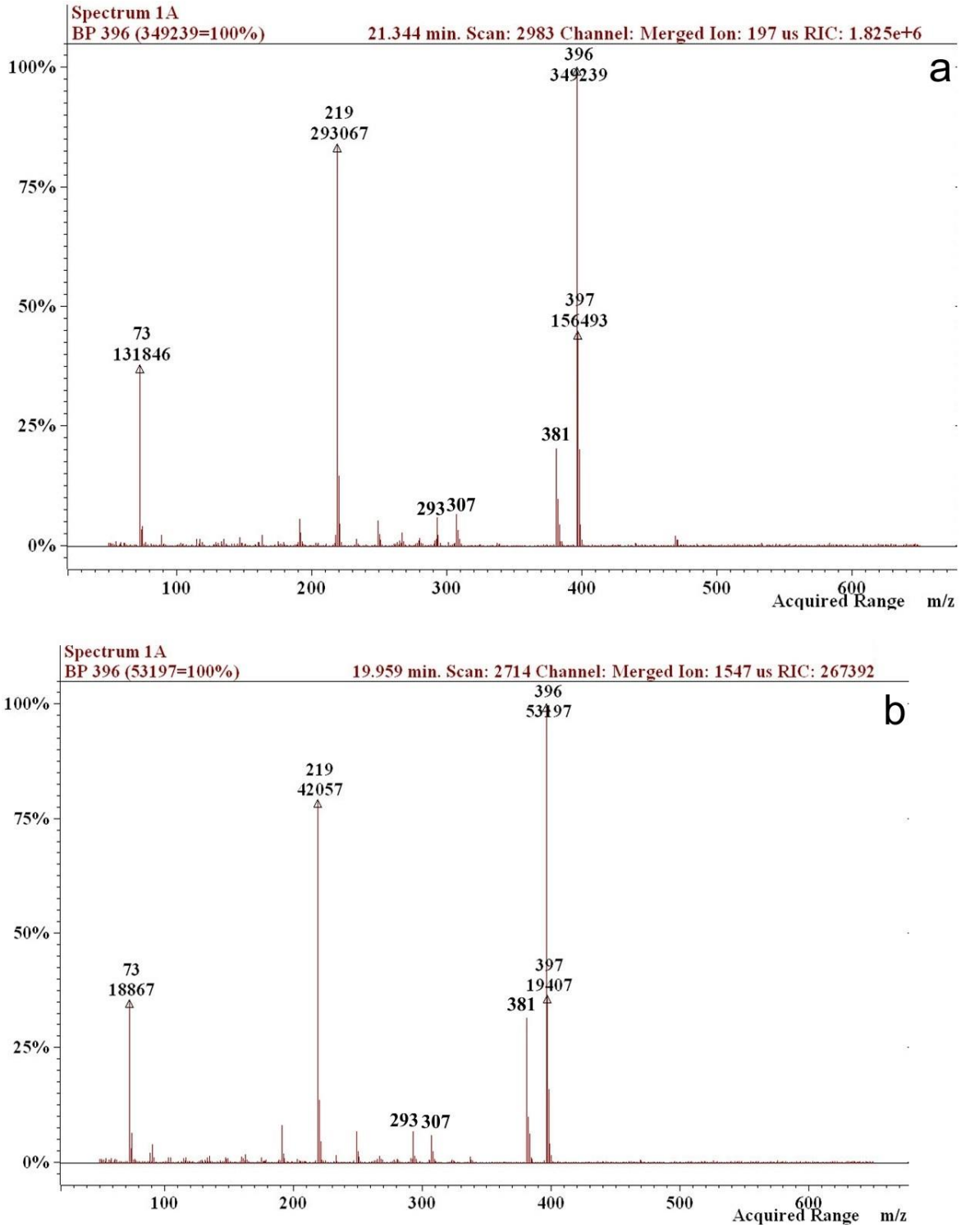


Figure 3-3. EI mass spectra of: a) *trans*-CA-3-TMS derivative; b) *cis*-CA-3-TMS derivative.

The proposed fragmentation pathway for the TMS derivative of CA are presented in **Figure 3-4** and are also in accordance with the available literature data [176].

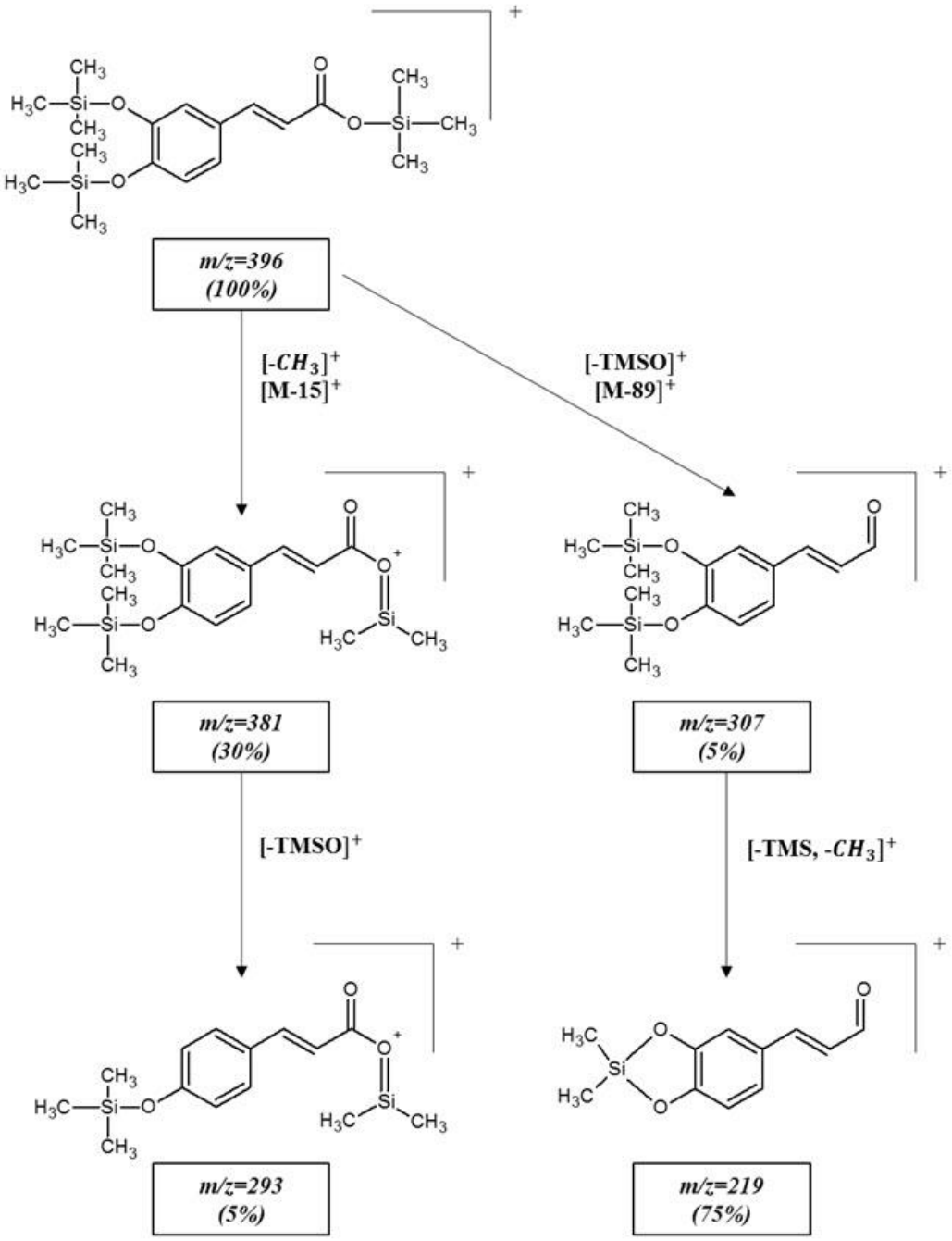


Figure 3-4. Proposed fragmentation pathway of the *trans*-CA-3-TMS derivative obtained under positive EI+ conditions.

3.1.4.2 Stability of *trans*-FA

The results proved that the reduction in *trans*-FA content dissolved in two different organic solvents (THF and MeOH) and exposed to varying storage conditions was also in accordance with the increase in the *cis*-FA amount. Markedly promoted isomerization was detected in the MeOH-solutions exposed to RT and daylight for 30 days (42.8%). After having been frozen at -18 °C for 1 day, the content of *trans*-FA in THF decreased insignificantly (about 5%). *Cis*-FA was the only degradant of *trans*-FA identified by GC-MS. Results of all stability tests performed on *trans*-FA dissolved in MeOH and THF are presented in **Table 3-2.**

Table 3-2. Isomerization of *trans*-FA dissolved in THF or MeOH under the influence of different storage conditions.

Solvent	Storage conditions	RT				Freezing at -18 °C	
		Darkness		Daylight			
		Trans (%)	*Cis (%)*	*Trans (%)*	*Cis (%)*	*Trans (%)*	*Cis (%)*
THF	1 day	92.8±2.8	7.3±0.1	74.6±1.0	25.4±0.2	94.8±1.3	5.2±0.2
	10 days	79.3±4.3	20.7±0.5	66.7±0.6	33.3±0.6	78.9±4.2	21.1±0.4
	20 days	88.5±5.8	11.5±0.2	66.9±2.1	33.1±1.6	88.5±3.4	11.5±0.3
	30 days	80.3±1.8	19.7±0.4	63.4±2.0	36.6±0.6	80.7±1.2	19.3±0.9
MeOH	1 day	94.7±1.7	5.3±0.4	57.5±2.1	42.5±1.1	89.3±2.9	10.7±0.3
	10 days	79.5±1.9	20.5±0.3	60.4±1.0	39.6±0.3	78.4±0.4	21.6±0.4
	20 days	80.9±2.0	19.1±1.0	58.9±0.7	41.1±0.8	83.9±4.5	16.1±1.9
	30 days	73.6±1.7	26.1±2.0	57.9±2.3	42.8±0.9	79.2±0.4	20.8±0.2

The geometric isomers *trans*-FA and *cis*-FA can be well distinguished by the order of elution during GC (*cis*-FA was eluted from the column about 1 min earlier than its *trans*-form), and by the relative intensities of certain fragment ion signals in their similar mass spectra. EI mass spectras of *trans*-FA-2-TMS derivative and *cis*-isomer is available in **Figure 3-5**. Differences are significant since intensities for the fragment ion signals at *m/z* 323, *m/z* 308, *m/z* 293 and *m/z* 219, are more intense for *cis*-FA than for *trans*-FA. The loss of a methyl group from the molecular ion resulted in the fragment ion *m/z* 323, and further loss of the methoxy group produced the ion at *m/z* 293. The loss of the methoxy group from the main molecule led to the formation of the ion *m/z* 308. The loss of the TMSO group from the molecular ion provided the *m/z* 249 ion, and further elimination of the -OCH_3 group led to the

fragment ion at *m/z* 219. The proposed fragmentation mechanism of *trans*-FA is presented in **Figure 3-6**.

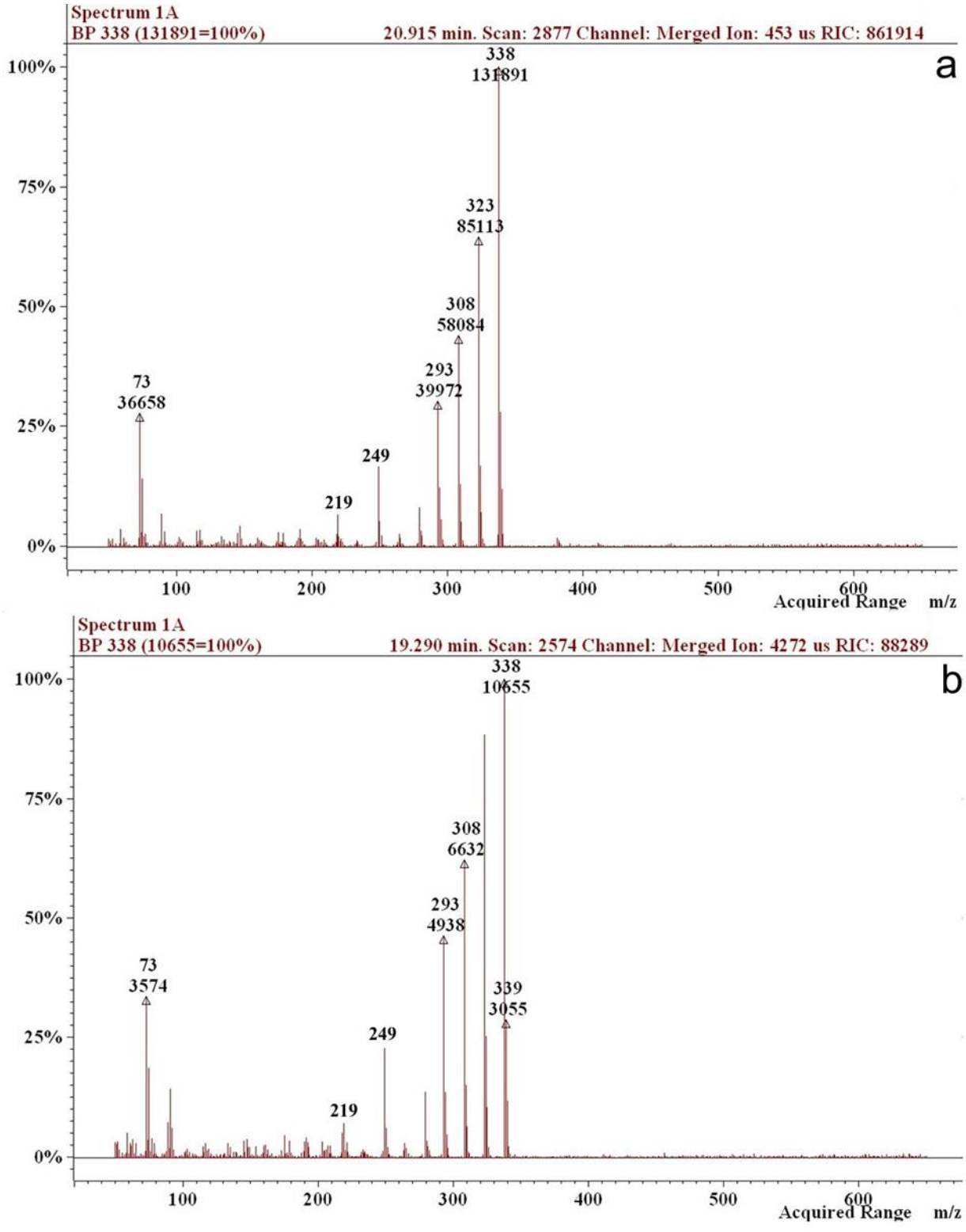


Figure 3-5. EI mass spectra of: a) *trans*-FA-2-TMS derivative; b) *cis*-isomer.

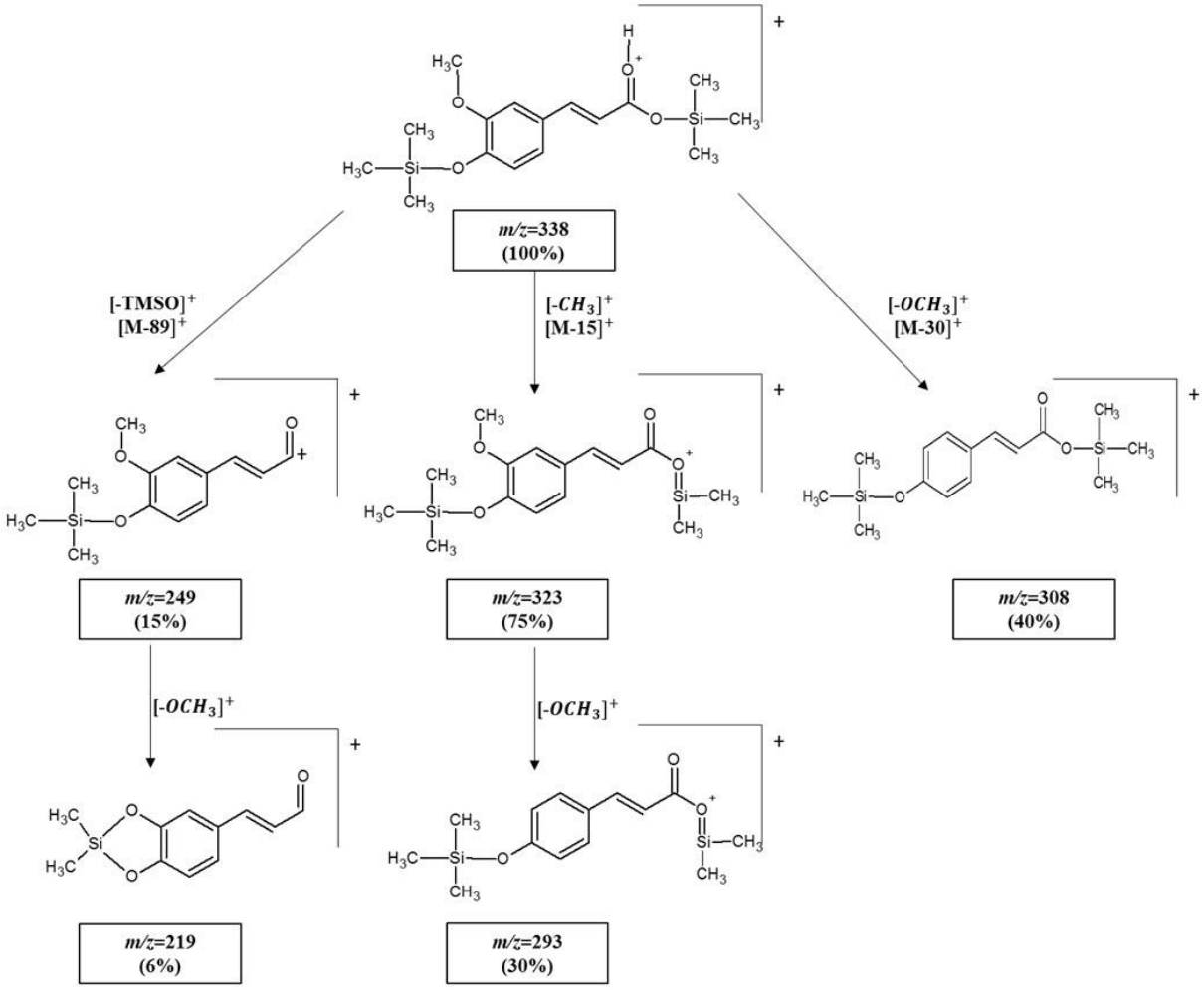


Figure 3-6. Proposed fragmentation pathway of the *trans*-FA-2-TMS derivative obtained under positive EI+ conditions.

3.1.4.3 Stability of *trans*-CA and *trans*-FA under the unfluence of UV radiation

Additionally, short term stability tests of CA and FA dissolved in organic solvents (MeOH and THF) and exposed to UV radiation at two different wavelengths (254 nm and 366 nm) were carried out. According to the results (**Table 3-3**), it can be concluded that maximum isomerization of *trans*-CA and *trans*-FA to their *cis*-forms (31.1% and 33.6%, respectively) was achieved in the THF solutions that were exposed to UV radiation at 366 nm, for 6 h. Isomerization took place to a greater extent at 366 nm than at 254 nm, in both solvents. This result pointed out that the isomerization of *trans*-PA seemed to be influenced by the corresponding absorption bands (λ_{max} values in MeOH solvents are 312 and 325 for CA and FA, respectively). Namely, Istasse et al. suggest that isomerization of *trans*-hydroxycinnamic acids to their *cis*-forms can occur even with very low light exposure [179]. This can be

explaned by the fact that light energy can unpair the electrons in the π bond so free rotation around C-C double bond can occur. Consequently, after rotation through 180° the unpaired electrons can pair up again forming the other geometric isomer [58].

Table 3-3. Isomerization of *trans*-CA and *trans*-FA dissolved in THF or MeOH under the influence of UV radiation at two wavelengths (254 nm and 366 nm).

Solvent	**Wavelength (nm)**	**Exposure time**	**CA**		**FA**	
			Trans (%)	*Cis (%)*	*Trans (%)*	*Cis (%)*
THF	254	2 h	94.9±3.1	5.1±3.0	90.6±1.2	9.4±1.1
		4 h	87.5±0.4	12.5±0.6	86.8±1.0	13.2±0.8
		6 h	74.7±0.5	25.3±0.4	73.0±0.7	27.0±0.5
	366	2 h	79.6±1.1	20.4±1.0	74.1±2.4	25.9±1.6
		4 h	73.2±2.7	26.8±1.6	69.4±3.8	30.6±3.2
		6 h	68.9±0.8	31.1±0.7	66.4±1.3	33.6±1.6
MeOH	254	2 h	96.3±1.2	3.7±1.2	94.2±2.1	5.8±1.0
		4 h	93.8±0.2	6.2±0.3	95.2±1.2	4.8±0.6
		6 h	90.8±1.0	9.2±1.1	98.7±1.3	1.3±0.9
	366	2 h	85.5±1.7	14.5±1.7	75.2±0.7	24.8±1.0
		4 h	82.3±0.9	17.7±1.0	74.1±1.5	25.9±0.8
		6 h	87.4±1.6	12.6±1.5	71.3±2.3	28.7±2.1

Ultimately, it can be concluded that this study has proven the reversible isomerization process of *trans*-PAs to their *cis*- forms, a process that was solvent, temperature and time-dependent. It was found that the *cis*- isomers were confirmed as the only degradation products in all the experiments.

3.2 Simultaneous GC-MS determination of free and bound PAs in Slovenian red wines and its chemometric characterization

Wine is a widely consumed beverage throughout the world and represent an important product of food industry with relatively high commercial value. As a complex matrix containing several hundreds of different chemical compounds [180] wine presents an analytical challenge, especially for identification and quantification of compounds in low concentration ranges [181]. The chemical composition of red wines includes minerals, vitamins, proteins, sugars and PCs.

The total amount of PCs in red wines has been estimated in the range from 2 000 to 6 000 mg L^{-1} (GAE equivalent) [182], and they are generally classified as flavonoids including: anthocyanins, flavon-3-ols, flavonols and dihydroflavonol and non-flavonoids such as PAs and stilbenes. The concentration levels of PCs in wine are influenced by several oenological factors such as origin, vine variety, winemaking practices, ageing and vintage. Their quantitative determination is of considerable importance, since it is known that they are responsible for the wine's taste, colour (anthocyanins), and for beneficial health effects including antioxidant and anti-inflammatory activities [180,183]. Red wines are considered to have more protective function than white or rosé wines, because of their higher content in antioxidant substances released from the grape skin and seeds [184]. Recent studies showed that daily consuption of normal amounts of red wine reduce health disease realted to the heart and atherosclerosis [185].

Except for the positive effect on human health, PAs from the wine are also one of the important factors for its quality. Namely, from the literature it is known that lactic acid bacteria (LAB) are responsible for the occurrence of malolactic fermentation (MLF), a secondary fermentation which is considered to be beneficial in most red wines [186]. Consequently, the content of PAs in grapes and wines can positively or negatively affect the rate of MLF [187]. For example, gallic acid at low concentrations has stimulatory effects on the growth and malolactic activity of LAB [188]. On the other hand, some PAs, especially those from the hydroxycinnamic class, delayed the conclusion of malolactic fermentation by this bacterium. Hydroxycinnamic acids (particularly *p*-coumaric acid) are also known to inhibit the growth of

a variety of microorganisms, including wine-spoiling strains of *L. collinoides*, *L. brevis* and *L. hilgardii* [189].

Although Slovenia is a small country, its wine production has a significant role in the economy. In Slovenia there are more than 28 000 wineries, producing between 80 and 90 million litres annually, of which 25% is red wine. Altogether, 22 000 hectares of vineyard area is divided among three major regions (Drava Valley-Podravje, Lower Sava Valley-Posavje and the Littoral-Primorska) with further division into sub-regions [181], [190]. The Slovenian Littoral is Slovenia's most widely known and prominent wine region of both white and red wines. Slovenian vineyards are planted with different vine varieties, including Merlot, Cabernet Sauvignon, Chardonnay, Pinot Noir, Syrah, Barbera, and many others [181].

Wine classification is a very important topic for detecting possible frauds and establishing wine authenticity, which is an important consideration in international markets [191]. This process consists of building mathematical-statistical models based on quantitative and qualitative information about the wine's natural constituents such as content of trace elements [192], organic wine constituents such as volatile compounds [193], sugars [194], PCs [195] and etc. Slovenian wine legislation also [196] prescribes that all wines should be submitted to chemical and sensory-organoleptic analysis before being released to the market. After wines pass the tests, they are assigned a quality level according to the »*Zaščiteno geografsko poreklo* (ZGP)«, which is similar to the European Union's quality wines produced in specified regions (QWPSR) system. Several articles about different analytical techniques, including HPLC, high-performance ion chromatography exclusion (HPICE), inductively coupled plasma emission spectroscopy (ICP-OES), isotope ratio mass spectrometry (IRMS), site-specific natural isotopic fractionation nuclear magnetic resonance (SNIF-NMR) and inductively coupled plasma mass spectrometry (ICP-MS) for the determination of different compounds and chemometric classification of Slovenian wines have been published [190,197,198]. Consequently, the focus of this research was on the development of an acceptable statistical-mathematical model for the classification of Slovenian red wines based on their content of PAs.

In the nature, PAs are present in their free forms or as glycosylated and esterified derivatives [199–201]. As mentioned in the theoretical part of this thesis, acidic, basic and enzymatic hydrolysis are the most commonly used methods for the extraction of PAs from natural materials. In this part of work basic hydrolysis was optimized.

From the literature, it was obvious that the most commonly used technique for the determination of PCs from wine samples is HPLC with UV or DAD detection or GC-MS [202]. Usually, for HPLC analysis, the wine sample can be directly injected into the system without any purification while GC-MS methods require LLE or SPE extraction followed by derivatization of the target compounds prior to the GC analysis. Due to the longer sample preparation process for the analysis, GC-MS is not often used. But compared to the HPLC method, GC-MS offers several advantages, including complete and high-resolution separation, sensitive detection, unambiguous identification and quantitation of a wide range of phenolics (including all isomers) in one chromatographic run.

3.2.1 Chemicals

Standard compounds: *trans*-caffeic acid (99%), vanillic acid (97%), syringic acid (97%), *trans*-p-coumaric acid (98%), *trans*-o-coumaric acid (98%) and *trans*-ferulic acid (98%) were supplied by Merck (Germany).

Solvents: THF (99.5%) and pyridine (99.9%) were supplied by Merck (Germany). HPLC-grade MeOH was purchased from Sigma (USA). GC-grade toluene (99.5%) and HCl (36.5%) were purchased from Carlo Erba (Italy), while dichloromethane (DCM) was purchased from JT Baker (Germany). The water used was obtained from a Milli-Q water purification system.

Other chemicals: derivatization reagent MSTFA and NaOH (99%) were purchased from Sigma (USA). L-ascorbic acid (99.7%) was purchased from Alkaloid (Macedonia), while EDTA was purchased from Kemika (Croatia).

3.2.2 Wine samples

The developed GC-MS method was tested using Slovenian red wine samples. Twelve red wines from different Slovenian wineries and different varieties were purchased from local supermarkets. All the tested wine samples orginated from four vintages (2011-2015). **Table 3-4** summarized the most important information about wines used in this study such as: varieties, wineries, year of production and percentage of alcohol. Wines were stored in a refrigerator at the temperature of +4 °C until analyzed.

Table 3-4. Characteristics of the analyzed wine samples.

Sample code	Variety	Variety code	Winery	Wine region	Year of production	% alcohol*
SW1	Cabernet Sauvignon	1	„Vina Koper“	Primorska	2014	13.0
SW2	Modra Frankinja	2	„Stari Hram“	Posavje	2014	10.5
SW3	Cabernet-Sauvignon	1	„Vipava“	Primorska	2014	11.0
SW4	Modri Pinot (Pinot noir)	2	Štajerska Slovenia-Ptuj	Podravje	2011	12.5
SW5	Cabernet Sauvignon	1	Goriška Brda	Primorska	2013	12.5
SW6	Refošk	3	Srednje Škofije	Primorska	2014	11.0
SW7	Refošk	3	„Vina Koper“	Primorska	2014	12.5
SW8	Modra Frankinja	2	Štajerska Slovenia-Ptujska Klet	Podravje	2011	11.5
SW9	Modri Pinot (Pinot Noir)	2	„Vipava“	Primorska	2013	12.0
SW10	Portugalka	3	Bela Krajina	Posavje	2015	11.0
SW11	Cabernet Merlot	1	Jeruzalem-Ormož	Podravje	2013	12.5
SW12	Metliška Črnina	2	Bela Krajina	Posavje	2012	11.5

*According to the declaration on the wine bottle.

3.2.3 Extraction of free PAs

In order to determine the extraction recoveries, optimization of the extraction process was performed on the synthetic wine spiked with known concentration of standard compounds. Synthetic wine represent hydroalcoholic solution of 5 g L^{-1} tartaric acid with 12% of ethanol, and pH value of 3.2 [203].

A standard solution of PAs mixture (in a concentration of 1 000 mg L^{-1}) was prepared in MeOH. Solutions of PAs mixture in synthetic wine were prepared by pipetting 30 and 100 µL of standard solution, respectively, in a 10 mL volumetric flask, and diluted with synthetic wine up to the mark. 1 mL of each solution was transferred into a 50 mL conical flask, spiked with 50 µL of ISTD (1 000 mg L^{-1}), diluted with 1 mL of ultra-pure water and acidified with 6 M HCl to a pH value of 2. Prepared samples were added to pre-conditioned HLB Supelco® SPE cartridges (3 mL, 60 mg stationary phase made from hydrophilic modified styrene). A schematic procedure of the sample extraction is shown in **Table 3-5**. The free PAs fraction was eluted with 2 x 2 mL of THF. The eluate was collected and dried in a rotary evaporator

(at 40 °C) to absolute dryness. Then the sample was derivatized by adding 100 μL of MSTFA and 50 μL piridine, heated at 80 °C for 1 h, diluted with toluene, and analyzed by GC-MS. The analyses were carried out in triplicate. The accuracy of the extraction process was determined through the recovery value in % of the PAs.

Table 3-5. Sample extraction by SPE (using HLB Supelco® cartridges).

Sample extraction by SPE
1. pre-washing of cartridge with 2 x 2 mL DCM
2. column conditioning: 2 x 2 mL of MeOH and 2 x 2 mL acidified water (pH=1-2)
3. sample application: 2 mL of the acidified sample
4. column washing: 2 x 2 mL ultra-pure water
5. elution: 2 x 2 mL THF

For the determination of free PAs in selected red wines, the samples were prepared according to the same procedure. 1 mL of homogenized wine sample was spiked with 50 μL of ISTD, diluted with 1 mL of ultra-pure water and acidified with 6 M HCl to a pH value of 2 followed by the previously described steps.

3.2.4 Alkaline hydrolysis and extraction of bound PAs

The stability of the studied compounds and their recovery in % after alkaline hydrolysis was also firstly determined on the standard compounds and later an optimized procedure was applied on the real wine samples. Standard solution of PAs mixture (at a concentration of 1 000 mg L^{-1}) was prepared in MeOH. Solutions of PAs mixture in synthetic wine were prepared by pipetting 30 and 100 μL of standard solution, respectively, into the 10 mL volumetric flask, and diluted with the synthetic wine up to the mark. 1 mL of each solution was transferred into a 50 mL conical flask, spiked with 50 μL of ISTD, and exposed to alkaline hydrolysis, according to the previously described method with some modifications [67]. Namely, 1 mL of the spiked synthetic wine was treated by adding 9 mL of 2 M NaOH (which contained 1% L-ascorbic acid and 10 mM EDTA as stabilizers) for 2 h at RT. Then the sample was acidified to pH=2 using 6 M HCl, and PAs were extracted with SPE HLB cartridges. The whole procedure with alkaline hydrolysis was repeated also without stabilizers.

3.2.5 Preparation of calibration curves

Standard stock solutions of caffeic acid, vanillic aicd, syringic acid, p-coumaric acid and ferulic acid, as well as of o-coumaric acid (ISTD) were prepared by accurately weighing 10 mg of each into a 10 mL volumetric flask, and then dissolving in THF [181]. Five calibration standard solutions were prepared by combining various volumes of PAs stock solutions with 50 μL of ISTD in a 50 mL conical glass flask. Each solution was derivatized by treating it with 100 μL of MSTFA and 50 μL of pyridine for 1 h at 80 °C in a sand bath [181].

After derivatization was finished, TMS derivatives were quantitatively transferred to 1 mL flasks and filled up to the mark with toluene. Five calibration standard solutions in concentration range from 1 to 100 mg L^{-1} were injected in triplicates. The calibration curves were constructed by linear regression of the peak-area ratio of individual PA standard to the ISTD (y), versus the concentration (mg L^{-1}) (x).

3.2.6 GC–MS instrumentation and data analysis

TMS derivatives of PAs were analyzed with a Varian 3900 GC, coupled to MS/MS Saturn 2100 ion trap MS. GC separation was performed using a Varian capillary column VF-5ms CP8944 (30 m × 0.25 mm, with the stationary phase 0.25 μm). 1 μL of the sample was injected in split mode (split ratio 1:10). Carrier gas was He (6.0 UHP) at a flow rate of 1.0 mL min^{-1}. The initial oven temperature was 40 °C, held for 1 min, and then the temperature was raised to 320 °C at a rate of 10 °C min^{-1}, and finally, held for 3 min. The total run time was 32 min. The injection-port and transfer-line were set to 250 °C and 170 °C, respectively. Mass spectra were recorded in SCAN or SIM mode in a range from 50 to 650 *m/z* using electron ionization energy at 70 eV. Peak identification was done by comparing retention times (t_R) and spectral properties with those of standard compounds or by library matching from NIST MS library containing the mass spectra of TMS derivatives of PAs. The method was validated for linearity, precision as repeatability, LOD and LOQ. For linearity determination, all calibration curves were constructed using the internal standard method. The curves were fitted to linear least-squares regression. The precision was evaluated through the within-day (WD) and between-days (BD) repeatability, and expressed as RSD. The LOD was calculated using the equation $(3 \cdot s_{y/x})/b_0$ and the LOQ was calculated from the equation $(10 \cdot s_{y/x})/b_0$ (where $s_{y/x}$ is

standard deviation of linear regression and b_0 is slope of the calibration line). The contents of free and total PAs were determined from the corresponding calibration curves using the ISTD method, taking into account the recovery of the extraction procedure. PAs from the hydroxycinnamic group exist in *trans*- and *cis*-forms, both found in plants. Although, *trans*-forms of PAs are naturally predominant isomers, for quantitative determination, the peak areas of the *trans*- and *cis*-forms of caffeic acid, p-coumaric acid and ferulic acid were summed.

Chemometrical data analysis was carried out in order to discover any statistically or other significant differences between the samples grouped according to two categorical variables-vine variety and Slovenian wine regions. Microsoft Excel was used for the data preparation and result outputs. Statistical data treatment was performed using SPSS Statistics version 22.

3.2.7 Optimization and validation of GC-MS method

In the first part of this study, GC-MS method for the quantitative determination of five target PAs (caffeic acid, vanillic acid, syringic acid, p-coumaric acid and ferulic acid) in red wine samples was otpimized and validated. All GC-MS SCAN parameters for TMS standard compounds, together with their retention times and characteristic fragment ions, are listed in **Table 3-6.**

Table 3-6. Retention times and fragmentation parameters for TMS PAs obtained after trimethylsilylation using the ion-trap mass detector.

Compound	t_R	Characteristic fragmentation ions *m/z* (relative intensity %)
cis-o-Coumaric acid	16.65	147 (100); 293; 308
Vanillic acid	17.70	253; 267; 282; 297(100); 312
cis-p-Coumaric acid	17.94	219; 249; 293 (100); 308
trans-o-Coumaric acid	18.18	147; 219; 293 (100); 308; 381
Syringic acid	19.11	298; 312; 328; 342 (100)
cis-Ferulic acid	19.32	249; 293; 308; 323; 338 (100)
trans-p-Coumaric acid	19.49	219; 250; 293 (100); 308; 381
cis-Caffeic acid	19.99	219; 381; 396 (100); 397
trans-Ferulic acid	20.95	249; 293; 323; 338 (100)
trans-Caffeic acid	21.38	73; 219; 381; 396 (100)

Linear regression analysis proved that the responses for all of the investigated compounds were linear over the tested concentration range (1−100 mg L^{-1}), and R^2 were above 0.999. The results of the regression analysis and calibration data are shown in **Table 3-7**. **Table 3-7** also

shows the WD and BD repeatability expressed as RSD, and it gives RSD below 2% in all cases. The determined values of LODs and LOQs for all selected PAs are also summarized.

Table 3-7. Validation parameters for investigated PAs.

PA	Linear correlation	R^2	[1]WD-RSD	[2]BD-RSD	LOD*	LOQ*
Vanillic acid	y=0.0477x + 0.0771	0.9999	0.11	0.72	0.05	0.17
Syringic acid	y=0.0231x + 0.0921	0.9999	0.95	1.81	0.06	0.20
p-Coumaric acid	y=0.0398x + 0.0555	0.9996	0.38	1.47	0.06	0.20
Caffeic acid	y=0.0558x + 0.0986	0.9999	1.36	1.97	0.07	0.23
Ferulic acid	y=0.0324x + 0.0718	0.9996	1.01	1.81	0.03	0.10

[1]Within-day PA/ISTD peak-area ratio repeatability of individual PAs at the concentration 10 mg L^{-1}, expressed as %RSD.

[2]Between-days PA/ISTD peak-area ratio repeatability of individual PAs at the concentration 10 mg L^{-1}, expressed as %RSD.

*LOD and LOQ are in mg L^{-1}.

3.2.8 Optimization of the extraction procedures

Some authors have evaluated PCs in wine after direct injection of the samples (appropriately diluted with ultra-pure water) into a chromatographic system, usually HPLC-UV [204–206]. This practise can give satisfactory results for the determination of high-content PCs, such as anthocyanins.

However, for the quantitative determination of other subclasses of PCs (PAs and others) present in lower concentrations in the wines, extraction, hydrolysis and concentration by different extraction techniques are required. Formerly, the most used extraction technique for the isolation of wine PCs was LLE using different non-polar organic solvents. Now, this method is not as accepted, mainly due to the use of large quantities of toxic organic solvents, low recoveries of target analytes and poor repeatability. SPE using different cartridges is a good alternative. Consequently, the focus in the present study was the development of an easy and fast SPE procedure for the extraction of target PAs from the wine samples.

The first step in the development of the SPE method was the selection of the most appropriate SPE SF. Cartridges with C18 SF are the most commonly used in PCs separation [91,207], but it has been proved that this SF has some disadvantages, such as low recoveries for some highly polar compounds and PAs, along with the fact that care must be taken to avoid drying the conditioned sorbent because its efficiency is reduced [208]. Additionally, it has been shown that anthocyanin-type pigments can interfere in the chromatographic separation and identification of non-anthocyanin PCs [209]. Because the focus of the present study was

on the determination of PAs, all the disadvantages of C18 cartridges were solved by using the HLB stationary phase.

The HLB water SF is a co-polymer made of two monomers divinylbenzene and N-vinylpyrrolidone. The researchers have been proved this SF as the effective for both polar and nonpolar compounds, with good retention [210]. Another advantages of HLB cartridges over conventional used C18 are that more polar interferences (e.g. sugars) can be eliminated with water without losing analytes, higher sensitivity, good repeatability, reproducibility, and high recovery percentages. The optimal separation protocol for the sample preparation by using HLB cartridges is shown in **Table 3-5**. As can be seen from the protocol, THF was selected as the eluent solvent, instead of the most commonly used MeOH. Namely, PAs showed good solubility in THF and this solvent is more suitable for the analysis by GC-MS.

Accuracy of SPE in measuring free PAs was evaluated by spiking a synthetic wine with the standard solution at two different concentration levels (30 and 100 mg L^{-1}). After extraction and derivatization (MSTFA), the extract was submitted to the GC-MS analysis and the concentrations were obtained using the coresponding calibration curves. The recoveries of free PAs ranged from 93% to 114% (**Table 3-8**). These results are in agreement with the results reported by other authors [211,212].

The same SPE protocol was used for the isolation of bound PAs after alkaline hydrolysis (without or with stabilizers), and the recovery of the standard compounds were also determined (**Table 3-8**). The results prove that hydrolysis without stabilizers (L-ascorbic acid and EDTA) led to a complete loss of caffeic acid [67] and promoted isomerization of *trans*-p-coumaric acid to its *cis*-form (**Figure 3-7**). For all other investigated compounds, the recoveries were above 96%.

Table 3-8. Determination of the method accuracy expressed as recovery (%).

Recovery of extraction procedure (%)					
Phenolic acid	**Free PAs**		**Bound PAs**		
			In the presence of stabilizer		**Without of a stabilizer**
	Concentration (mg L^{-1})				
	30	100	30	100	100
Vanillic acid	105.5±4.8	98.9±0.2	114.2±6.8	105.2±3.2	113.7±2.8
Syringic acid	106.8±1.3	93.6±3.8	107.5±6.4	101.4±2.8	105.4±1.8
p-Coumaric acid	101.7±2.2	106.6±2.5	110.5±5.6	103.0±2.7	135.1±10.3
Ferulic acid	94.5±3.5	96.2±1.4	97.4±3.7	104.4±2.2	133.7±0.8
Caffeic acid	102.7±2.4	106.5±2.1	105.9±3.2	96.3±2.4	NQ[a]

[a]NQ-not quantified. Concentration (mg L^{-1})<LOQ (for details see **Table 3-7**).

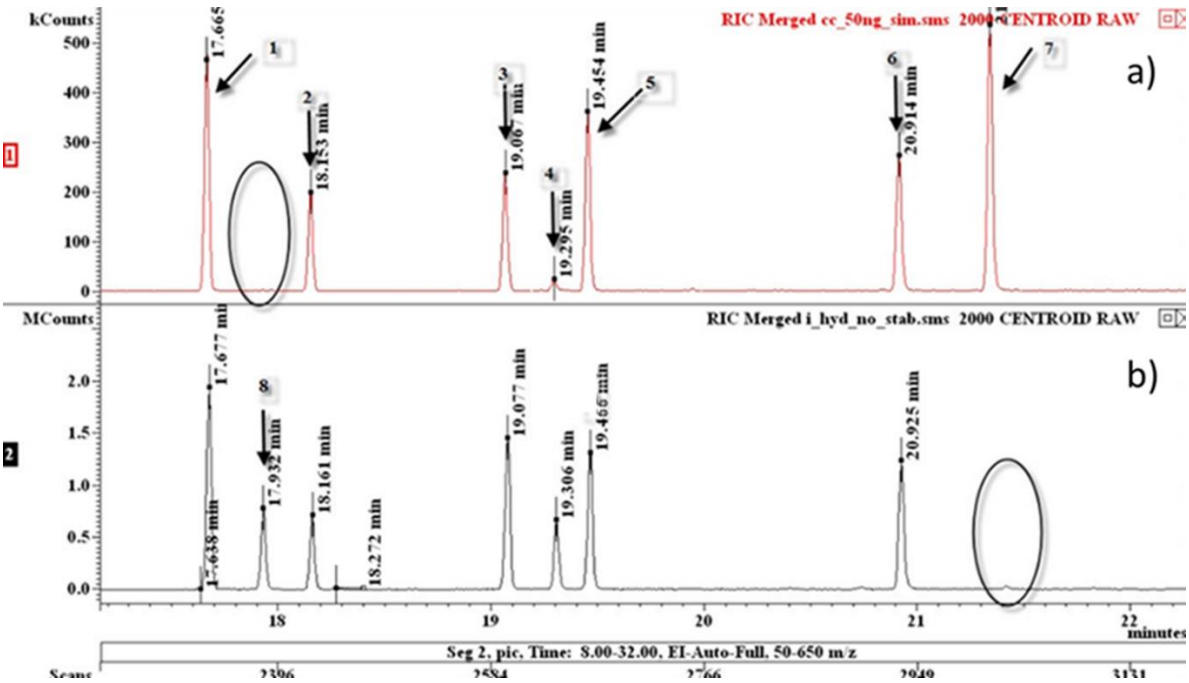


Figure 3-7. Chromatograms of standard solutions after; a) hydrolysis in presence of stabilizer (1.vanillic acid; 2. o-coumaric acid; 3. syringic acid; 4. *cis*-ferulic acid; 5. *trans*-p-coumaric acid; 6. *trans*-ferulic acid; 7. *trans*-caffeic acid); b) hydrolysis without stabilizer (8. *cis*-p-coumaric acid).

3.2.9 Application of developed methods on real wine samples

The method developed was then used for the determination of selected PAs in twelve Slovenian red wine samples. **Figure 3-8c** presents a typical chromatogram of wine extract. Contents of five different PAs present in red wine samples are shown in **Table 3-9** (mean value ± sd). From these results it can be concluded that caffeic acid and p-coumaric acid are the most important of total PAs, with contents ranging from 17.1 to 71.9 mg L^{-1}. In all of the wines investigated ferulic acid, is present at the lowest concentration level. It was measured only in wines from the Posavje region and in two samples from the Primorska region.

It is also worth to mention that red wine sample SW12 represent a mixture of red wine varieties Modra Frankinja, Žametovka, Portugalka and Šentlovrentka and therefore is very specific red wine sample. This fact was confirmed by the obtained results (**Table 3-9**) as the contents of all investigated PAs in the sample SW12 were comparable with contents in the samples SW2 and SW10, both of them belonging to varieties Modra Frankinja and Portugalka, respectively. The largest proportion of PAs was present in bound form.

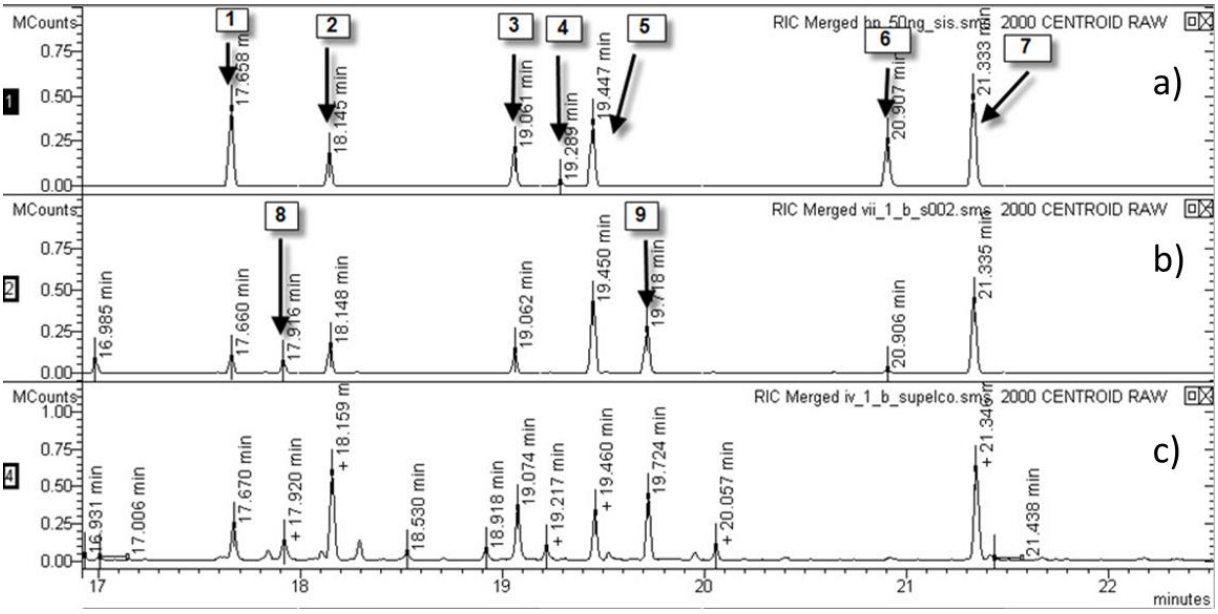


Figure 3-8. Typical chromatograms of selected PAs recorded in: a) SIM mode of standard mixture; b) SIM mode of red wine extract and c) SCAN mode of red wine extract (1. vanillic acid; 2. o-coumaric acid; 3. syringic acid; 4. *cis*-ferulic acid; 5. *trans*-p-coumaric acid; 6. *trans*-ferulic acid; 7. *trans*-caffeic acid; 8. *cis*-p-coumaric acid; 9. L-ascorbic acid (stabilizer)).

Table 3-9. Content (mg L^{-1}) of free and total PAs in Slovenian red wines.

Wine	Form	Phenolic acid[a]				
		Vanillic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Caffeic acid
SW1	Free	NQ[b]	3.79±0.42	7.72±0.16	ND[c]	3.24±0.18
	Total	3.79±0.42	19.81±0.35	32.61±0.30	ND	17.13±0.59
SW2	Free	1.00±0.08	3.23±0.31	0.73±0.08	NQ	5.39±0.91
	Total	14.47±2.85	29.84±3.13	28.05±1.37	0.10±0.00	49.75±2.76
SW3	Free	NQ	NQ	NQ	NQ	NQ
	Total	5.40±0.01	14.93±1.42	37.45±2.12	ND	17.80±0.41
WW4	Free	2.07±0.16	10.66±0.16	1.95±0.11	ND	11.79±1.15
	Total	12.58±1.85	25.86±0.52	31.01±1.07	ND	44.52±1.99
SW5	Free	0.88±0.09	5.06±0.11	1.58±0.07	ND	3.98±0.31
	Total	7.26±0.63	21.87±2.57	48.37±0.86	ND	29.32±0.45
SW6	Free	1.61±0.13	4.79±0.36	0.71±0.06	NQ	4.79±0.09
	Total	9.51±1.06	25.23±2.84	42.76±3.57	2.50±0.78	41.00±0.07
SW7	Free	2.46±0.43	3.39±0.71	1.36±0.13	NQ	3.63±0.36
	Total	10.33±1.36	29.95±3.04	71.86±0.16	4.90±0.13	48.09±1.10
SW8	Free	3.92±0.78	4.41±0.40	2.13±0.18	NQ	3.54±0.36
	Total	14.58±1.02	19.96±0.01	9.52±0.71	NQ	36.87±0.49
SW9	Free	5.12±0.11	5.55±0.18	NQ	ND	0.67±0.00
	Total	10.97±0.57	19.66±0.31	20.94±0.08	NQ	38.42±0.62
SW10	Free	4.92±0.31	7.67±0.26	NQ	NQ	NQ
	Total	7.73±0.80	29.46±2.22	54.23±1.18	0.71±0.08	64.04±0.61
SW11	Free	1.97±0.07	4.64±0.27	2.95±0.06	ND	4.13±0.00
	Total	11.84±0.31	29.67±2.48	63.10±0.87	ND	33.66±0.33
SW12	Free	6.03±0.59	10.32±0.83	NQ	NQ	NQ
	Total	14.55±0.21	27.58±1.09	47.23±0.61	0.20±0.01	64.42±0.62

[a]Each value is the mean (mg L^{-1}) of three independent replicates ± standard deviation.
[b]NQ-not quantified. Concentration (mg L^{-1})<LOQ (for details see **Table 3-7**).
[c]ND-not detected. Concentration (mg L^{-1})<LOD (for details see **Table 3-7**).

3.2.10 Discussion and chemometric classification of Slovenian red wines

Exploratory data analysis was performed using the SPSS program. In the first step we searched for outliers but no outliers were found in the dataset. Departures from the normal distribution were demonstrated by the Q-Q plots and tested with the Kolmogorov-Smirnov test. Significance value for all tested variables was above 0.05, which indicates normal distribution of data. A direct examination of any inter-relation between two continual variables is mostly realized by correlation analysis determining the extent to which the values of the two variables are mutually dependent [213]. The Pearson (pair) and Spearman tests are the most commonly used methods for that purpose. The Pearson correlation test (0.01 and 0.05 significance levels) was applied to determine any inter-relation between two variables in our research (**Table 3-10**). Statistically significant positive correlations were found only between

caffeic acid and syringic acid at the 0.01 level (0.744), and between caffeic acid and vanillic acid at the 0.05 level (0.592).

Table 3-10. The parametric Pearson corelation test.

	Vanillic acid	Siringic acid	p-Coumaric acid	Ferulic acid	Caffeic acid
Vanillic acid	1				
Siringic acid	0.477				
p-Coumaric acid	-0.212	0.574*			
Ferulic acid	-0.035	0.386	0.573*		
Caffeic acid	0.592*	0.744**	0.248	0.239	1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Principal component analysis (PCA) is an unsupervised multidimensional method used for reducing the number of variables along with preserving the information contained in the data table. Projection of the wines on the first two principal components (accounting for 79.8% of the total data variability) demonstrates a clear separation of the samples according to the vine variety (**Figure 3-9**). The first principal component (PC1) explained 53.7% of the variation between the samples, and the second (PC2) explained 26.1% of the variation. Wines from the Cabernet-Sauvignon variety (group 1 on the biplot) were separated from the other samples, and formed a group in the positive part of PC2, while the Modri Pinot variety (group 2 on the biplot) formed a group in the negative part of PC2.

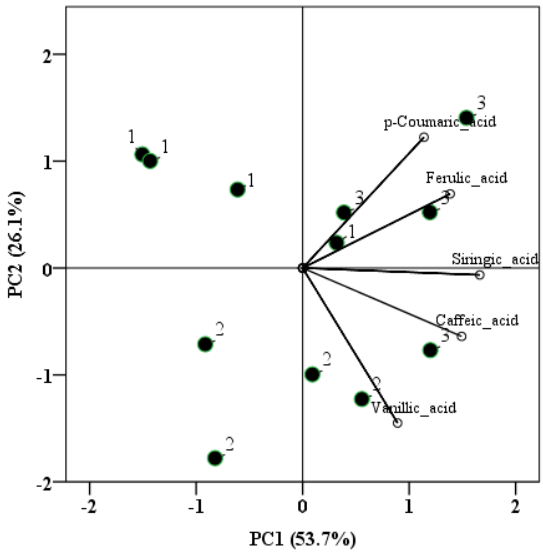


Figure 3-9. PCA bi-plot in the plane PC2 vs. PC1. The objects are labelled by vine varieties. The first principal component (PC1) explained 53.7% of the variation between the samples, and the second (PC2) explained 26.1% of the variation.

For linear discriminant analysis (LDA) classification of the samples according to their vine varieties, all parameters were taken as statistical important variables. The graphic output of the classification is shown in **Figure 3-10**. The classification rates for the categories mentioned were acceptable and good; overall, the correct classification ratio was 100% for the training set and almost 85% for the validation set. This analysis showed a strong similarity between the Modri Pinot and Modra Frankinja vine varieties, and together, they formed a group in the negative part of DF1. The similarity between these two varieties, according to some other parameters, was also shown. Using the selected descriptors, the Cabernet-Sauvignon variety was distinguished from the other classes and, and formed a group in the positive part of DF1.

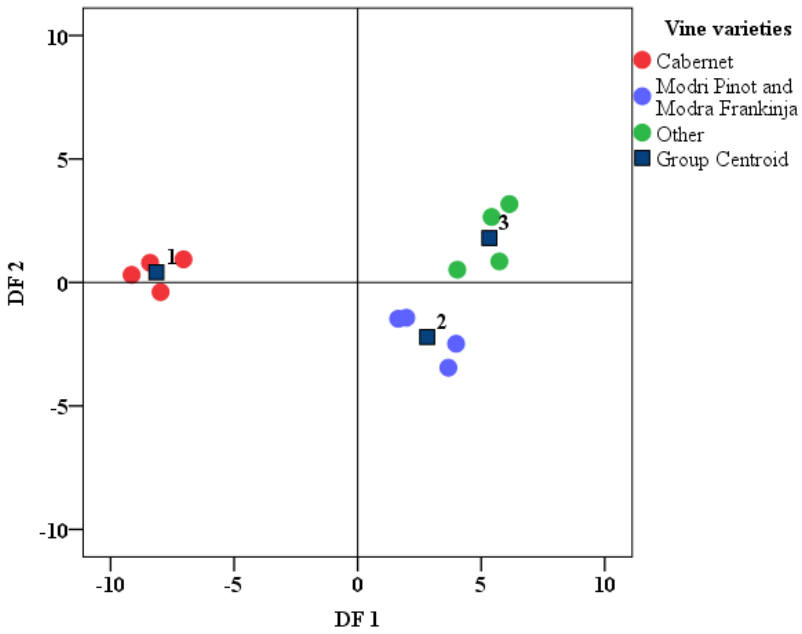


Figure 3-10. Graphic output of LDA in the plane of the first two discriminant functions. Classification according to the vine varieties.

LDA was also used to categorise wine samples according to the Slovenian wine regions by employing optimally selected variables: caffeic acid, ferulic acid, vanillic acid and syringic acid content; the results are presented in **Figure 3-11.** The classification ratio was 91.7% for the training set and 75% for the validation set. Using the aforementioned descriptors, the wine samples from the Primoska region could be distinguished from the other two regions, and formed a group in the positive part of DF1.

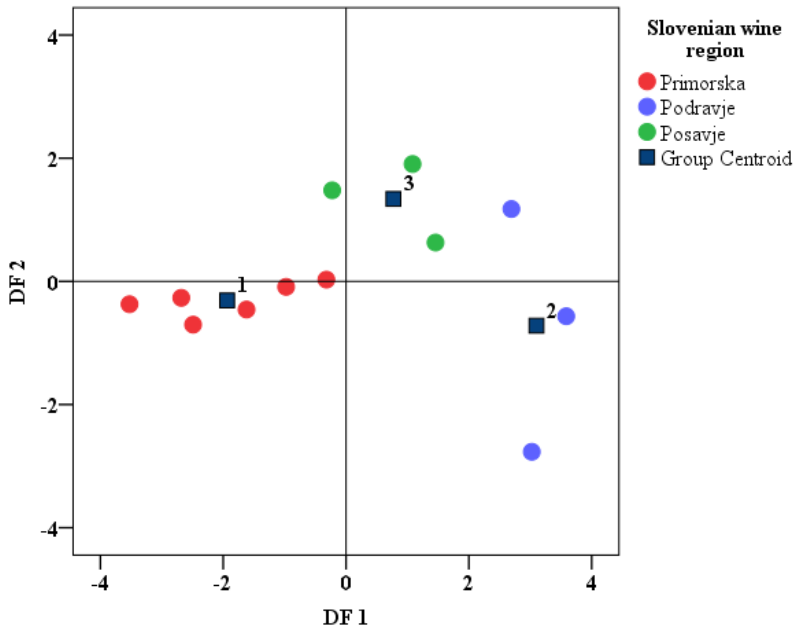


Figure 3-11. Graphic output of LDA in the plane of the first two discriminant functions. Classification according to Slovenian wine regions.

Cluster analysis (CLU) is one of the unsupervised multidimensional procedures that involve measuring the distances or similarities between the objects (or variables) to be clustered. In the present work, agglomerative hierarchical cluster analysis was performed in order to classify the wines tested according to variety type or wine region. Dissimilarities between the samples were determined based on the squared Euclidean distance, and the objects were clustered using Ward′s method.

A CLU dendrogram is presented in **Figure 3-12** and suggests three groups of clusters. The first cluster group consisted of wine marked as SW2, SW4, SW8 and SW9. All of these wines belong to the Modri Pinot and Modra Frankinja varieties. Samples marked as SW1, SW3, SW5 and SW6 comprised the second group of wines. Three of these wine samples belong to the Cabernet-Sauvignon variety, and sample SW6 belongs to the Refošk variety. All were produced in the Littoral region. Samples marked as SW7, SW10, SW11 and SW12 comprise the third cluster. These results are in accordance with those observed using PCA, and confirm that variety has more influence than wine regions on the content of PAs in Slovenian red wines.

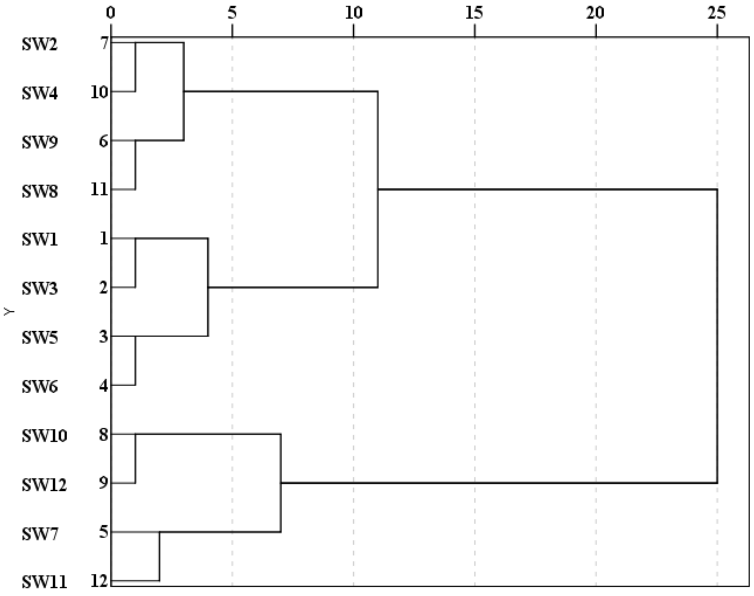


Figure 3-12. Dendrogram constructed with minimum linkage method for twelve Slovenian red wines.

3.3 Optimization of extraction conditions for the isolation of PCs from *Coriandrum sativum L.*

Aromatic plants are a rich source of essential oils, vitamins, minerals, proteins, fibre and phytochemicals, especially PCs [214]. Although the daily intake of aromatic plants is relatively low, their biological impact cannot be ignored. One of these medicinal plants is coriander (*Coriandrum sativum L.*), which belongs to the *Apiaceae* family, widely grown in North Africa and the Middle East, with increasing interest being shown in Western Europe [215]. *Coriandrum sativum L.* provides two types of herbal raw materials: fruits and leaves. Coriander fruits are widely used as a condiment to improve the flavour of foods. In traditional medicine, both parts of the coriander have been utilised for the treatment of many diseases. Recently, several authors have also confirmed the health benefits of consuming the various parts of the coriander plant [216,217].

The content of the pharmaceutically important compounds in coriander was reported in a review by Sahib et al. [218]. Although methanolic, ethanolic and acetone coriander extract were investigated by some authors, the relevant data are often scattered and fragmented [219,220]. Generally, TPC in coriander varies from 0.95 mg GAE g^{-1} DW for the Egyptian variety [219] up to 15.60 mg GAE g^{-1} DW for the Canadian type [221]. Currently, 21 individual PCs, including 11 PAs (gallic, chlorogenic, caffeic, vanillic, *p*-coumaric, ferulic, rosmarinic, *o*-coumaric, *trans*-hydroxycinnamic, salicylic and *trans*-cinnamic acids) and 10 flavonoids (quercetin-3-rhamnoside, rutin trihydrate, luteolin, quercetin dihydrate, resorcinol, kaempferol, naringin, apigenin, flavone and coumarin) have been identified in different coriander varieties [219]. The same authors have also shown that ratio between PAs and flavonoids in the plant is highly dependent on the investigated coriander variety. Namely, PAs were predominant (81.5%) in the Tunisian coriander variety, whereas the Syrian one was characterised by the predominance of the flavonoid subclass (61.3%) [219]. Additionally, the phenolic composition in coriander can be affected by the analytical procedure for extraction and determination, harvesting time and storage conditions.

For the isolation of PCs from the different parts of coriander, conventional extraction methods using 70% MeOH as the extraction solvent [220] and subcritical water have been utilised [222]. However, as previously mentioned, only a small amount of PAs in nature exist

in their free form while most are linked to lignin through their hydroxyl groups or ester linkages through their carboxylic group with carbohydrates and proteins [223]. This part of coriander PAa can be quantitatively determined after application one of the conventional hydrolysis methods (alkaline, acid and enzymatic hydrolysis). Additionally, the hydrolysis of PAs can be significantly improved by different external effects, such as sonication, microwave irradiation, far-infrared radiation (FIR) and the application of pulsed electric field (PEF). Recent studies showed that using an ultrasound can enhance the extraction efficiency through acoustic cavitation and mechanical effects [37,75,100,224]. Some of the advantages of UAE for the extraction of plant bioactive components over the conventionally used methods are shorter extraction time, lower solvent consumption and increased extraction yields [225].

Additionally, to reduce the cost and analysis time, DoE, one of the most powerful tools in the scientific and engineering disciplines, was used. Namely, one variable at a time (OVAT) optimization can give satisfactory results only in the cases where the tested parameters are independent each from each other. The application of DoE in process optimization can result in increased process yields, reduced process variability and reduced overall costs [13]. However, for optimal results, all factors influencing the observed process need to be taken into account in the experiment's planning. The independent variables and their tested ranges need to be selected according to the preliminary results and literally available data [226].

The aim of this part of PhD thesis was sistematical characterization of different PCs in coriander fruits. Firstly, TPC and TFC in methanolic extracts of coriander fruits, depending on the different extraction conditions, were determined. Furthermore, BBD combined with RSM was performed. In this way, alkaline hydrolysis in combination with UAE was optimized for extraction of *trans*-cinnamic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid and caffeic acid from coriander fruits. BBD was used to test the influence of three different factors (sonication time, temperature and concentration of NaOH) on the hydrolysis process, and to determine maximum PAs yields. The extraction recoveries of active compounds according to the hydrolysis conditions were also taken into account.

3.3.1 Chemicals

Standard compounds: *trans*-caffeic acid (99%), vanillic acid (97%), syringic acid (97%), *trans*-p-coumaric acid (98%), *trans*-o-coumaric acid (98%), *trans*-ferulic acid (98%) and *trans*-cinnamic acid (99%) were supplied by Merck (Germany). Protocatechuic acid (99%) and rutin (99%) were were purchased from Sigma (USA). Gallic acid (96%) was supplied by Carlo Erba (Italy).

Solvents: THF (99.5%) and pyridine (99.9%) were supplied by Merck (Germany). THF was additionally distilled before use. HPLC-grade MeOH was purchased by Sigma (USA). GC-grade toluene (99.5%) and HCl (36.5%) were purchased from Carlo Erba (Italy). DCM was purchased from JT Baker (Germany). Water (resistivity above 18 MΩ cm) used was obtained from a Milli-Q water purification system.

Other chemicals: Folin-Ciocalteu phenol reagent (2 M) and CH_3COONa were supplied by Merck (Germany). MSTFA, NaOH, $AlCl_3$ and Na_2CO_3 were purchased from Sigma (USA). L-ascorbic acid was purchased from Alkaloid (Macedonia) and EDTA was purchased from Kemika (Croatia).

3.3.2 Preparation of calibration curves

Standard stock solutions of caffeic acid, ferulic acid, p-coumaric acid, vanillic acid, syringic acid, as well as of o-coumaric acid (ISTD) were prepared by accurately weighing 10 mg of each into a 10 mL volumetric flask, and then dissolving in THF. Working calibration solutions were prepared by combining various volume of PAs stock solutions with 50 μL of ISTD in a 50 mL conical glass flask. Each solution was derivatized by treating it with 100 μL of MSTFA and 50 μL of pyridine for 1 h at 80 °C in a sand bath. After derivatization was finished, TMS derivatives were quantitatively transferred into 1 mL flasks and filled up to the mark with toluene. Five working solutions in concentration range from 1 to 100 mg L^{-1} were injected in triplicate. The calibration curves were constructed by linear regression of the peak-area ratio of individual PA standard to the ISTD (y), versus the concentration (mg L^{-1}) (x). The working solutions were prepared daily fresh.

3.3.3 Extraction of PCs from coridander fruits using methanol

Coriander sample used in this work (cultivated in Romania) was purchased from the local supermarket in Maribor, Slovenia. The fruits were milled in electric blender (Gorenje, Slovenia), packaged in glass vessels and kept in a dark place at RT before analysis.

The UAE of the miled coriander fruits was performed in the ultrasound bath (Model-LWB 106D, Daihan Labtech Co. Ltd, Korea). For UAE three parelel homogenized sub-samples of 1.0 g were weighed into separate centrifuge tubes. The samples were extracted separatly three times by sonication with 10 mL of appropriate solvent (80% or 100% MeOH) for different lengths of time (15, 30 and 45 min). After each extraction, extracts were centrifuged for 10 min at 6 000 rpm, the supernatants were combined, and evaporated to the absolute dryness by rotary evaporation (at 40 °C) (BÜCHI, Germany). The dry extracts were kept at 4 °C until analysis.

Beside UAE also conventional extraction (CE) techinque was performed and the results were compared. For CE 1.0 g of the sample was homogenized with aqueous MeOH solution (80%), as well as with pure MeOH (100%). The homogenate was stirred with magnetic stirrer at 900 rpm min^{-1} at RT for 30 min or for 24 h. Each extraction was done in triplicate.

3.3.4 Alkaline hydrolysis and extraction of bound PAs

The powdered sample (1.0 g) of coriander fruits was mixed with 20 mL of NaOH (which contained 1% L-ascorbic acid and 10 mM EDTA as stabilizers) at three different concentration (2, 3 and 4 M) in 100 mL round-bottom flask [181]. These samples were mixed properly for 1-2 min and kept in ultrasound bath for varying lengths of time (15, 30 and 45 min) at various temperatures (20, 40 and 60 °C). The temperature, sonication time and NaOH concentration were based on the experimental design (**Table 3-11**). After hydrolysis, the samples were acidified to pH=2 using 6 M HCl. Prepared samples were added to pre-conditioned (2 x 3 mL of MeOH, and 2 x 3 mL of acidified water (pH=2)) HLB Supelco® SPE cartridges [181]. Cartridges were washed with ultra pure water (2 x 3 mL) to remove sugars and other polar compounds. The free PAs fraction was eluted with 2 x 2 mL of THF. The eluate was collected and dried in a rotary evaporator (at 40 °C) to absolute dryness. Then the sample was derivatized by adding 100 μL of MSTFA and 50 μL pyridine, heated at 80 °C for 1 h, diluted with toluene up to 1 mL, and analyzed using GC-MS. The analyses were carried out in duplicates.

At the same time, the extraction recovery for every performed experiment was determined. For this purpose, 1.0 g of the powdered sample was spiked with standard compounds mixture at exactly known concentration (100 μL, 1000 mg L^{-1}). The spiked samples were exposed to the same conditions, as well as unspiked samples, whereby differences between spiked and unspiked samples were used for determination of extraction recovery (%).

3.3.5 Experimental design

In order to optimize the hydrolysis conditions for extraction of target PAs from coriander fruits, an experimental design was applied. Design-expert software (Design Expert 10, free trail version) was used for the experimental design and statistical analysis of the data. A three level (-1, 0, +1) three factor BBD combined with RSM were conducted to the design experimental project.

The influence of three major factor: temperature (X_1), sonication time (X_2) and NaOH concentraction (X_3) as the independent variables were tested. Temperature extraction was evaluated in the range 20-60ºC, sonication time covered the range from 15 to 45 min and NaOH concentration evaluated were from 2 to 4 M. The actual and coded values of operational variables are shown in **Table 3-11**. Total of 12 experiments with three replication at the central point with the different combinations of three factors were carried out.

The extraction yields of caffeic acid (Y_1), ferulic acid (Y_2), vanillic acid (Y_3), p-coumaric acid (Y_4) and syringic acid (Y_5) as well as extraction recoveries (%) of caffeic acid (Y_6), p-coumaric acid (Y_7), ferulic acid (Y_8) and *trans*-cinnamic acid (Y_9) were selected as dependent variables. The experimental data were fitted to a second-order polynomial models to obtain the regression coefficients.

The generalized second-order polynomial model used in the response surface analysis was as follow:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \beta_{ij} X_i X_j \qquad (3.1)$$

where, Y is the response variable, X_i and X_j are the independent variables, and k is the number of tested variables (k = 3). Regression coefficient is defined as β_0 for intercept, β_i for linear, β_{ii} for quadratic and β_{ij} for cross product term.

Table 3-11. Experimental variables (factors and respective levels).

Independent variable	Unit	Symbol	Values (uncoded and coded)		
Temperature	°C	X_1	20 (-1)	40 (0)	60 (1)
Sonication time	min	X_2	15 (-1)	30 (0)	45 (1)
NaOH concentration	mol L^{-1} (M)	X_3	2 (-1)	3 (0)	4 (1)

3.3.6 Total phenolic and total flavonoid content

TPC of the methanolic coriander extracts were determined according to the standard spectrophotometric method, with some modifications [227]. Briefly, 40 μL of properly diluted methanolic extract was mixed with 3160 mL of ultra pure water and 200 μL of Folin-Ciocalteu's phenol reagent. After 6 min, 600 μL of Na_2CO_3 (0.2 g mL^{-1}) was added. The tubes were allowed to stand for 2 h in a dark place at RT. Additionally, standard solutions of gallic acid in the concentration range from 50-500 mg L^{-1} were prepared in the 80% MeOH. For construction of the calibration curve, absorbances were measured at the wavelenght of 765 nm (Shimadzu UV-VIS spectrophotometer, Kyoto, Japan). Samples were measured under the same conditions. The TPC of coriander fruits was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW). All samples were analyzed in duplicates.

TFC were determined using the aluminium chloride colorimetric method. 500 μL of properly diluted crude methanolic extract was added to 1.5 mL of pure MeOH and mixed well. After that 0.1 mL of $AlCl_3$ (10%), 0.1 mL of CH_3COONa (1 M) and 2.8 mL of ultra-pure water were added. Standard solutions of rutin in the concentration range from 10-100 mg L^{-1} were prepared on the same way. The tubes were allowed to stand for 30 min in a dark place at RT, and the absorbances were measured at the 415 nm against a blank. The blank was prepared following the same procedure described for the sample, expect that destilation water was used insted of sample. The TFC was expressed as milligrams of rutin per gram of dry weight (mg RUT g^{-1} DW). All samples were analyzed in duplicates.

3.3.7 GC-MS conditions and data analysis

Chromatographic separation and identification of the target compounds were performed with the GC-MS system described in **Section 3.2.6**. Quantification of the identified compounds was carried out by using a previously developed and validated method (**Section 3.2.6.**).

All results in the text and tables were expressed as the mean values ± standard deviations. Microsoft Excel was used for the data preparation and result outputs. Statistical data treatment was performed using SPSS Statistics (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). One-way analysis of variance (ANOVA) at the 95% confidence level and Student-Newman-Keuls (S-N-K) post-hoc test was applied in order to determine difference between extraction methods.

3.3.8 Determination of TPC and TFC

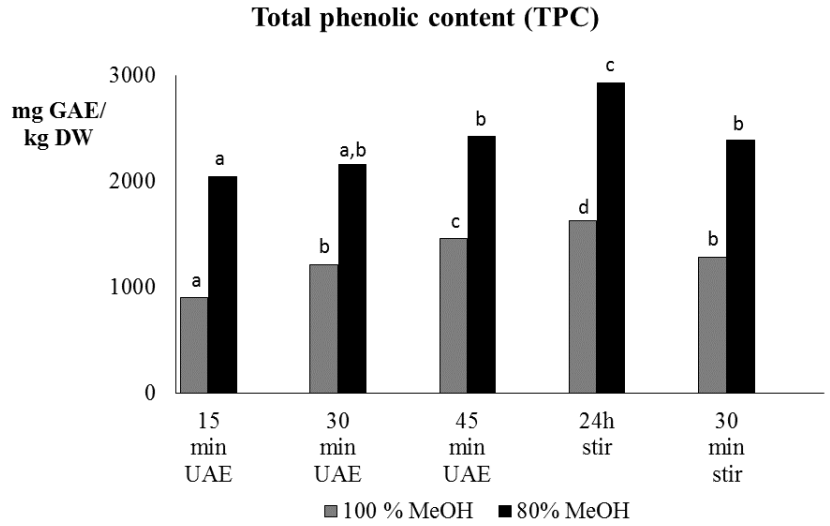
In the first step of our study, two extraction methods (CE and UAE) were used to extract total phenolic fraction from the coriander fruits under the previously described conditions (**Section 3.3.3.**). **Figure 3-13** present the extraction yields of two mentioned extraction techniques for TPC and TFC. Extraction techniques were compared regarding two influencing variables, namely type of the extraction solvent (80% aqueous MeOH solution and 100% MeOH) and sonication time (20, 30 and 45 min).

From **Figure 3-13** it can be observed that the obtained extraction rates were affected by both, the type of extraction as well as by solvent used. In general, the highest yields in TPC and TFC from coriander fruits were achieved by extraction using the aqueous MeOH (80%) under the 24 h long stirring and by 45 min of sonication using 100% MeOH, respectively. Namely, compared to UAE, CE method resulted in significantly lower content of TFC, but this not was case for TPC. This results are in agreement with the results of other authors, reported for the similar plants [228].

In the case of coriander fruits we confirmed that UAE was not effective for TPC recovery. This can be explaned by the fact that too long exposure of the same sample to ultrasound causes the degradation of some phenolics from the certain compound sub-classes [229]. On the other hand, when compared to the CE application of UAE, may contribute to lower solvent consumption and shorter extraction time. Namely, by the 45 min long UAE can achieve at

least 90% of TPC extracted with 24 h stirr CE technique. For the TFC, between 45 min sonication method and 24 h CE not statistically significant difference was observed (**Figure 3-13b**). The values for TPC and TFC in coriander fruits were varied among the extracts and ranged from 800 to 2 900 mg GAE g^{-1} DW and from 540 to 850 mg RUT g^{-1} DW, respectively.

A)



B)

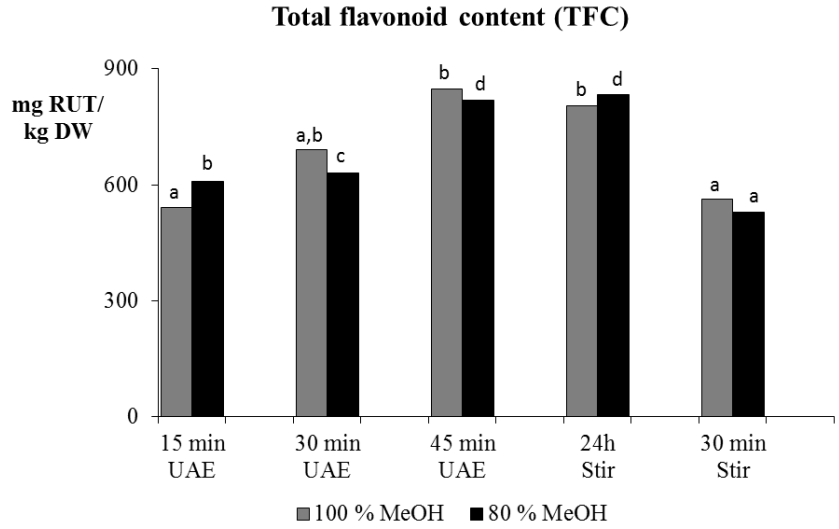


Figure 3-13. Effect of the extraction solvent and extraction time on the TPC (a) and on the TFC (b) in *Coriandrum sativum L.* fruits. Different superscripts for the same compound denoted significant differences according to the S-N-K method at $P < 0.05$.

3.3.9 Identification and quantification of bound PAs using GC-MS

As the mentioned before PAs in the plant materials can be present in free or bound form. Free PAs are extractable using different organic solvents, or their mixtures. On the other hand, bound PAs can be extracted after being released by alkaline, acidic and enzymatic hydrolysis. From the literature it is known that the most importan factor affecting efficiency of hydrolysis reaction are: concentration of hydrolysis reagent, temperature, reaction time, the mass of analyzed samples, the influence of microwaves or ultrasound, etc. [230,231]. In our preliminary study, a total of six different PAs (caffeic acid, ferulic acid, p-coumaric acid, vanillic acid, syringic acid and protocatechuic acid) including their geometric isomers were identified in the coriander fruits after alkaline hydrolysis (**Figure 3-14**).

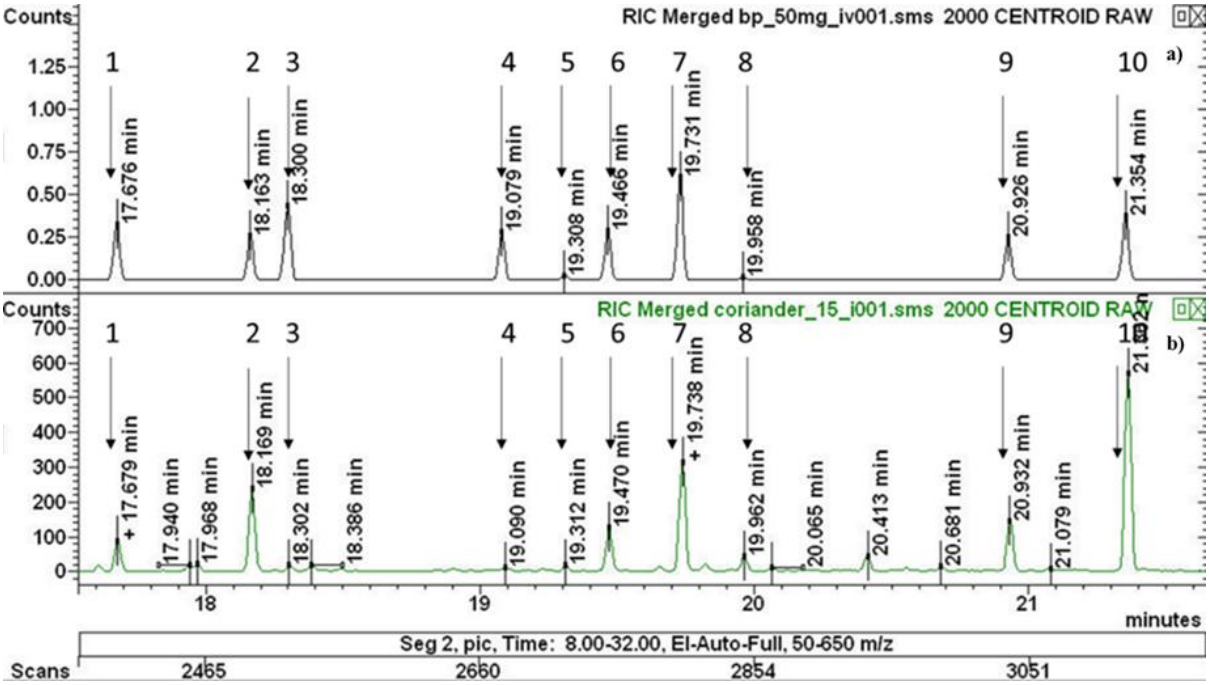


Figure 3-14. GC chromatograms of: a) silylated standard mixture of PAs (1. vanillic acid; 2. o-coumaric acid (ISTD); 3. protocatehuic acid; 4. syringic acid; 5. cis-ferulic acid; 6. *trans*-p-coumaric acid; 7. *trans*-cinnamic acid; 8. *cis*-caffeic acid; 9. *trans*-ferulic acid; 10. *trans*-caffeic acid); b) silylated PAs present in *Coriandrum sativum L.* extract obtained after SPE (1. vanillic acid; 2. o-coumaric acid (ISTD); 3. protocatehuic acid; 4. syringic acid; 5. cis-ferulic acid; 6. *trans*-p-coumaric acid; 7. L-ascorbic acid (stabilizer); 8. *cis*-caffeic acid; 9. *trans*-ferulic acid; 10. *trans*-caffeic acid).

The quantification of identified PAs, using corresponding calibration curves, were performed. Validation parameters of developed GC-MS method are summarized in the **Chapter 3.2.8.1**.

3.3.10 Box–Behnken experimental design with discussion

Additionally, BBD combined with RSM was used to optimize the alkaline hydrolysis and extraction conditions of previously identified bound PAs (**Figure 3-14**). For optimization the experiments were conducted by a 2^3 full factorial central composite design.

The yields of caffeic acid (Y_1), ferulic acid (Y_2), vanillic acid (Y_3), p-coumaric acid (Y_4), syringic acid (Y_5) as well as extraction recoveries of caffeic acid (Y_6), ferulic acid (Y_7), p-coumaric acid (Y_8) and *trans*-cinnamic acid (Y_9) were deremined as the dependent variables. The concentration of protochatehuic acid in all obtained extracts was below LOQ, and was therefore not used for method optimization. All the experimental data obtained from the 15-run experiments are shown in **Table 3-12**.

The F test and p-value were used to determine the significance of each coefficient. The result, values of ‘‘Prob > F’’ less than 0.05, indicates that the model terms are significant. The optimized conditions were validated for the maximum PAs yields and extraction recoveries based on the values obtained using RSM. The experimental values were compared with predicted values based on CV% in order to determine the validity of the model. For the graphical presentation of influence of tested conditions on the extraction yields the three dimensional (3-D) surface response plots were generated by varying two variables within the experimental range and by holding one variable constant at the central point. On the other hand, contour plots were generated for the graphical description of influence of tested variables on the extraction recoveries. The test of statistical significance was based on the total error criteria with a confidence levels of 95.0%, 99.0% and 99.9%.

Table 3-12. Mean responses obtained for investigated PAs from the experimental design.

Run	Temperature (°C)	Sonication time (min)	NaOH con. (mol L^{-1})	Caffeic acid	Ferulic acid	Vanillic acid	p-coumaric acid	Syringic acid	Caffeic acid	Ferulic acid	p-coumaric acid	*Trans*-cinnamic
	Unit			mg g^{-1}	mg g^{-1}	mg g^{-1}	mg g^{-1}	mg g^{-1}	%	%	%	%
	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8	Y_9
1	40 (0)	30 (0)	3 (0)	1.94	0.55	0.25	0.38	$5.13 \cdot 10^{-3}$	55	70	72	71
2	40 (0)	45 (+1)	4 (+1)	1.43	0.54	0.15	0.27	NQ	70	61	69	62
3	60 (+1)	45 (+1)	3 (0)	1.66	0.63	0.17	0.32	$2.13 \cdot 10^{-3}$	82	81	83	65
4	60 (+1)	30 (0)	4 (+1)	1.68	0.47	0.15	0.12	NQ	83	82	95	61
5	20 (-1)	30 (0)	2 (-1)	1.53	0.24	0.16	0.05	NQ	86	102	101	87
6	20 (-1)	45 (+1)	3 (0)	1.51	0.38	0.19	0.21	$1.33 \cdot 10^{-3}$	81	83	77	78
7	60 (+1)	30 (0)	2 (-1)	1.41	0.60	0.19	0.30	NQ	89	80	96	84
8	40 (0)	15 (-1)	2 (-1)	1.43	0.37	0.17	0.11	NQ	99	102	88	95
9	40 (0)	30 (0)	3 (0)	1.94	0.56	0.25	0.38	$5.13 \cdot 10^{-3}$	55	70	72	71
10	40 (0)	45 (+1)	2 (-1)	1.72	0.47	0.18	0.29	NQ	77	91	93	89
11	40 (0)	15 (-1)	4 (+1)	1.68	0.47	0.15	0.15	NQ	90	91	95	75
12	40 (0)	30 (0)	3 (0)	1.94	0.56	0.25	0.38	$5.13 \cdot 10^{-3}$	55	70	72	71
13	20 (-1)	30 (0)	4 (+1)	1.47	0.58	0.14	0.15	NQ	92	79	88	67
14	20 (-1)	15 (0)	3 (0)	1.52	0.32	0.17	0.12	$4.8 \cdot 10^{-4}$	100	105	102	76
15	60 (+1)	15 (-1)	3 (0)	1.40	0.52	0.19	0.27	NQ	94	96	97	74

NQ-not quantified.

Effect of the independent variables on the PAs yield

To study the interactive effects of operational parameters on the extraction yields, the three-dimensional (3D) profiles of multiple non-linear regression models were depicted in **Figure 3-15**. The profiles present the interaction of two process factors, while the third factor was fixed at its middle level.

Optimization of extraction process for the PAs yield was carried out by applying second order polynomial equation. Analysis of variance (ANOVA) showed that this model adequately represented the experimental datas. The coefficient of multiple determinations (R^2) for PA yields (caffeic acid, ferulic acid, p-coumaric acid, syringic acid and vanillic acid) were 0.9701; 0.9848; 0.9897; 0.9791 and 0.9927, respectively. Analyses of variance for response surface polynomial models are compiled in **Table 3-13.**

For caffeic acid (Y_1), X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant model terms ($p<0.05$) (**Table 3-13**). It means that only combinations of two factor influenced the extraction yield of caffeic acid significantly. Non-significant variables were removed and the following second order polynomial equation was found to represent the extraction yield adequately:

$$Y_1 = 0.29 + 0.02X_1X_2 + 0.02X_1X_3 - 0.04X_2X_3 - 0.06X_1^2 - 0.05X_2^2 - 0.05X_3^2 \qquad (3.2)$$

The highest positive effect on the extraction yield of caffeic acid showed interaction (X_1X_3) followed by interaction (X_1X_2) with the maximum achived when the factors were controled to the ultrasound influence for 30 min at 40 °C and NaOH concentration of 3 M. Caffeic acid was the most abundant between PAs presented in the coriander fruits with concentrations ranging from 1.43 to 1.94 mg g^{-1}DW.

F-value of 36.02 for ferulic acid indicated that the model was significant. X_1, X_2, X_3, X_1^2, X_2^2 and X_3^2 were significant model terms ($p<0.05$). It means that all tested parameters influenced the extraction yield of ferulic acid significantly. Meanwhile, the p value of X_1X_3 was lower than 0.05, the interaction of temperature and NaOH concentration also had a significant influence on the extraction yield of ferulic acid. The reduced second order polynomail equation for ferulic acid was:

$$Y_2 = 1.34 - 0.17X_1 - 0.07X_2 - 0.11X_3 + 0.69 \cdot 10^{-3}X_1X_2 + 0.11X_1^2 + 0.06X_2^2 + 0.07X_3^2 \qquad (3.3)$$

The maximum yield of the ferulic acid (0.63 mg g^{-1} DW) was achived when the factors were adjusted to the central point values: temperature (60 °C); sonication time (45 min) and NaOH concentration (3 M).

The developed model was significant for the vanillic acid, with the F-value of 53.60. There is only a 0.02% chance that an F value this large could occur due to noise. In this case X_1, X_3, X_1X_2, X_1^2, X_2^2 and X_3^2 were significant model terms, with the probability values less than 0.05. Those results indicated that ultrasound were not influenced the extraction yield of vanillic acid. Namely, the ultrasound influence occurs only in the combination with temperature ($P<0.001$). Reduced second order equation which represent the extraction yield of vanillic acid:

$$Y_3 = -1.39 + 0.04X_1 + 0.08X_3 - 0.06X_1X_2 - 0.17X_1^2 - 0.15X_2^2 - 0.28X_3^2 \quad (3.4)$$

The model F-value of 26.04 for p-coumaric acid implies the model was significant. In this case X_1, X_2, X_1X_3, X_1^2 and X_3^2 were significant model terms (equation 3.5.):

$$Y_4 = -0.42 + 0.15X_1 + 0.12X_2 - 0.22X_1X_3 - 0.21X_1^2 - 0.27X_3^2 \quad (3.5)$$

The optimal conditions for the maximum p-coumaric acid extraction yield were BBD central point (0.38 mg g^{-1}DW).

The high F-value (75.46) for the syringic acid implies to the significance of the model. There is only a 0.01% chance that an F-value this large could occur due to noise. In this case the influence of significant model terms (X_1^2, X_2^2 and X_3^2) can be represent by folowing reduced second order polynomail equation:

$$Y_5 = -0.40 - 5.93 \cdot 10^{-5}X_1^2 - 1.06 \cdot 10^{-4}X_2^2 - 0.03X_3^2 \quad (3.6)$$

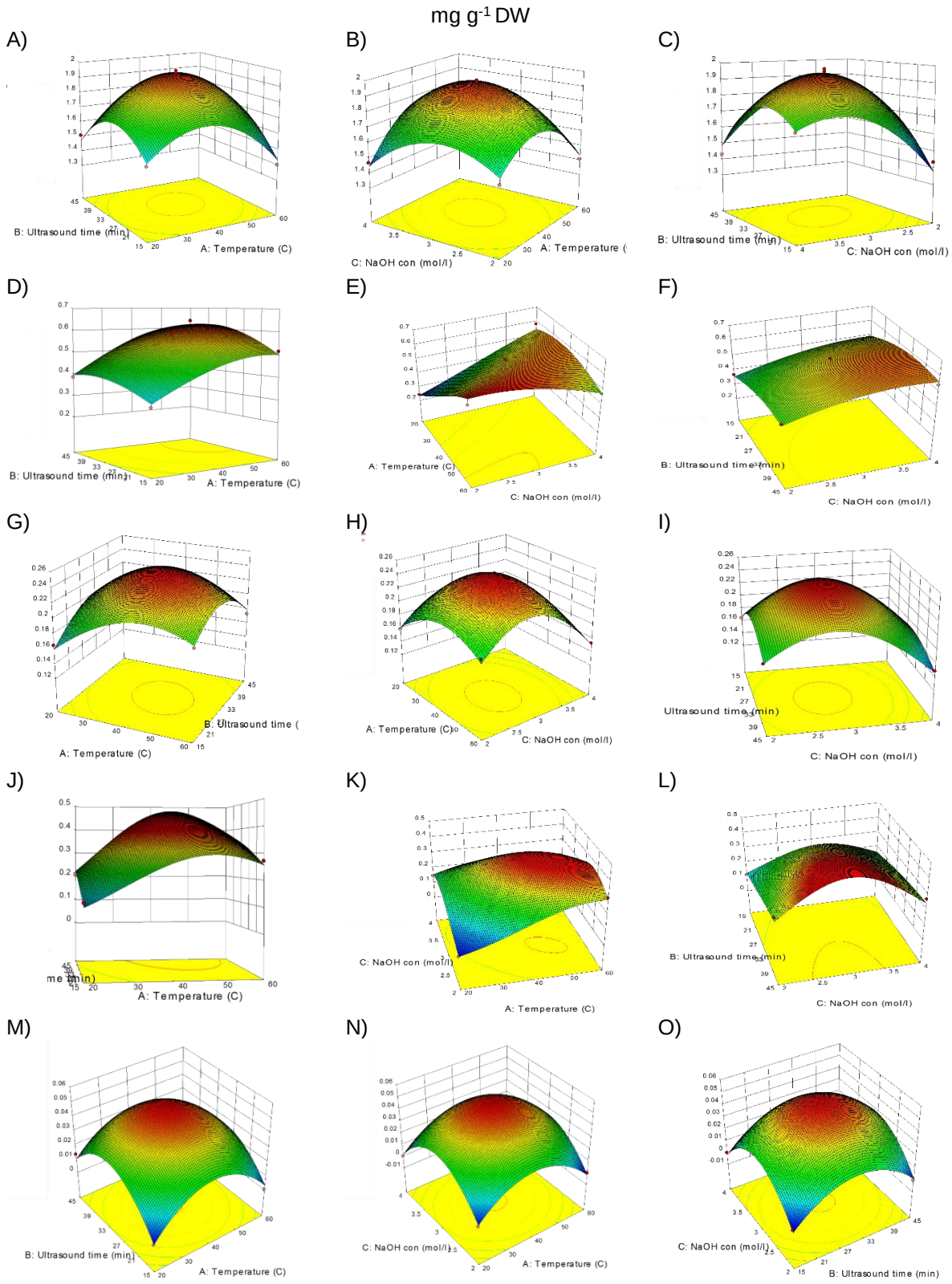


Figure 3-15. 3D plots for intraction between three extrction variable on the caffeic acid (A-C), ferulic acid (D-F), vanillic acid (G-I), p-coumaric acid (J-L) and syringic acid (M-O).

Table 3-13. Analysis of variance (ANOVA) of response surface second order polynomial models for PAs yields.

Variables	Responses (mg g^{-1} DW)					
	Caffeic acid			Ferulic acid		
	Mean of square	F value	*p* value	Mean of square	F value	*p* value
Model	0.04	18.00	0.003**	0.07	36.02	<0.001***
X_1	$8.76 \cdot 10^{-5}$	0.39	0.559	0.23	116.20	<0.001***
X_2	$7.59 \cdot 10^{-4}$	3.39	0.125	0.04	20.83	0.006**
X_3	$2.90 \cdot 10^{-4}$	1.30	0.307	0.09	48.22	0.001***
X_1X_2	$1.55 \cdot 10^{-3}$	6.90	0.047*	$1.92 \cdot 10^{-4}$	0.10	0.766
X_1X_3	$2.23 \cdot 10^{-3}$	9.95	0.025*	0.20	102.14	<0.001***
X_2X_3	$5.40 \cdot 10^{-3}$	24.13	0.004**	$2.68 \cdot 10^{-4}$	1.38	0.293
X_1X_1	0.01	57.38	< 0.001***	0.05	23.01	0.005**
X_2X_2	$8.46 \cdot 10^{-3}$	37.77	0.002**	0.01	7.28	0.043*
X_3X_3	$8.59 \cdot 10^{-3}$	38.35	0.002**	0.02	9.89	0.026*
R^2	**0.9701**			**0.9848**		
Adjusted R^2	**0.9162**			**0.9575**		
Adeq. precision	**12.162**			**21.713**		

Table 3-13. Continued.

Varibales	Responses (mg g^{-1} DW)								
	Vanillic acid			p-Coumaric acid			Syringic acid		
	Mean of square	F value	*p* value	Mean of square	F value	*p* value	Mean of square	F value	*p* value
Model	0.06	53.60	<0.001***	0.10	26.04	>0.001**	$6.80 \cdot 10^{-4}$	75.46	< 0.001***
X_1	0.02	13.96	0.014*	0.19	48.08	0.001***	$1.64 \cdot 10^{-5}$	1.82	0.236
X_2	$1.16 \cdot 10^{-3}$	1.10	0.343	0.12	31.37	0.003**	$2.87 \cdot 10^{-5}$	3.18	0.135
X_3	0.05	46.33	0.001***	$3.38 \cdot 10^{-3}$	0.87	0.393	$8.67 \cdot 10^{-19}$	$9.62 \cdot 10^{-14}$	1.000
X_1X_2	0.02	15.40	0.011*	$7.04 \cdot 10^{-3}$	1.82	0.235	$3.22 \cdot 10^{-5}$	3.57	0.117
X_1X_3	$8.71 \cdot 10^{-4}$	0.82	0.406	0.19	49.18	<0.001***	$8.67 \cdot 10^{-19}$	$9.62 \cdot 10^{-14}$	1.000
X_2X_3	$2.20 \cdot 10^{-3}$	2.07	0.210	$5.11 \cdot 10^{-3}$	1.32	0.302	0.00	0.00	1.000
X_1X_1	0.10	97.12	<0.001***	0.16	41.93	>0.001**	$2.08 \cdot 10^{-3}$	230.88	< 0.001***
X_2X_2	0.09	81.57	<0.001***	$5.23 \cdot 10^{-3}$	1.36	0.297	$2.08 \cdot 10^{-3}$	230.88	< 0.001***
X_3X_3	0.29	277.26	<0.001***	0.26	67.32	<0.001***	$2.80 \cdot 10^{-3}$	310.97	< 0.000***
R^2	**0.9897**			**0.9791**			**0.9927**		
Adjusted R^2	**0.9713**			**0.9415**			**0.9795**		
Adeq. precision	**20.904**			**17.059**			**21.697**		

Level of significance $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$

Effect of the independent variables on the PAs extraction recoveries

Contour plots (**Figure 3-16**), which are the graphical representations of the quadratic polynomial regression equation, illustrate the significant ($p<0.05$) interaction effects of two tested factors on the recovery of investigated compounds, while thirdh factor was fixed at its middle level.

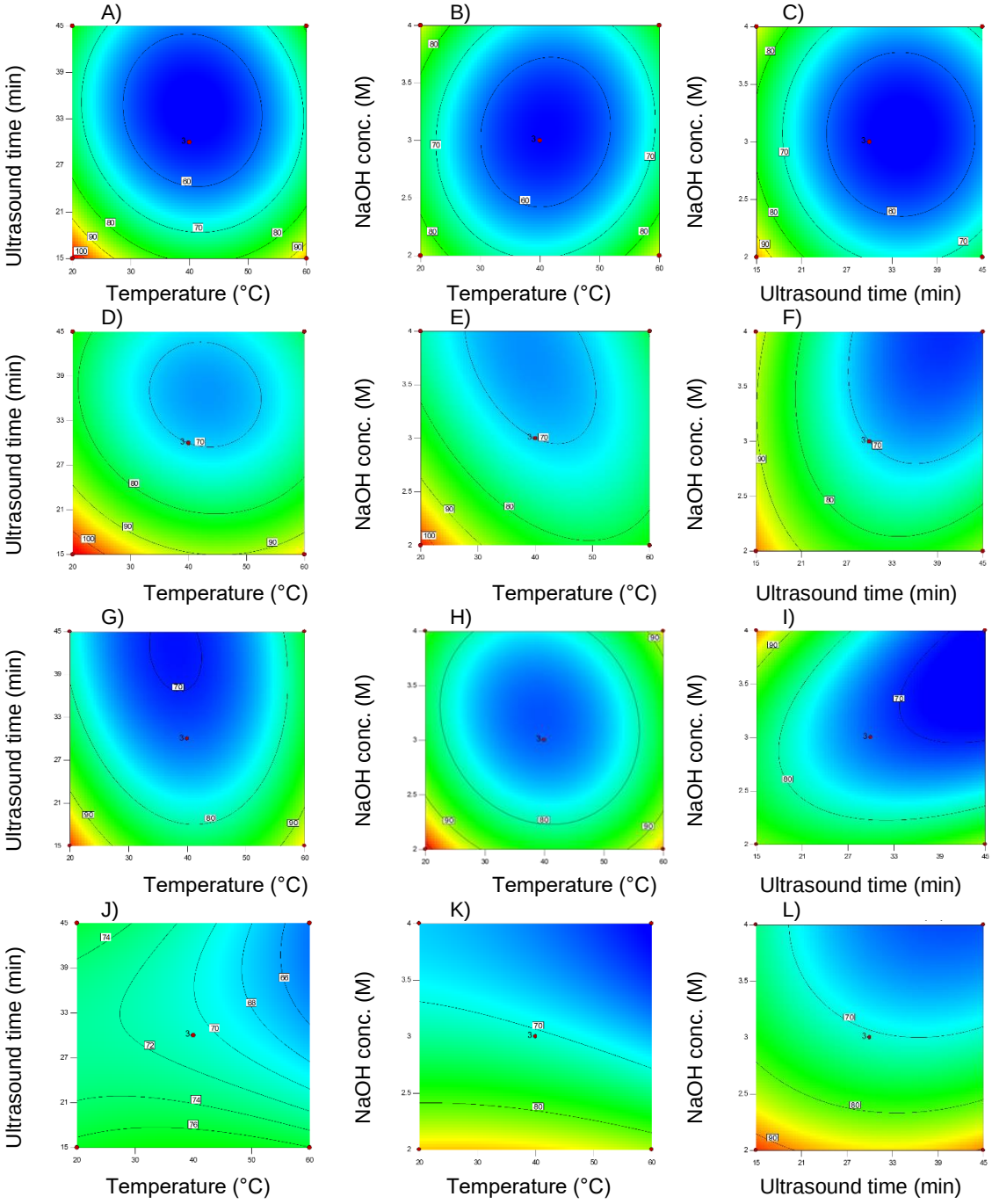


Figure 3-16. Contour plots for interactions between three parameters on the extraction reoveries of PAs from coraiander fruits: A-C) caffeic acid; D-F) ferulic acid; G-I) p-coumaric acid; J-L) *trans*-cinnamic acid.

The results of this study also confirmed a great influence of studied conditions on the PAs extraction recoveries, especially those from the hydroxycinnamic acid derivative group. *Trans*-cinnamic acid was not identified in the tested coriander samples, but their stability under tested extraction conditions has also been a subject of the study.

The coefficient of multiple determinations (R^2) for caffeic aid, feruli acid, p-coumaric acid and *trans*-cinnamic acid were 0.9895; 0.9760; 0.9834 and 0.9865, respectively (**Table 3-14**). The model showed high significant ($p<0.001$) value with the experimental data for all tested responses. ANOVA test showed significant ($p<0.001$) negative linear (X_2) effect on the extraction recoveries. It can be explained by the fact that too long application of ultrasound to the same matrix causes the degradation of PAs with the double bound in their structures [229].

The F-value of 52.43 implies the model was significant for the extraction recovery of caffeic acid. There is only a 0.02% chance that an F-value this large could occur due to noise. In this case X_2, X_1^2, X_2^2 and X_3^2 are significant model terms, and can be represented by the following equation:

$$Y_6 = 7.42 - 0.52X_2 + 1.14X_1^2 + 0.93X_2^2 + 0.80X_3^2 \quad (3.7)$$

That mean that only ultrasound time ($p<0.001$) has a significant influence on the extraction recovery of the caffeic acid. The negative influence of NaOH concentration on the stability of caffeic acid was probably eliminated by adding L-ascorbic acid and EDTA as a stabilizers [232]. A adeq. precision ratio greater than 4 is desirable. In this case the value of 22.15 indicates an adequate signal, and this model can be used to navigate the design space.

Based on regression coefficient (β) values, influence of ultrasound (X_2) showed major negative effect on the extraction recovery of ferulic acid followed by NaOH concentration (X_3), interaction (X_1X_3), and temperature (X_1). After removing a non-significant variables the following second order polynomial equation was found to represent the extraction recovery of ferulic acid adequately:

$$Y_7 = 70 - 3.75X_1 - 9.75X_2 - 7.75X_3 + 6.25X_1X_3 - 4.75X_2X_3 + 10.38X_1^2 + 10.88X_2^2 + 5.37X_3^2 \quad (3.8)$$

For the p-coumaric acid the F-value was 32.93, (there is only a 0.06% chance that an F-value this large could occur due to noise). It was found that the major negative influence on this PA recovery has interaction (X_2X_3) followed by influence of ultrasound (X_1) and NaOH concentration (X_3). Those interaction can be represented by following reduced equation:

$$Y_8 = 4.28 - 0.09X_2 - 0.05X_3 - 0.09X_2X_3 + 0.16X_1^2 + 0.06X_2^2 + 0.12X_3^2 \quad (3.9)$$

Trans-cinnamic acid has also been in focus of this research, and it proved that that it stability was also affected by tested conditions, with the major negative effect of NaOH concentration. The model was significant with the F-value 40.67. The equation that described the influence of independent variables on the *trans*-cinnamic acid recovery, can be written as:

$$Y_9 = 4.26 - 0.04X_1 - 0.04X_2 - 0.15X_3 - 0.04X_1X_2 + 0.05X_2^2 + 0.06X_3^2 \quad (3.10)$$

Generally, due to satisfactory statistical parameters (R^2 and CV) and ANOVA test, it could be concluded that developed second-order polynomial models provided adequate mathematical description of the UAE extraction yields. Further, according to the results obtained it could be concluded that all tested parameters had a significant impact on the extraction yields of PAs, but sonication time was the most critical factor. With the aim to maximize the extraction yields of all independed variables the following extraction conditions were determined as the optimal: sonication time of 17.4 min at 35.3 °C and NaOH concentration of 2.02M.

Table 3-14. Analysis of variance (ANOVA) of response surface second order polynomial models for PAs recoveries.

Variables	Responses (%)					
	Caffeic acid			Ferulic acid		
	Mean of square	F value	*p* value	Mean of square	F value	*p* value
Model	1.28	52.43	<0.001***	271.38	22.61	>0.001**
X_1	0.07	3.03	0.142	112.50	9.38	0.028*
X_2	2.14	87.63	<0.001***	760.50	63.38	<0.001***
X_3	0.10	3.94	0.104	480.50	40.04	>0.001**
X_1X_2	0.08	3.20	0.134	12.25	1.02	0.359
X_1X_3	0.10	4.22	0.095	156.25	3.02	0.015*
X_2X_3	$7.47 \cdot 10^{-4}$	0.03	0.868	90.25	7.52	0.041*
X_1X_1	4.79	196.72	<0.001***	397.44	33.12	0.002**
X_2X_2	3.20	131.49	<0.001***	436.67	36.39	0.002**
X_3X_3	2.34	96.20	<0.001***	106.67	8.89	0.031*
R^2	**0.9895**			**0.9760**		
Adjusted R^2	**0.9706**			**0.9329**		
Adeq. precision	**22.150**			**21.713**		

Table 3-14. Continued.

Variables	Responses (%)					
	p-Coumaric acid			*Trans*-cinnamic acid		
	Mean of square	F value	*p* value	Mean of square	F value	P value
Model	0.03	32.93	<0.001***	0.03	40.67	<0.001***
X_1	$3.19 \cdot 10^{-4}$	0.36	0.573	0.02	22.62	0.005**
X_2	0.06	69.91	<0.001***	0.02	23.48	0.005**
X_3	0.02	19.47	0.006**	0.18	269.21	<0.001***
X_1X_2	$3.93 \cdot 10^{-3}$	4.46	0.088	$6.06 \cdot 10^{-3}$	9.15	0.029*
X_1X_3	$4.05 \cdot 10^{-3}$	4.60	0.085	$1.12 \cdot 10^{-3}$	1.69	0.250
X_2X_3	0.04	39.96	0.002**	$3.51 \cdot 10^{-3}$	5.30	0.070
X_1X_1	0.09	105.48	<0.001***	$1.50 \cdot 10^{-3}$	2.27	0.193
X_2X_2	0.01	12.86	0.016*	$8.85 \cdot 10^{-3}$	13.36	0.015*
X_3X_3	0.05	57.88	<0.001***	0.01	19.08	0.007**
R^2	**0.9834**			**0.9865**		
Adjusted R^2	**0.9536**			**0.9623**		
Adeq. precision	**16.626**			**21.044**		

Level of significance *p<0.05, **p<0.01, ***p<0.001

3.4 Deep eutectic solvents (DESs) for the extraction of PCs from different plant materials

The new trend in chemistry for environmental protection has led to increased interest for the use of non-toxic, cheap and biodegradable 'green' solvents. Namely, the traditionally used organic solvents are not suitable for use in green technology due to their high toxicity, high volatility and poor biodegradability.

Consequently, a new generated solvent class, ionic liquids (ILs), has gained much attention from the scientific community in different research fields. Although the exact definition of these solvents is still questionable, generally, they can be defined as a mixture of solid compounds with poorly adjusted ions and unique and promising properties that are liquid even at temperatures lower than 100 °C [233]. The most important advantages of ILs over the traditionally used organic solvents are their negligible vapour pressure, good thermal properties, broad liquid range, wide range of solubility and miscibility, credential properties for chemical reactions and good recycling properties [234]. However, nowadays, the 'green' nature of ILs has become questionable for many researchers, who take into account all the ILs' proved shortcomings, such as high preparation cost for large-scale applications (in some cases, 10 times higher than the conventional organic solvents), similar or higher toxicity than organic solvents and low biodegradability [235].

In the last few years, the new generation of ILs, named deep eutectic solvents (DESs), has garnered much attention. The first pioneering study about DESs as a possible alternative for ILs was reported by Abbott et al. in 2001 [236]. In this papre, the authors described the preparation of DESs based on different quaternary ammonium salts in combination with $ZnCl_2$. In their study, DES based on choline chloride as the ammonium salt, with a melting point of 23–25 °C, was confirmed as the most promising.

Generally, DESs are composed of two or more solid organic or inorganic compounds whose mixing under the optimal conditions (temperature and stirring time) leads to the formation of a stable eutectic liquid mixture. The melting point of the new prepared mixture is far less than for the individual composition. In the case of the simple binary mixture A + B, the melting point directly depends on the composition of individual compounds A and B and interaction between them (**Figure 3-17**) [237].

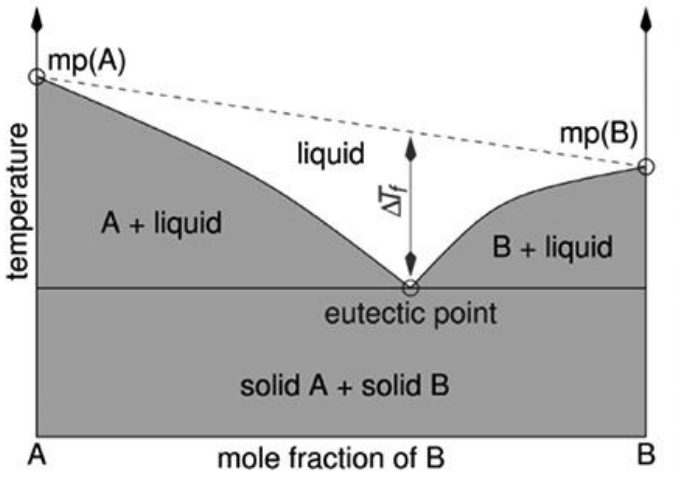


Figure 3-17. Schematic representation of eutectic mixture formation in a two-component system [238].

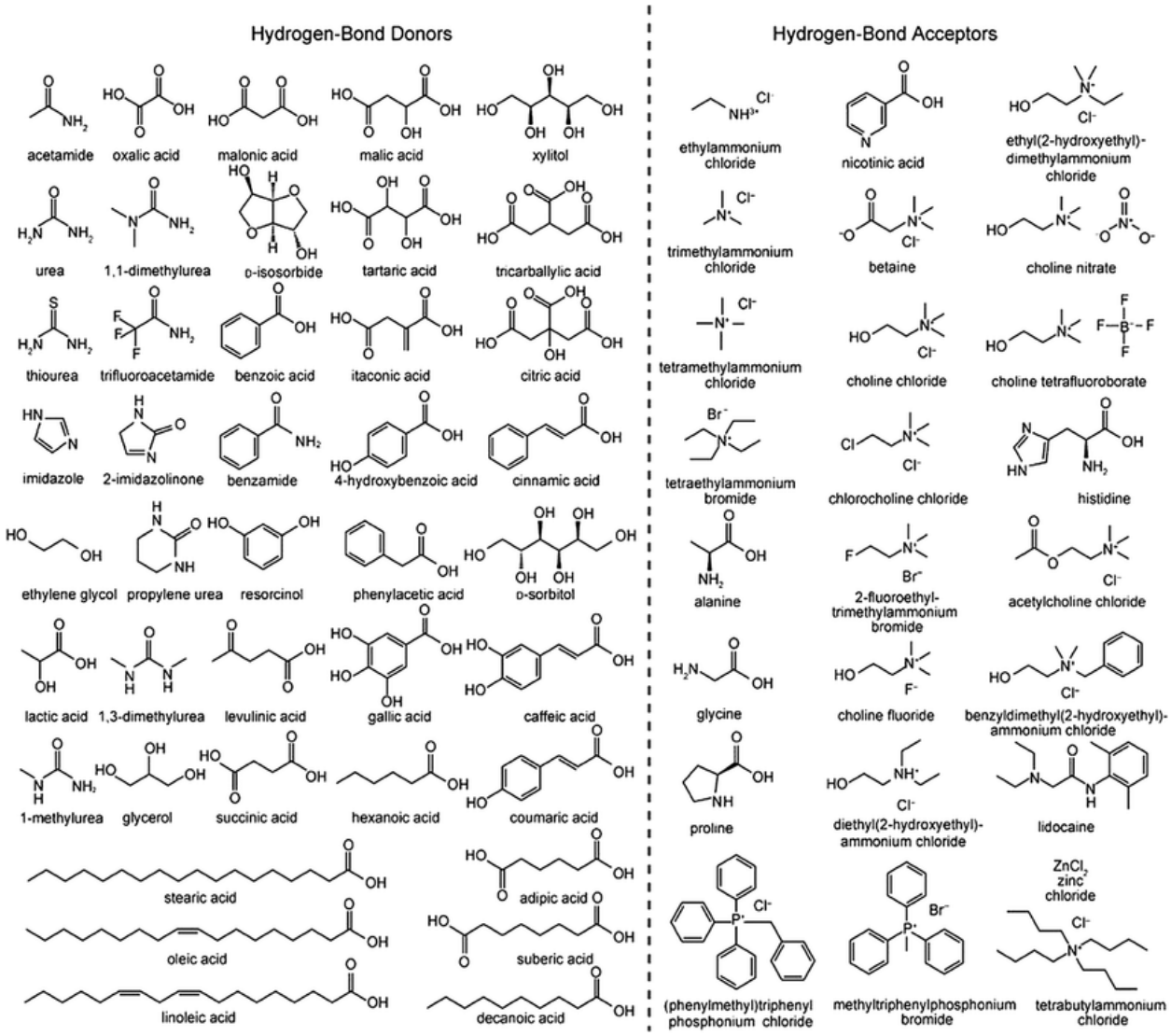
According to the Smith et al., DESs can be classified into four groups (**Table 3-15**) [238].

Table 3-15. Classification of DESs [238].

Type	Composition	Genaral formula	Terms
Type I	Quaternary ammonium salt and metal chloride	$Cat^+X^-zMCl_x$	M= metal ions like Zn, Sn, Fe, Al, Ga, In
Type II	Quaternary ammonium salt and metal chloride hydrate	$Cat^+X^-zMClx{\cdot}yH_2O$	M= metal ions like Cr, Co, Cu, Ni, Fe
Type III	Quaternary ammonium salt and hydrogen bond donor	Cat^+X^-zRZ	Z= groups like $CONH_2$, COOH, OH
Type IV	Metal chloride hydrate and hydrogen bond donor	$MCl_x + RZ = MCl_{x-1}^+.RZ + MCl_{x+1}^-$	M= Al, Zn and Z= $CONH_2$, OH

DES type III is the most commonly used; here, choline chloride (HBA) is commonly selected as a quaternary ammonium salt, and the typical HBDs are urea, polyalcohols, sugars, organic acids and others. In the case where the compounds that form DESs have abundant cellular constituents, these mixtures are called natural deep eutectic solvents (NADESs). **Table 3-16** summarises some of the most used HBDs and HBAs for the synthesis of DESs type III, which is further discussed in this doctoral dissertation. As can be noted from **Table 3-16**, some simple PCs are also classified as potential HBDs for DESs preparation.

Table 3-16. Chemical structures of the most commonly used HBDs and HBAs [239].



There are three possible procedures for the preparation of DESs, as follows:

1. Heating and stirring method [240]. The constituents of DESs (two or more) are weighted and placed in a bottle with the stirring bar and cap. The mixture is heated in a water bath between 50 °C and 80 °C with agitation for the appropriate period of time (usually up to 3 h).

2. Evoporating methods [240]. In the first step, the compounds of the DESs are dissolved in the water. In the second step, the water is evaporated at 50 °C with a rotary evaporator.

3. Freeze drying method described by Gutierrez et al. [241]. This method is based on freeze-drying the aqueous solution of the DESs' compounds.

Generally, DESs have similar physical characteristics to ILs, such as low vapor pressure, thermal stability and availability, but also, they have several advantages over traditionally used ILs, including biodegradability, a simple preparation process and low cost, which makes them potentially useful for application in large-scale processes.

A number of published papers related to DESs and their use in different scientific areas have increased exponentially over the last 10 years. DESs have been used in different fields of chemistry, including organic synthesis [242], electrochemistry [243], preparation of inorganic materials and biochemistry [238].

Interest for DESs is also rapidly increasing for separation processes, especially in the case of PCs extraction, indicating their great potential in the preparation of plant extracts for direct use for human consumption. One of the first articles on this was published by Dai et al. [244].

Radošević et al. [11] used NADES for the extraction of PCs from grape skins and tested the biological activity of the obtained extract. The results of their study confirmed the mixture of choline chloride and malic acid as the DESs with the best performance for extraction efficiency of TPC (91 mg GAE g^{-1} FF).

Bosiljkov et al. [9] focused on the extraction of anthocyanins from wine using ultrasound. Considering the maximum yield of total anthocyanins, the authors described the following conditions as optimal: extraction time of 30.6 min under the influence of ultrasound power of 341.5 W and water content in NADES of 35.4% (w/w).

On the other hand, Wei et al. tested the influence of different MAE parameters for the extraction of 14 PCs from *C.cajan* leaves using different types of DESs [8]. The optimized conditions were as follows: choline-chloride-maltose-based DES with 20% water, temperature of 60 °C under the influence of irradiation for 12 min and a solid-to-liquid ratio of 1:30 g mL^{-1}. Preparation of the solid phase for the SPE cartridges based on the DESs for the extraction of PCs was recently reported by Fu et al. [10].

Consequently, the last part of this doctoral dissertation focused on the optimization of the parameters for the isolation of bioactive compounds from different plant materials based on the type of DESs. The study of the different DESs was divided into two major segments.

The first segment involved the development of an HPLC-UV method for the quantitative determination of 21 target PCs from dried chokeberries. In the initial phase, the HPLC-UV method was optimized for the composition of the mobile phase, mobile phase flow, temperature and wavelength. The method developed was validated for linearity, precision as repeatability, LOD and LOQ. Additionally, efficiencies of five different DESs for the extraction of selected PCs from dried chokeberry were determined as the recoveries. All experiments were carried out by UAE.

In the second segment, a simple, inexpensive and eco-friendly MAE method involving DESs for the extraction of PCs from olive leaves was optimized. After extraction, a total of 48 PCs were quantitatively determined by the HPLC-DAD-ESI-TOF-MS method. The results were compared with those obtained by the CE method using 80% MeOH as the extraction solvent. According to the experimental results and the multivariate data analysis, optimal DES was selected. Additionally, a BBD and RSM were applied with the aim of optimizing the main parameters involved in the MAE procedure.

3.4.1 Optimization of DESs based extraction procedure for the isolation of PCs from chokeberries (*Aronia melanocarpa*)

3.4.1.1 Chemicals

Standard compounds: ethyl-o-vanillin (99%), diosmin (95%), rutin (99%), chrysin (99%), morin hydrate (95%), myricetin (>96%), apigenin (97%), (-)-epicatechin (99%), (+)-catechin, vanillic acid (97%), syringic acid (97%), *trans*-p-coumaric acid (98%), *trans*-o-coumaric acid (98%), *trans*-ferulic acid (98%), and rosmarinic acid (97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chlorogenic acid (99%), *trans*-cinnamic acid (98%), quercetin hydrate (99%) and flavone (99%) were supplied by Acros Organics (Belgium). Protocatehuic acid (99%), *trans*-caffeic acid (99%) and kaempferol were supplied by Merck (Germany). Gallic acid (99%) was purchased by Carlo Erba (Italy).

Solvents: HPLC-grade MeOH, HPLC-grade acetonitrile (ACN) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water (resistivity above 18 MΩ cm) used was obtained from a Milli-Q water purification system.

Other chemicals: Na_2CO_3 (>99%), $AlCl_3$ (>99%), D-(+)-glucose, DL-lactic acid and choline chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu phenol reagent (2 N), D-(-)-fructose and CH_3COONa were supplied by Merck (Germany).

3.4.1.2 Samples

The chokeberry or aronia (*Aronia melanocarpa*) belong to *Rosaceae* family, widely spread in North America, but in recent time it is very interesting also in Europe [245]. Cultivation of aronia is rapidly increasing in Slovenia since 2010. Nowadays, except a fresh aronia fruits different products based on this fruit such as juices, jams, jellies as well as extracts and dietary supplements are available.

It is proved that chokeberries are a rich source of dietary PCs, including anthocyanins, proanthocyanidins, hydroxycinnamic acids and flavonoids [246], with multiple health-promoting properties. Namely, the positive effect of diet rich in aronia is reflected in the prevention and treatment of various health diseases such as: colone cancer [247], blood pressure [248], reduction of total plasma cholesterol [249] and other.

Air dried chokeberries sample used in study was purchased from specialized chokeberry market (Aronija, Slovenia), milled in electric blender (Gorenje, Slovenia) during 45 s, packed in glass vessels and stored in the dark place at RT until analysed.

3.4.1.3 Preparation of DESs

All chemicals used for the preparation of five different DESs (**Table 3-17**) were vacuum oven dried (at 60 °C) for 24 h before use. HBA-choline chloride and different HBDs (urea, lactic acid, fructose and glucose) were weighted in certain molar ratios and mixtures were stirred in the sealed flasks at the 80 °C until the transparent, colorless liquids were formed (between 1 h and 3 h). Preparation of sugar based DESs was not possible without addition of a certain amount of water (1 mol).

Table 3-17. Composition of DESs tested for the extraction of PCs from dried chokeberries.

DES composition	Abbreviation	Molar ratio
Choline chloride:urea	CCU	1:1
Choline chloride:DL-lactic acid	CCLac	1:2
Choline chloride:DL-lactic acid	CCLac1	1:1
Choline chloride: D-(-)-fructose: water	CCFruc	2:1:1
Choline chloride: D-(+)-glucose: water	CCGluc	2:1:1

The main constraint since hinders the handling and efficiency of DESs as extraction solvents compared with conventional ones represents their relatively high viscosity. Thus, the addition of a certain amount of water is necessary for decreasing the viscosity and surface tension [239]. Optimization of dilution ratio in this research was carried out on CCLac. Therefore, different CCLac/water ratios (v/v=4/1, 2/1 and 1/1) were tested and an optimal ratio was 2/1 (v/v). All other DES were subsequently diluted with water at the same ratio.

3.4.1.4 Extraction procedure

1.0 g of milled aronia sample was weighted into centrifuge tube-15 mL, spiked with 50 μL of ISTD (ethyl-o-vanillin) and 5 mL of aqueous solution of specific DES was added. Extraction process was performed using ultrasonic bath for 20 min at the temperature of 35 °C. After sonication, the sample was centrifuged at 7 000 rpm for 5 min and the supernatant was accurately transferred into 10 mL glass flask using glass Pasteur pipette. Extraction procedure was repeated twice, supernatants were combined and diluted to exactly 10 mL with the appropriate DES.

In order to control the effectiveness of DESs for the extraction of PCs from chokeberries, the most used conventional methanolic extraction (using 80% MeOH) was carried out. For that purpose, 1.0 g of the sample was weighted into centrifuge tube-50 mL, 15 mL of 80% MeOH was added, and sample was twice exposed to the same extraction procedure as described previously. The methanolic extract (cca. 30 mL) was evaporated under the vacuum at 40 °C to approximately 8 mL and reconstituted to exactly 10 mL with 80% MeOH. All extractions were performed in duplicates.

The recoveries were obtained by spiking the chokeberry samples with the known concentrations of investigated standard compounds (10 mg L^{-1} each) and by their extraction following one of the above described procedures (DES or 80% MeOH).

3.4.1.5 Total phenolic and total flavonoid content

TPC in aronia extracts were determined according to the slightly modified Folin–Ciocalteu method, detailed described in **Section 3.3.6** [250].

TFC were measured by a standard spectrophotometric method, described in details in **Section 3.3.7**.

3.4.1.6 Total anthocyanin content

TAC were determined by the pH differential method [119]. Two appropriate aliquots of each extract were diluted with two different buffers (pH=1 and pH=4.5). The absorbances of so prepared solutions were measured at the two different wavelengths: 520 nm and 700 nm. TAC, expressed as mg cyanidin-3-glucoside equivalents per liter (mg Cya-3-Glu L^{-1}), was calculated using the following equation:

$$TAC = \frac{A \cdot MW \cdot DF \cdot 10^3}{\varepsilon \cdot 1} \quad (3.11)$$

where $A = (A_{520nm} - A_{700nm})pH_{1.0} - (A_{520nm} - A_{700nm})pH_{4.5}$; MW (molecular weight) = 449.2 g mol^{-1} for Cya-3-Glu; DF = dilution factor; 1 = pathlength in cm; ε= 26 900 molar extinction coefficient, in L $mol^{-1}cm^{-1}$, for cyanidin-3-glucoside and 10^3 = conversion factor from g to mg. The final results were expressed as mg cyanidin-3-glucoside equivalents per gram of dry weight (mg Cya-3-Glu g^{-1} DW). All measurements were performed in duplicates.

3.4.1.7 HPLC-UV instrumentation and data analysis

HPLC analysis were performed on Varian (ProStar) chromatographic system fitted with an auto sampler Varian (ProStar 410), a binary solvent pump Varian (ProStar 210), and an UV-ViS detector Varian (ProStar 310). The separation was achieved on an Agilent XDB-C18 chromatographic column (150 mm x 4.6 mm I.D., 5 μm particle size). The mobile phase consisted of 100% ACN (solvent A) and 1% aqueous solution of acetic acid (solvent B). The gradient program was as follow: 0-1 min 100% (B), 1–5 min 90% (B), 5–45 min 41% (B), 45–45:01 min 100% (B), and reequilibrium time was 10 min. Injection volume was 10 μL, and the flow rate was 1 mL min^{-1} at RT. The detection wavelength was set at 280 nm.

Additionally, developed method was validated for linearity, precision as repeatability, LOD and LOQ. For linearity determination, all calibration curves were constructed using the internal standard method. The curves were fitted to linear least-squares regression (R^2). The precision was evaluated through intraday and interday repeatability expressed as relative standard deviation in percentages (RSD %). The LOD was determined as the minimal concentration of analyte required to obtain S/N≥3, and the LOQ was determined as the minimal concentration of analyte required to obtain S/N≥10.

The identification of PCs from the samples was established by comparing their retention times to the retention times obtained for standard compounds, measured under the same chromatographic conditions.

Quantification was performed using standard calibration curves and internal standard method. The concentrations were expressed as mg of target compound per g^{-1} of dry weight (mg g^{-1} DW). All solvents and solutions were filtered through 0.45 μm polytetrafluorethylene (PTFE) filters prior to injection.

All results in the text and tables were expressed as the mean values ± standard deviations. Microsoft Excel was used for the data preparation and result outputs. Statistical data treatment was performed using SPSS Statistics (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). In order to determine the significant differences between extraction recoveries with conventional and different DESs, one-way ANOVA at the 95% confidence level was applied.

3.4.1.8 Discussion of results

HPLC-UV-VIS method optimization

One of the main objective of this part of the study was optimization of chromatographic separation of 21 PCs from different classes in a single chromatographic run. For that purpose, the following compounds were analysed: 11 different PAs (caffeic acid, ferulic acid, syringic acid, vanillic acid, p-coumaric acid, protocatehuic acid, o-coumaric acid, gallic acid, *trans*-cinnamic acid, rosmarinic acid and chlorogenic acid) and 10 flavonoids devided into sub-groups: flavonols (morin, quercetin, kaempferol and myricetin, catechin and (-)-epicatechin) and flavones (flavone, apigenin, chrysin and diosmin. In all performed analysis ethyl-o-vanillin was used as an internal standard. Ethyl-o-vanillin is a synthetic PC which could not be found in the nature and at the same time it has properties similar to those of the target compounds.

Optimization of HPLC method was performed on mixtures of pure standards. In the first step of method optimization different mobile phases consisting of acidified MeOH-water or ACN-water mixtures under isocratic conditions were tested for the separation and elution of the compounds. Obtained results were completely or partially overlapping peaks with poor resolution. Therefore the optimization was continued by gradient method.

Experiments during the development stage showed that the optimal chromatographic separation was achieved by selecting ACN rather than MeOH as an organic solvent (solvent A). For acidification, acetic and formic acid at various percentages were tested. The best separation was achived by using 1% acetic acid (solvent B). Additionaly, different flow rates were tested (0.7 mL min^{-1}, 0.8 mL min^{-1}, 1 mL min^{-1} and 1.2 mL min^{-1}), and flow of 1 mL min^{-1} was selected as the optimal one. At the end of optimization process the best separation of all tested analytes was obtained by the next gradient conditions: 0-1 min 100% (B), 1–5 min 90% (B), 5–45 min 41% (B), 45–45:01 min 100% (B) within total run time of 40 min. All of the compounds were properly detected at a wavelength of 280 nm, although other wavelength (240 nm, 285 nm and 320 nm) were tested. **Figure 3-18** present a HPLC-UV chromatogram of 21 standard compounds mixture (40 mg L^{-1}) recorded at 280 nm.

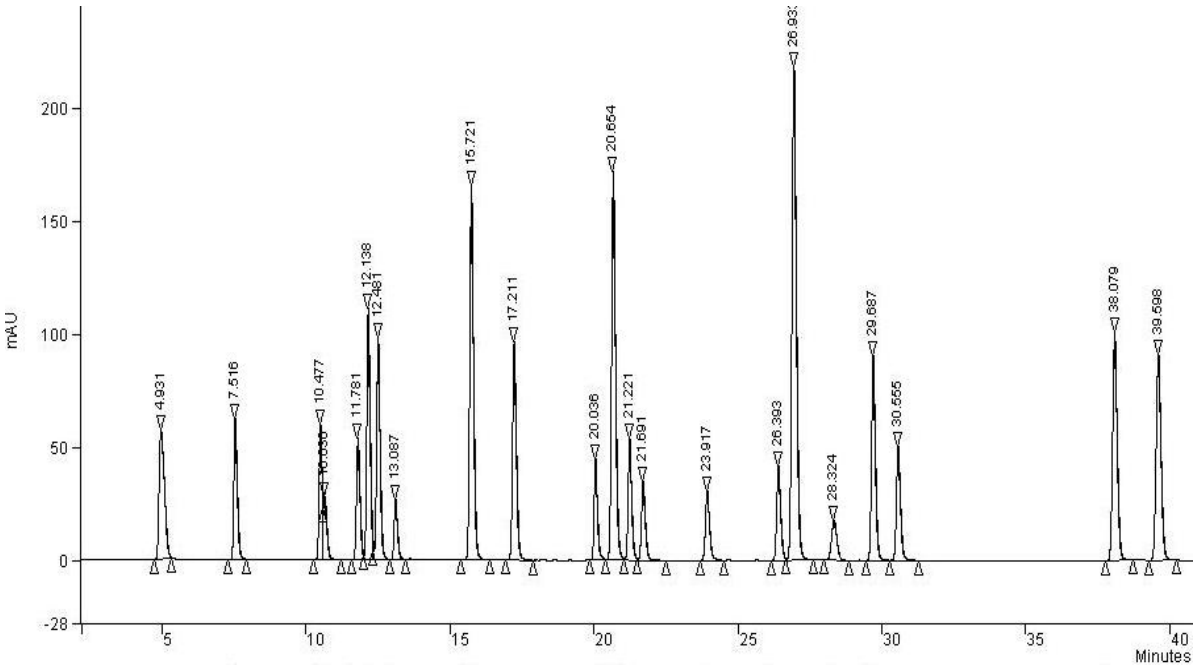


Figure 3-18. HPLC-UV chromatogram of standard PCs mixture (40 mg L^{-1}) recorded at 280 nm under optimal chromatographic conditions. Compounds order listed in **Table 3-18**.

The linearity of the method developed was tested within the concentration range from LOQ's to 50 mg L^{-1}. Good method linearity was confirmed for almost all of the investigated compounds (R^2>0.9993) except for the catechin (R^2=0.9967). The linearity of the proposed method was further tested by one-way ANOVA, which confirmed significant linear regression and non-significant deviation from linearity (P<0.05).

The intraday repeatability (system precision) was established by three successive injections of the one calibration solution (30 mg L^{-1}) on the same day and RSD's were calculated of the peak-area ratios between the PCs and ISTD. RSD's varied between 0.12% and 2.72%. Interday repeatability was determined after replicate injections of standard solutions on three different days, and RSD's ranged between 0.42% and 4.45%.

Average LOD and LOQ values were 0.30 mg L^{-1} and 0.80 mg L^{-1}, respectively. All validation parameters for investigated compounds including average retention times are summarized in **Table 3-18.**

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Table 3-18. Validation parameters for HPLC-UV method: average retention time (t_R); correlation coefficient (R^2); limit of detection (LOD); limit of quantification (LOQ); intraday and interday repeatability (%RSD).

No	Compound	t_R	R^2	LOD[a]	LOQ[a]	Repeatability (%RSD)	
						Intraday	Interday
1	Gallic acid	4.93	0.9999	0.27	0.91	0.25	2.14
2	Protocatehuic acid	7.52	0.9995	0.18	0.61	0.25	1.14
3	Chlorogenic acid	10.36	0.9993	0.18	0.61	2.66	1.19
4	Catechin	10.48	0.9967	0.36	1.20	1.72	2.34
5	Vanillic acid	11.63	0.9996	0.45	1.52	0.17	1.26
6	Caffeic acid	11.78	0.9996	0.27	0.91	2.49	2.50
7	Syringic acid	12.14	0.9996	0.36	1.21	0.12	1.43
8	(-)-Epicatechin	13.09	0.9998	0.18	0.61	0.25	2.28
9	p-Coumaric acid	15.72	0.9996	0.09	0.30	0.28	1.91
10	Ferulic acid	17.21	0.9996	0.18	0.61	1.18	1.89
11	Diosmin	20.04	0.9997	0.36	1.21	0.58	4.45
12	o-Coumaric acid	20.65	0.9996	0.09	0.30	0.36	2.17
13	Rosmarinic acid	21.22	0.9997	0.27	0.91	0.34	1.23
14	Myricetin	21.69	0.9998	0.27	0.91	1.99	1.20
15	Morin	23.92	0.9996	0.27	0.91	0.46	0.42
16	Quercetin	26.39	0.9994	0.18	0.61	0.62	1.22
17	*trans*-Cinnamic acid	26.93	0.9998	0.09	0.30	0.40	1.47
IS	Ethyl-o-vanillin	28.32	NC	NC	NC	1.01	1.31
18	Apigenin	29.69	0.9999	0.27	0.91	0.83	1.70
19	Kaempferol	30.56	0.9998	0.27	0.91	0.28	1.20
20	Chrysin	38.08	0.9998	0.18	0.61	2.72	1.28
21	Flavone	39.60	0.9995	0.18	0.61	1.16	1.93

[a] LOD and LOQ are in mg L^{-1}.
NC-not calculated.

Optimization of DESs based extraction method

As mentioned in the experimental part (**Section 3.4.1.3**), three groups of HBDs (amide e.g.urea, organic acid e.g. lactic acid and two sugars- fructose and glucose) were used in combination with choline chloride as the HBA to produce different DESs. All of the prepared DESs were found to be stable, transparent, viscous liquids without forming any precipitates during the preparation, extraction and analysis time (**Figure 3-19)**.

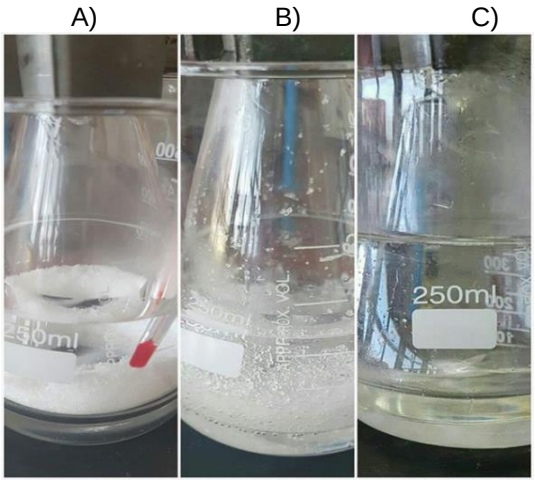

Figure 3-19. Preparation of DESs. A) mixing of individual compounds; B) mixture after 30 min at 80 °C; C) final product.

In order to decrease viscosity and surface tension as well as to increase extraction efficiency, aqueous solutions of all synthesized DES solvents were prepared. Optimization of this dilution process was carried out using different CCLac/water ratios (v/v=4/1, 2/1 and 1/1). Higher water content was not tested due to fact that large excess of water could cause breaking of hydrogen bonds between DES components and losing of eutectic nature of prepared solvents [241]. Three representative phenolic indices (dependent variables) were compared, namely TPC, TFC and TAC (**Figure 3-20**).

As can be observed from **Figure 3-20,** TPC was maximal at CCLac/water ratio 2/1 (27.40 mg GAE g^{-1} DW). The minimal extraction yield of TPC was found in the chokeberries extract obtained with CCLac/water 1/1. Similar patterns were observed for the TFC and TAC where the highest yields were also achived using CCLac/water ratio 2/1 (v/v), 2.06 mg RUT g^{-1} DW and 0.92 mg Cya-3-Glu g^{-1} DW, respectively. According to these preliminary results all other DESs were diluted with water at the same volume ratio.

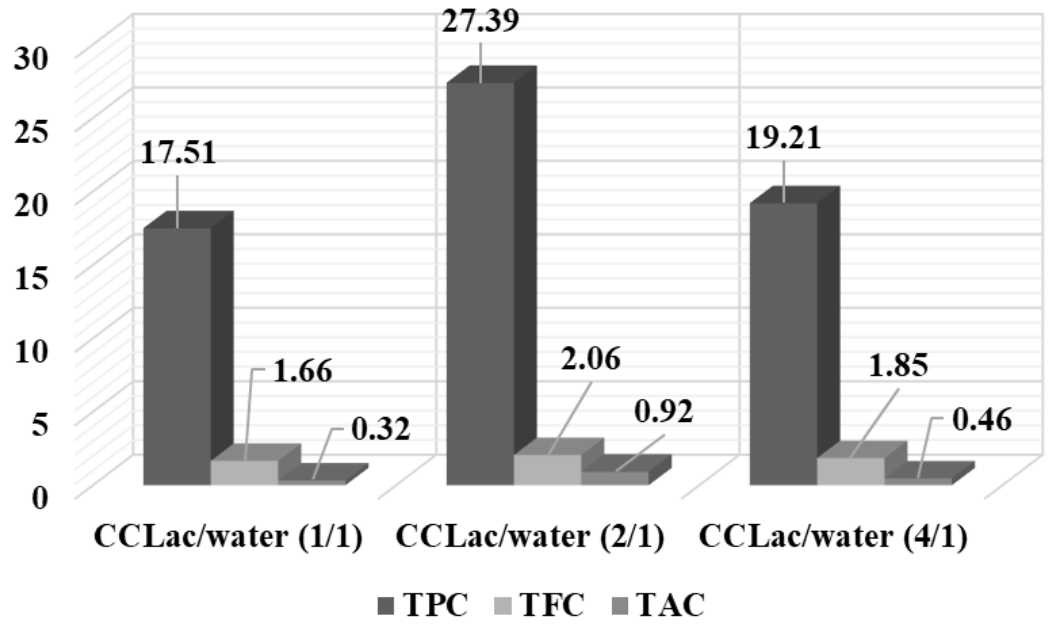

Figure 3-20. TPC, TFC and TAC in chokeberries extracts obtained affter extraction with different CCLac aqueous solutions. TPC were expresed as mg GAE g^{-1} DW; TFC were expresed as mg RUT g^{-1} DW and TAC were expresed as mg Cya-3-Glu g^{-1} DW.

The extraction efficiencies of synthesized DESs and 80% MeOH for extraction of different PCs sub-classes from chokeberry were compared. TPC, TFC and TAC were determined in all obtained extracts and results are presented in **Table 3-19**.

Table 3-19. Average extraction yields for TPC, TFC and TAC obtained after extraction with different DESs (DES/water = 2/1 v/v) and with 80% MeOH. Different superscripts for the same response denoted significant differences among solvents tested according to the S-N-K method at $p<0.05$.

Solvent	**TPC[1,2]**	**TFC[1,3]**	**TAC[1,4]**
80% MeOH (control sample)	27.11±1.37^{b}	3.37±0.25^{b}	1.25±0.06^{e}
CCU	17.32±0.61^{a}	2.50±0.14^{a}	0.52±0.01^{a}
CCLac	27.52±0.14^{b}	2.13±0.10^{a}	0.93±0.05^{c}
CCLac1	23.84±1.23^{b}	1.99 ±0.12^{a}	0.57±0.02^{a}
CCFruc	36.15±3.39^{c}	4.71±0.33^{c}	1.01±0.03^{d}
CCGluc	23.60±0.02^{b}	3.25±0.08^{b}	0.83±0.01^{b}

[1]Mean values ± standard deviation.
[2]TPC expresed as mg GAE g^{-1} DW.
[3]TFC expresed as mg RUT g^{-1} DW.
[4]TAC expresed as mg Cya-3-Glu g^{-1} DW.

From **Table 3-19** it is evident that the highest value of TPC was found in the extract obtained with CCFruc (36.15±3.39 mg GAE g^{-1} DW). No statistically significant difference was found between extract obtained using 80% MeOH, CCLac1, CCLac and CCGluc as the extraction solvents. While, the lowest extraction efficiency for the TPC was shown by urea based DES (CCU), with the mean value 17.32 ± 0.61 mg GAE g^{-1} DW.

For TFC, CCFruc showed the highest extraction efficiency, followed by CCGluc and 80% MeOH. The lactic acid based DESs (CCLac and CCLac1) had the lowest efficiency in the extraction of total flavonoids from chokeberries.

The efficiency order for the extraction of TAC from the dried chokeberries was as follow: 80%MeOH>CCFruc>CCLac>CCGluc>CCLac1≈CCU. The results confirmed that fructose-based DES (CCFruc) generally showed the highest capacity for extracting different PCs from *Aronia* berries (even 33% higher than with 80% MeOH under the same extraction conditions). This results are comparable with the similar results reported by other authors [251].

The lowest extraction capacity generally showed urea-based DES (CCU).

Among lactic acid based DES, higher extraction efficiency showed CCLac, the solvent with the higher molar ratio of lactic acid. It can be assumed that lower viscosity and higher polarity of CCLac led to better extraction when compared with CCLac1 [252].

Determination of selected PCs in real chokeberries samples

Plant samples exhibit matrix effects in since other sample components interfere with the studied compounds. Therefore matrix spikes and comparing the spiked samples with non-spiked samples enable the best measurement of this effect. Matrix spikes also provide the best overall assessment of accuracy of the developed method. The accuracy after extraction with DES or 80% MeOH was determined through the calculation of recovery values expressed in percentages. Therefore the chokeberry samples were spiked with the known concentrations of investigated standard compounds mixture (10 mg L^{-1} each compound) and extracted either by methanolic or DES extraction. Results were compared with those after extraction of non-spiked samples. The calculated recovery values for each compound are summarized in **Table 3-20**.

Table 3-20. Extraction recoveries (%) for analysed PCs obtained after extraction with 80% MeOH and after extraction with different DESs.

Compound	MeOH[a]	CCLac[a]	CCLac1[a]	CCFruc[a]	CCGluc[a]
Gallic acid	35.6±2.2	58.2±2.2	85.2±0.2	50.9±3.1	45.3±1.7
Protocatehuic acid	73.8±4.6	108.8±1.2	96.4±4.5	ND	68.6±1.8
Chlorogenic acid	93.2±1.8	91.2±4.3	99.6±0.4	99.8±1.8	99.8±2.0
Vanillic acid	38.1±0.7	94.6±0.6	73.5±3.2	75.0±1.5	79.5±1.9
Caffeic acid	49.6±2.3	79.7±4.7	83.1±0.1	88.7±2.1	97.6±2.4
Syringic acid	56.6±5.0	52.3±4.1	51.4±1.6	93.8±0.1	100.5±2.5
(-)-Epicatechin	93.8±1.3	115.3±1.9	52.3±5.0	101.3±0.6	103.7±2.5
p-Coumaric acid	61.1±2.0	100.2±4.3	70.0±5.0	80.4±3.6	58.3±2.2
Ferulic acid	ND	119.0±6.2	69.0±5.0	88.9±4.5	60.9±2.8
Diosmin	76.2±1.4	75.9±1.8	66.8±4.3	70.5±5.0	49.1±0.9
o-coumaric acid	62.3±4.9	98.8±3.5	78.5±5.0	86.0±4.9	98.5±1.1
Rosmarinic acid	60.8±0.7	90.0±3.7	67.7±5.1	99.0±1.3	105.1±4.1
Myricetin	82.3±4.0	98.1±0.6	89.0±2.8	102.3±3.0	103.5±2.4
Morin	58.0±3.7	99.5±5.0	85.5±3.8	57.7±3.8	60.2±0.2
Quercetin	65.4±2.7	93.6±0.6	88.6±3.4	71.9±3.5	88.7±1.8
trans-Cinnamic acid	65.5±4.9	99.1±3.9	99.5±1.7	96.6±2.1	101.3±1.7
Chrysin	80.9±4.7	88.8±0.8	74.7±4.9	35.8±4.6	36.6±3.3
Flavone	89.7±1.4	104.9±1.5	83.9±10	49.3±4.1	38.5±4.9

[a]The results are expresed as mean values ± standard deviations of two individual measerments, in percentages.
ND-not defined. Co-elution was appeared, and correct integration was not possible.

From **Table 3-20** it is evident that the extraction efficiencies were highly dependent on the type of DES solvent as well as on the PCs class, ranging from 36% up to 119%. Generally, high extractability of PCs with DES may be attributed to H-bonding interactions between DES molecules and PCs [253]. Further, from our results it is obvious, that for all of the analysed PAs, DESs showed higher extraction efficiency in the comparison to the 80% MeOH. This is probably related to the fact that different PAs (gallic acid, caffeic acid, *trans*-cinnamic acid and coumaric acids) can also be used as the hydrogen bond donors for the preparation of DES [239], [253]. Our study further confirmed high extraction capacity of lactic acid based DES (CCLac) for flavonoids such as myricetin, morin and quercetin.

Finally, the concentrations of individual PCs in the dried chokeberries were determined by optimized HPLC-UV method, and the results are summarized in **Table 3-21.**

Table 3-21. Content of PCs in real dried chokebbery samples. Effect of DESs type (CCLac and CCFruc). Comparison of the results with those obtained after extraction with 80% aqueous MeOH.

No.	Compound	MeOH[a]	CCLac[a]	CCFruc[a]
1	Gallic acid	NQ	3.47±0.28	2.59±0.04
2	Protocatehuic acid	109.20±6.40	136.31±8.72	89.12±6.03
3	Chlorogenic acid	1339.80±13.81	2006.91±117.04	1965.04±1.12
4	Catechin	NQ	NQ	NQ
5	Vanillic acid	NQ	29.12±4.20	NQ
6	Caffeic acid	14.17±0.73	17.83±1.10	19.19±1.80
7	Syringic acid	15.81±1.75	9.31±0.09	NQ
8	(-)-Epicatechin	NQ	NQ	NQ
9	p-Coumaric acid	NQ	2.47±0.45	4.62±0.26
10	Ferulic acid	NQ	NQ	NQ
11	Diosmin	NI	NI	NI
12	o-Coumaric acid	NI	NI	NI
13	Rosmarinic acid	NI	NI	NI
14	Myricetin	NI	NI	NI
15	Morin	NI	NI	NI
16	Quercetin	8.62±0.77	28.93±1.48	11.14±1.70
17	*trans*-Cinnamic acid	NQ	NQ	NQ
18	Apigenin	NI	NI	NI
19	Kaempferol	NI	NI	NI
20	Chrysin	NI	NI	NI
21	Flavone	NI	NI	NI

[a]The results are expresed as mean values±standard deviations (mg kg^{-1} DW).
NI-not identified. Concentration below LOD (see **Table 3-18**).
NQ-not quantified. Concentration below LOQ (see **Table 3-18**).

3.4.2 Optimization of DESs based extraction procedure for the isolation of PCs from olive leaves (*Olea europaea)*

3.4.2.1 Chemicals

Standard compounds: hydroxytyrosol (99%), tyrosol (99%), luteolin (99%) and apigenin (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while oleuropein was provided by Extrasythese (Lyon, France). Gallic acid (95%) was purchase by Merck (Darmstadt, Germany).

Solvents: HPLC-grade MeOH and ACN were purchased from Panreac (Barcelona, Spain) and Labscan (Dublin, Ireland), respectively. Acetic acid of an analytical grade (>99.5%) was acquired from Fluka (Switzerland). Water was purified through a Milli-Q system (Millipore, Bedford, MA, USA).

Other chemicals: choline chloride, lactic acid, oxalic acid, tartaric acid, 1,4-butanediol, ethylene glycol, xylitol, 1,2-propanediol, maltose and urea were purchase from Sigma-Aldrich (St. Louis, MO, USA). Folin Ciocalteu's phenol reagent and Na_2CO_3 were obtained from Merck (Darmstadt, Germany).

3.4.2.2 Samples

Olive leaves, the by-products of olive farming, are recognised as a rich source of bioactive compounds, among them secoiridoids, phenylethanoids and flavonoids [254]. Recent scientific research has classified olive leaves as providing potential health benefits and demonstrated their biological and pharmaceutical activities, such as the ability to aid in antihypertensive, anticarcinogenic, anti-inflammatory, hypoglycaemic, antimicrobial, antiviral, antitumour, antithrombotic and hypocholesterolaemic effects [255,256].

The olive leaves used in this research were from the *Hojiblanca* variety grown in Seville (Spain). The samples were collected randomly from different parts of several trees in which no phytosanitary products were applied. Fresh leaves were immediately transferred to the CIDAF laboratory (Granada), washed with distilled water, and dried inside under a controlled temperature at 22ºC until constant weight was achieved. All collected samples were ground and stored at -20ºC until their use.

3.4.2.3 Preparation of DESs

Nine different DESs based on choline chloride as HBA with different HBDs were prepared (**Table 3-22**), as described previously in section 3.4.1.2.

Table 3-22. Composition of DESs used for the extraction of PCs from olive leaves.

DES composition	Abbreviation	Molar ratio
Choline chloride:lactic acid	CCLac	1:2
Choline chloride:oxalic acid	CCOx	1:1
Choline chloride:tartaric acid	CCTart	2:1
Choline chloride:1,4-butanediol	CCBut	1:6
Choline chloride:ethylene glycole	CCEtg	1:2
Choline chloride:xylitol	CCXy	2:1
Choline chloride:1,2-propanediol	CCProp	1:1
Choline chloride:maltose	CCMalt	3:1
Choline chloride:urea	CCU	1:2

3.4.2.4 Microwave-assisted extraction procedure

MAE of the olive leaves was conducted in closed microwave extractor vessels (Anton Paar GmbH, Graz, Austria). For the preliminary screening of DESs, 200.0 mg of powdered sample was accurately weighted, and 1.5 mL of the selected DES was added. The following initial conditions were set up: extractions were carried out with the DES mixed with 25% of water at 65ºC with an irradiation time of 20 min. All experiments were temperature-controlled and monitored by the software while microwave power and pressure were fixed to 700 W and 18 bar, respectively. After extraction was finished, 1.5 mL of water was added, and the sample was quantitatively transferred into a falcon tube to be centrifuged at 13 000 rpm for 15 min at 20ºC. Further, the supernatant was filtered through a 0.20 μm PTFE filter and diluted up to 10 mL with water. The most promising DES was selected, and the extraction procedure was further optimized by applying the experimental design.

To control the effectiveness of DESs for the extraction of PCs from olive leaves, the most commonly used methanolic extraction (using 80% MeOH) was carried out. For this purpose, exactly 200.0 mg of the sample was weighted into MAE vessels, 1.5 mL of 80% MeOH was added, and the sample was exposed twice to the same extraction procedure. All extractions were performed in duplicates.

3.4.2.5 Experimental design

To optimize the DES based extraction procedure for the isolation of PCs from olive leaves, an experimental design was applied. Design-expert software (Design Expert 10, free trial version) was used for the experimental design and statistical analysis of the data. A three-level (-1, 0, +1), three-factor BBD combined with RSM was conducted. The influence of three major factors, temperature (X_1), microwave irradiation time (X_2) and water content in DES (X_3), were tested as the independent variables.

Temperature extraction was evaluated in the range 40–80ºC, and irradiation time ranged from 10–40 min; the percentage of water evaluated was from 0–70%. A total of 12 experiments with three replications at the central point with different combinations of three factors were carried out. The selected variables were both the sum of the content of the phenolic compounds (Σ phenolic compounds) and their individual yields, as well as the TPC measured by Folin-Ciocalteu method. The experimental data were fitted to second-order polynomial models to obtain the regression coefficients; the generalised equation can be written as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j=1}^{k} \beta_{ij} X_i X_j \quad (3.12)$$

where Y is the response variable, X_i and X_j are the independent variables, and k is the number of tested variables (k = 3). The regression coefficient is defined as β_0 for the intercept, β_i for the linear, β_{ii} for the quadratic and β_{ij} for cross-product term.

3.4.2.6 Total phenolic content

TPC in olive leave extracts was determined according to the Folin-Ciocalteu procedure [257]. Here, 10 µL of properly diluted extracts were mixed with 600 µL of deionised water and 50 µL of Folin-Ciocalteu reagent. After 10 min, 150 µL of Na_2CO_3 solution 20% (w/v) and 190 µL of deionised water were added. After 2 h of incubation at RT in the dark, 200 µL were transferred into a microplate with 96 wells, and the absorbance was measured at 760 nm in a BioTec spectrophotometer microplate reader (Winooski, Vermont, EEUU). The results were expressed as mg GAE g^{-1} DW. The analyses were performed in triplicates.

3.4.2.7 HPLC-DAD-ESI-TOF-MS instrumentation and data analysis

Analysis of the phenolic fractions of olive leaves were performed on an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA) coupled to a micrOTOF (Bruker Datonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) [258].

The chromatographic separation was carried out on a Poroshell 120 EC-C18 analytical column (4.6 mm x 100 mm, 2.7 μm) supplied by Agilent Technologies. Satisfactory HPLC separation was achieved by using previously optimized and validated method [258]. The mobile phases used were water with acetic acid 1% (phase A) and acetonitrile (phase B). The linear gradient for solvent B was as follows: 0 min, 5% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 38% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 35 min, 100% B; 37 min, 5% B. The flow rate used was set at 0.8 mL min^{-1}. The column temperature was set at 25ºC and a sample volume of 10 μL was injected.

The effluent from the HPLC column was split using a T type phase separator before introducing it into the mass spectrometer (split ratio 1:3). The final flow arrived to the ESI-TOF-MS detector was 0.2 mL min^{-1}. The UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240 and 280 nm. Parameters for ESI-TOF-MS were set using negative ion mode with spectra acquired over a mass range from *m/z* 50 to 1500. The optimum values of the ESI-TOF-MS parameters were as follows: capillary voltage, +4.5 kV; drying gas (N_2, 5.0) temperature, 190ºC; drying gas flow, 9.0 L min^{-1}; and nebulizing gas pressure, 2 bar [259]. The accurate mass data of the molecular ions were processed through the software Data Analysis 3.4 (Bruker Daltonik GmgH, Bremen, Germany), which provided a list of possible elemental formulas by using the Generate Molecular Formula (GMF) Editor. The GMF Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula (Bruker Daltonics Technical Note 008, Molecular formula determination under automation). All of the spectra were calibrated, prior to compound characterization, by using a sodium acetate cluster

containing 5 mM sodium hydroxide and 0.2% acetic acid in water:isopropanol (1:1, v/v), injected at the beginning of each run with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL) directly connected to the interface [258].

To check the significant differences between the yields after extraction with 80% MeOH and different DESs, a one-way ANOVA test at a confidence level of 95% was applied to the chemical data. The S-N-K t test was also applied to discriminate among the means of the chemical data. All statistical analyses were carried out using IBM SPSS Statistics 19 for Windows statistical package.

3.4.2.8 DES for the extraction of PCs from the olive leaves

The first aim of this part of the study was to select the most suitable DES for the extraction of PCs from olive leaves. Nine different DESs were prepared and evaluated. As mentioned before, the viscosity of DESs differs significantly according to the DES's composition. Water addition and temperature are the most crucial factors that can change the properties of a DES and decrease the viscosity and surface tension. Thus, to select the best DES to extract PCs with, the following initial conditions were set up: extractions were carried out with the DES mixed with 25% of water under 65ºC with an irradiation time of 20 min. With the aim of comparing the extraction efficiency exhibited by DES, 80% MeOH was selected because of its excellent performance, as described in other studies [258,260].

The phenolic profile of olive leaves was determined by HPLC-DAD-ESI-TOF-MS. The identification of PCs was carried out based on the interpretation of their mass spectra, which was determined by TOF-MS and from previously reported data. A total of 49 compounds were identified and quantified in all extracts. **Table 3-23** summarises all the compounds identified in the control (80% MeOH) sample, including retention times, wavelengths of the maximum absorption and the most important TOF-MS identification parameters. These results revealed the presence of a wide range of PCs from different classes.

Table 3-23. PCs identified in the olive leaves extract.

Peak	RT (min)	λ (nm)	*m/z* experimental	*m/z* calculated	Tolerance (*ppm*)	Error (*ppm*)	mSigma	Molecular formula	Proposed Compound
1	3.26	240	375.1303	375.1297	10	-0.6	15.3	$C_{16}H_{24}O_{10}$	(Epi)loganic acid isomer 1
2	3.43	240	389.1081	389.1089	10	2.2	24.4	$C_{16}H_{21}O_{11}$	Oleoside /Secologanoside isomer 1
3	3.61	240	389.1085	389.1089	10	1.2	22.5	$C_{16}H_{21}O_{11}$	Oleoside /Secologanoside isomer 2
4	3.96	240	389.1095	389.1086	10	-1.3	22.3	$C_{16}H_{21}O_{11}$	Oleoside /Secologanoside isomer 3
5	4.52	280	315.1075	315.1085	10	3.2	18.7	$C_{14}H_{20}O_{8}$	Hydroxytyrosol-glucoside isomer 1
6	4.78	280	315.1073	315.1085	10	3.9	9.4	$C_{14}H_{20}O_{8}$	Hydroxytyrosol-glucoside isomer 2
7	5.00	280	153.0550	153.0557	10	4.7	15.7	$C_{8}H_{10}O_{3}$	Hydroxytyrosol
8	5.10	240	389.1100	389.1089	10	-2.9	21.1	$C_{16}H_{22}O_{11}$	Oleoside/Secologanoside isomer 4
9	5.55	240	375.1288	375.1297	10	2.4	17.8	$C_{16}H_{24}O_{10}$	(Epi)loganic acid isomer 2
10	7.36	280	339.0722	339.0722	10	0.0	55.6	$C_{15}H_{16}O_{9}$	Esculin
11	8.01	280	341.0866	341.0878	10	3.5	30.9	$C_{15}H_{18}O_{9}$	Caffeoylglucoside
12	9.28	240	389.1095	389.1086	10	-1.3	22.3	$C_{16}H_{22}O_{11}$	Oleoside/Secologanoside isomer 5
13	10.05	240	389.1139	389.1089	20	-12.8	27.2	$C_{16}H_{22}O_{11}$	Olesoide/Secologanoside isomer 6
14	11.29	240	403.1267	403.1246	10	-5.2	8.9	$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer 1
15	12.32	240	377.1466	377.1453	10	-3.4	14.1	$C_{16}H_{26}O_{10}$	Oleuropein/oleuroside aglycone
16	13.15	240	609.1479	609.1461	10	-2.9	28.0	$C_{27}H_{30}O_{16}$	Glucosyl rhamnosylquercetin (rutin) isomer 1
17	16.30	240	403.1254	403.1246	10	-2.0	3.1	$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer 2
18	13.68	240	415.1612	415.1610	10	-0.6	5.8	$C_{19}H_{28}O_{10}$	Phenethyl primeveroside isomer 1
19	13.88	240	415.1607	415.1610	10	0.7	7.3	$C_{19}H_{28}O_{10}$	Phenethyl primeveroside isomer 2
20	14.58	240	403.1973	403.1974	10	0.0	3.3	$C_{19}H_{32}O_{9}$	Ethyl-glucopyranosyloxy-oxopropyl-cyclohexaneacetic acid
21	15.35	240	555.1804	555.1719	20	-15.3	58.7	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein/hydroxyoleuroside isomer 1
22	15.52	240	555.1810	555.1719	20	-16.3	55.3	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein/hydroxyoleuroside isomer 2
23	15.69	240	609.1485	609.1461	10	-3.9	27.7	$C_{27}H_{30}O_{16}$	Glucosyl rhamnosylquercetin (rutin) isomer 2
24	15.82	240	593.1551	593.1512	10	-6.5	36.3	$C_{27}H_{29}O_{15}$	Luteolin rutinoside
25	16.50	240	447.1014	447.0933	20	-18.1	66.6	$C_{21}H_{20}O_{11}$	Luteolin glucoside isomer 1
26	16.72	240	623.1979	623.1981	10	0.3	11.2	$C_{29}H_{36}O_{15}$	Verbascoside
27	16.86	240	555.1727	555.1719	10	-1.4	4.3	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein/hydroxyoleuroside isomer 3
28	18.06	240	577.1604	577.1563	10	-7.2	12.0	$C_{27}H_{30}O_{14}$	Apigenin rutinoside
29	18.23	240	701.2301	701.2298	10	-0.3	9.9	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 1

Table 3-23. Continued.

30	18.50	240	701.2307	701.2298	10	-1.2	9.2	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 2
31	18.88	240	701.2336	701.2298	10	-5.4	30.4	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 3
32	18.98	240	431.1017	431.0984	10	-7.7	15.0	$C_{21}H_{20}O_{10}$	Apigenin glucoside
33	19.11	240	447.0974	447.0933	10	-9.2	30.5	$C_{21}H_{20}O_{11}$	Luteolin glucoside isomer 2
34	19.45	240	461.1129	461.1089	10	-8.7	15.3	$C_{22}H_{22}O_{11}$	Diosmetin glucoside
35	19.80	240	701.2307	701.2298	10	-1.3	12.3	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 4
36	20.35	240	701.2345	701.2298	10	-6.7	39.7	$C_{31}H_{42}O_{18}$	Oleuropein/oleuroside glucoside isomer 5
37	20.07	240	569.1930	569.1876	10	-9.5	34.6	$C_{26}H_{34}O_{14}$	2”-methoxyoleuropein isomer 1
38	20.25	240	569.1941	569.1876	20	-11.5	52.5	$C_{26}H_{34}O_{14}$	2”-methoxyoleuropein isomer 2
39	20.60	240	539.1931	539.1770	30	-29.8	13.9	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 1
40	21.21	240	539.1791	539.1770	10	-3.8	22.1	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 2
41	21.57	240	539.1888	539.1770	30	-21.9	59.4	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 3
42	21.87	240	557.2253	557.2240	10	-2.5	8.3	$C_{26}H_{38}O_{13}$	(dimethyl hydroxy octenoyloxi)secologanoside isomer 1
43	22.26	240	601.2187	601.2138	10	-8.1	34.7	$C_{27}H_{38}O_{15}$	Unknown
44	22.44	240	523.1892	523.1821	20	-13.5	32.8	$C_{25}H_{32}O_{12}$	Ligstroside
45	23.00	240	285.0412	285.0405	10	-2.8	0.7	$C_{15}H_{10}O_{6}$	Luteolin
46	23.30	240	557.2243	557.2240	10	-0.6	17.3	$C_{26}H_{38}O_{13}$	(dimethyl hydroxy octenoyloxi)secologanoside isomer 2
47	23.50	240	553.1927	553.1927	10	-0.1	14.6	$C_{26}H_{34}O_{13}$	Oleuropein/oleuroside methyl ether
48	23.85	240	539.1778	539.1770	10	-1.4	17.2	$C_{25}H_{32}O_{13}$	Oleuropein/oleuroside isomer 4
49	25.45	240	269.0457	269.0455	10	-0.5	92.8	$C_{15}H_{10}O_{5}$	Apigenin

The quantification of all identified peaks using the peak areas and calibration curves of several available standards (**Table 3-24**) was performed. Among the identified compounds, oleuropein and its corresponding isomers were confirmed as the most abundant PCs in olive leaves. Oleuropein is a secoiridoid, previously described as one of the PCs with several pharmacological effects, including antioxidant, anti-inflammatory, anticancer, antiviral, antimicrobial and antiatherogenic effects; but it is also responsible for the characteristic bitterness of the olive fruit [261]. According to the results reported by other authors, the concentration of oleuropein can reach up to 140 mg g^{-1} DW in young olives and 60–90 mg g^{-1} of DW in olive leaves [262]. Alongside oleuropein, oleoside, elenolic acid, luteolin glucoside and ligstroside were also quantified at high concentration levels in all extracts. The concentrations of the identified compounds were expressed as the mg of compound per g of dried plant material (mg g^{-1} DW).

Table 3-24. Calibration curves for the quantification of PCs identified in the olive leaves.

Phenolic compounds	External standard	Wavelength	Regresion curve	R^2
Secoiridoids: oleoside/secologanoside, elenolic acid glucoside, oleuropein aglycone, hydroxyl-oleuropein, 2”-methoxyoleuropein, oleuropein, oleuropein glucoside and ligstroside	Oleuropein	240	Y = 29.103x + 2.7426	0.9999
Apigenin and its derivatives: apigenin rutinoside and apigenin glucoside isomer 1	Apigenin	240	Y = 80.372x – 148.88	0.9941
Luteolin and its derivatives: luteolin isomer 1, luteolin glucoside isomer 2, luteolin glucoside isomer 1 and luteolin rutioside isomer 2	Luteolin	240	Y=22.953x - 13.989	0.9999
Hydroxytyrosol and its derivatives: hydroxyltyrosol glucoside, esculine, caffeoylglucoside and verbascoside	Hydroxytyrosol	280	Y=20.238x + 2.1161	0.9998

Figure 3-21 represents the effect of different DESs on the extraction efficiencies of the PCs from the olive leaves compared to the conventional extraction methodology (80% MeOH).

Based on the results, significant differences regarding the yields of extracted PCs were found.

In relation to the organic acid-based DESs, three different acids (lactic acid, oxalic acid and tartaric acid) were used in a proper molar ratio to form eutectic mixtures with choline chloride. Among them, significant higher extraction yields for all considered compounds were achieved with CCLac.

On the other hand, oxalic acid and tartaric acid DESs showed the lowest extraction capacity for the Σ phenolic compounds, as well as for oleuropein compared to all other DESs tested. This could be attributable to the high viscosity of oxalic and tartaric acid DESs, which hindered their efficiency as extraction solvents due to the low mass transport. Comparing these two solvents (CCOx and CCTart), no statistically significant differences were found in the extraction efficiency for the Σ phenolic compounds. However, curiously, the extraction of oleuropein seemed to be better for CCTart than for CCOx. This result was probably caused by the extremely low pH value of oxalic acid (the pKa values of organic acids used are 1.25, 2.89 and 3.86 for oxalic acid, tartaric acid and lactic acid, respectively). Namely, other authors have confirmed higher extraction recovery for oleuropein extracted with the solvents under mild pH conditions [251].

From **Figure 3-21**, it could be noted that polyalcohol-based DESs (CCBut, CCEtg, CCXy and CCProp) showed the highest ability for extraction of PCs compared to the other tested solvents. This was in good agreement with the results reported by other authors who pointed out polyalcohol-based DESs are excellent extraction solvents for PAs and other PCs from several types of oils and plants, such as *Lonicerae japonicae* and *Pyrola incarnate* [251,263,264]. This can be explained by the low viscosity and the high hydrogen-bonding ability of these solvents.

CCEtg was found to be the best alcohol-based DES for the extraction of all individual PCs, including the Σ phenolic compounds. Compared to extraction using 80% MeOH, no significant differences were found in the extraction efficiency of the Σ phenolic compounds by means of CCEtg. On the other hand, for the extraction of oleuropein, 80% MeOH showed a slightly higher extraction yield. However, major recoveries of glycoside derivatives for some PCs, such as elenolic acid glucoside, luteolin glucoside and oleuropein glucoside, were detected when CCEtg was employed as an extraction solvent.

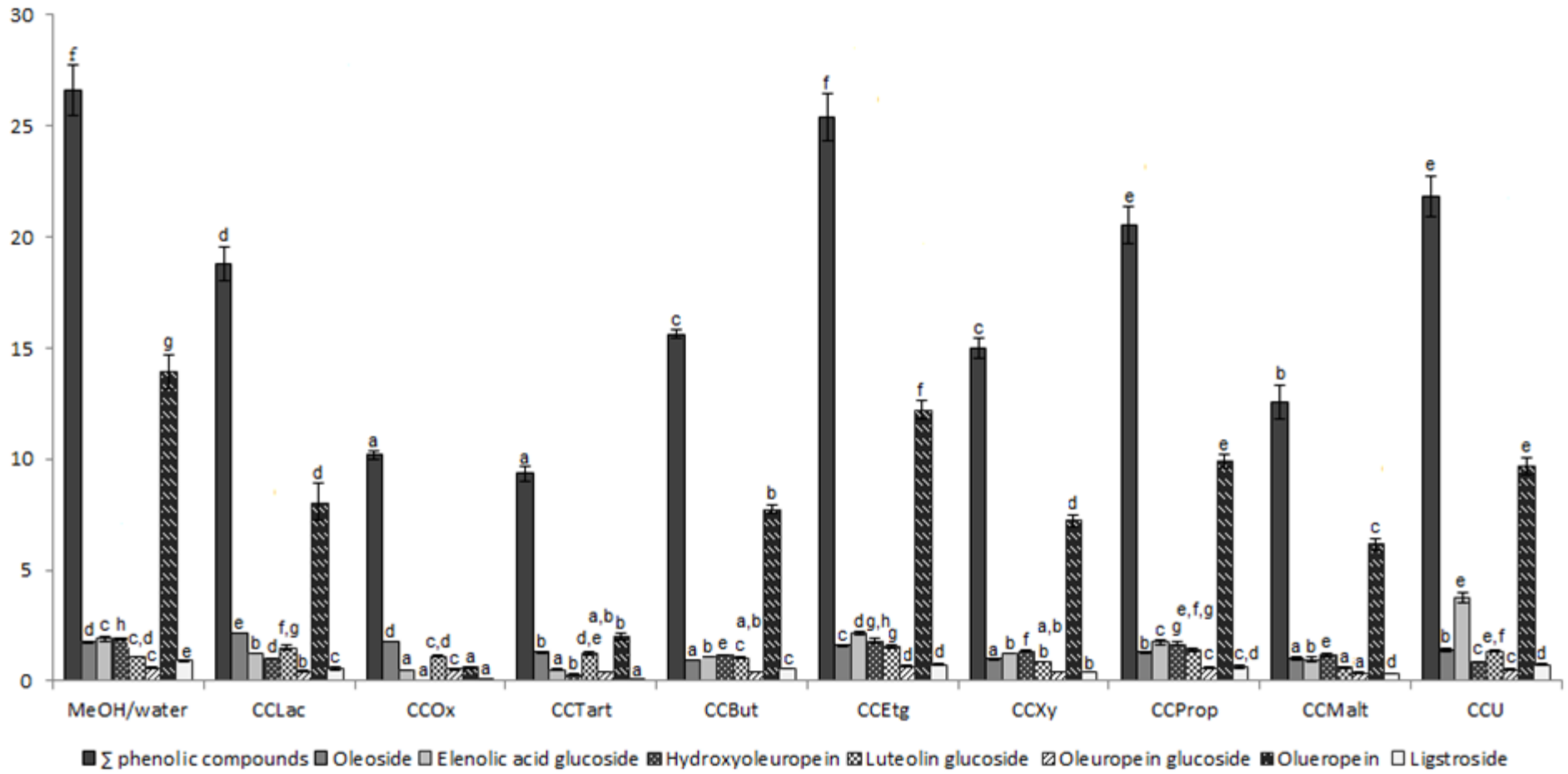

Figure 3-21. Comparison of the extraction efficiencies of DESs and 80% MeOH. Different superscripts for the same compound denote significant differences among the solvents tested according to the S-N-K method at $p<0.05$.

The results obtained are in line with similar results by Garcia at al. [251]. Namely, the authors found choline chloride-1,2-propanediol to be the most suitable DES for the extraction of less polar PCs from olive oil. In this research, ethylene–glycol-based DES was not considered as a possible solvent, and the present study's results are in favour of CCEtg compared to the CCProp, which can be explained. First, due to its polarity, ethylene glycol has significant polar interactions (dipole-type and hydrogen-bonding interactions) with polar compounds such as PCs. Furthermore, as has been mentioned several times in this section, PCs can be considered a type of HBD. Therefore, this alcohols and target PCs can be interacting with halide anion of choline chloride [264]. The branched structure of 1,2-propanediol has considerable steric hindrances on the molecule of choline chloride; consequently, the linear structure of ethylene glycol probably provides easier interaction for target compounds with choline chloride.

Finally, DESs based on urea (CCU) and maltose (CCMalt) as the HBAs were prepared. According to the results, CCU was also revealed as an excellent solvent for the extraction of PCs from olive leaves. CCU exhibited similar extraction capacity to CCProp for the Σ phenolic compounds and for almost all the individual compounds under consideration.

Compared to the rest of the DESs tested, CCMalt showed relatively low extraction efficiency for all compounds. However, sugar DESs have been the most investigated recently because they are primary metabolites, classifying them as NADES.

In conclusion, taking into consideration all the solvents, the order of efficiency for the extraction of PCs from olive leaves based on the Σ phenolic compounds was as follows: CCEtg>CCU≈CCProp>CCLac>CCBut≈CCXy>CCMalt>CCOx>CCTart.

3.4.2.9 Optimization of DES based extraction method using an experimental design

According to the preliminary results, CCEtg was selected as the most promising for the extraction of PCs from olive leaves. Additionally, to optimize the most important MAE extraction factors, an experimental design was applied. In this doctoral dissertation, the focus was on temperature, solvent composition and microwave irradiation time, which are recognised as the most important parameters affecting MAE efficiency [140].

In a closed-vessel MAE system, which was also used in this work, microwave power and pressure are parameters that directly depend on the selected temperature and were not chosen for method optimization.

To avoid the degradation of DESs and PCs, the extraction temperature tested ranged from 40–80ºC. Namely, according to the recently published results about DES thermal-stability, it was found out that the use of this solvents at the higher temperatures has some limitations [265,266].

The importance of the water content in DESs for extraction efficiency has already been discussed. On the other hand, adding too much water can break hydrogen bonds, consequently destroying the eutectic nature of DES solvents [267]. The water content evaluated in the present study ranged from 0–70%.

Finally, the extraction time varied from 10–40 min. These three independent variables were coded at three levels (-1, 0, +1), and a total of 15 experiments in random order (to avoid systematic error) with three replications in the central point were performed.

Σ phenolic compounds characterised by HPLC-DAD-ESI-TOF-MS, TPC and the extraction yield of oleuropein as the most represented PCs in olive leaves were selected as the main dependent variables**.**

Table 3-25 shows the coded and uncoded levels of the independent variables provided by the BBD, with the experimental values of the main responses expressed in mg g^{-1} and the predicted values provided by the statistical model.

TPC in the extracts ranged from 18.67–31.62 mg GAE g^{-1} DW. For the Σ phenolic compounds and oleuropein, the values were between 16.60–26.57 mg g^{-1} DW and between 8.11–11.56 mg g^{-1} DW, respectively.

Experimental results obtained were further mathematically processed to build quadratic models; this was done by applying second-order polynomial equations (without data transformations). **Table 3-26** summarises the all the important parameters about the ANOVA. ANOVA tests at the 95% confidence level showed that the models adequately represented the experimental data. The resulting R^2 (0.9961, 0.9877 and 0.9887 for TPC, Σ phenolic compounds and oleuropein, respectively) indicated that the responses were in the good agreement with the predicted extraction yields.

Adj-R^2 values were determined to be 0.9899, 0.9656 and 0.9684, very close to R^2, implying that all parameters included in the established model were necessary for optimiz

ation. Analysis of error indicated insignificant lack of fit values of 0.337, 0.550 and 0.072 for TPC, Σ phenolic compounds and oleuropein, respectively.

Due to complexity of the mathematical equations related to the number of factors that affect the response, only the parameters useful to predict the results were take into account. Consequently, following the results listed in **Table 3-26**, it is obvious that X_1, X_3, X_2X_2, X_3X_3, X_1X_2 and X_1X_3 were significant model terms for TPC ($p<0.05$). Microwave irradiation time at this confidence level did not influence the extraction processes. Avoiding non-significant variables, the following regression equation was found to represent the extraction yield of TPC:

$$Y= 30.35 + 1.60X_1 + 3.84X_3 – 2.17X_2X_2– 4.78X_3X_3 – 0.75X_1X_2 + 0.97X_1X_3 \quad (3.13)$$

In the case of the Σ phenolic compounds, X_1, X_3, X_1X_1, X_3X_3, X_1X_2 and X_1X_3 were found to be statistically significant, and the following reduced second order polynomial equation was written:

$$Y= 24.06 + 2.46X_1 + 2.33X_3 + 0.83X_1X_1 -3.55X_3X_3 – 0.96X_1X_2 + 0.86X_1X_3 \quad (3.14)$$

Finally, equation 3.15 summarises the influence of the extraction factors on the content of oleuropein in the dried olive leaves:

$$Y= 11.38 + 0.67X_1 + 0.79X_3 + 0.30X_1X_1 + 1.55X_3X_3 – 0.35\ X_1X_2 \quad (3.15)$$

The equations were written in coded terms, where: X_1 is temperature, X_2 is irradiation time, X_3 is the percentage water and Y is the dependent variable (response).

Table 3-25. BBD experimental design with the independent variables and experimental data for the TPC, Σ phenolic compounds and oleuropein content. The predicted values provided by the statistical model for the responses are also included.

Variables				Responses mg g^{-1}_{DW}								
				TPC[b]			Σ phenolic compounds[c]			Oleuropein		
Run	T, ºC	I.T[a], min	% water	Exp. values	Predicted values	Confidence interval, 95%	Exp. values	Predicted values	Confidence interval, 95%	Exp. values	Predicted values	Conficence interval, 95%
1	80 (1)	25 (0)	0 (-1)	22.28 ± 0.25	21.96	20.99-22.92	20.64 ± 1.36	20.60	19.24-21.97	10.25 ± 0.06	10.18	9.71-10.64
2	40 (-1)	25 (0)	70 (1)	26.10 ± 1.54	26.43	25.46-27.39	20.31 ± 0.99	20.34	18.98-21.70	10.35 ± 0.11	10.42	9.95-10.89
3	60 (0)	40 (1)	70 (1)	27.61 ± 1.19	27.66	26.69-21.37	22.62 ± 1.20	22.73	28.62-24.10	10.15 ± 0.39	10.27	9.80-10.73
4	80 (1)	40 (1)	35 (0)	28.55 ± 0.48	28.55	27.58-29.52	26.37 ± 0.63	25.83	24.47-27.19	11.56 ± 0.19	11.44	10.98-11.91
5	60 (0)	40 (1)	0 (-1)	18.67 ± 0.56	18.99	18.03-19.96	16.60 ± 0.23	17.17	15.81-18.54	8.11 ± 0.20	8.30	7.84-8.77
6	40 (-1)	40 (1)	35 (0)	27.21 ± 1.33	26.84	25.87-27.80	22.99 ± 0.39	22.83	21.47-24.20	11.00 ± 0.21	10.82	10.35-11.29
7	80 (1)	25 (0)	70 (1)	31.62 ± 0.91	31.57	30.61-32.54	26.57 ± 0.85	26.98	25.62-28.35	11.40 ± 0.17	11.40	10.94-11.87
8	40 (-1)	25 (0)	0 (-1)	20.64 ± 0.68	20.69	19.72-21.65	17.29 ± 0.36	17.40	16.04-18.77	8.50 ± 0.22	8.50	8.03-8.96
9	60 (0)	10 (-1)	0 (-1)	20.18 ± 0.57	20.13	19.17-21.10	18.56 ± 0.10	18.44	17.08-19.80	9.21 ± 0.10	9.10	8.63-9.56
10	80 (1)	10 (-1)	35 (0)	29.83 ± 2.94	30.20	29.24-31.17	27.97 ± 0.86	28.12	26.76-29.49	12.37 ± 0.05	12.56	12.09-13.03
11	60 (0)	10 (-1)	70 (1)	27.16 ± 2.21	26.83	25.87-27.80	22.77 ± 0.13	22.20	20.83-23.56	10.48 ± 0.02	10.29	9.82-10.76
12	40 (-1)	10 (-1)	35 (0)	25.50 ± 1.05	25.50	24.53-26.46	20.74 ± 0.45	21.27	19.91-22.64	10.40 ± 0.22	10.52	10.05-10.99
13	60 (0)	25 (0)	35 (0)	30.63 ± 1.26	30.63	29.71-31.00	24.44 ± 0.14	24.06	23.15-24.97	11.44 ± 0.52	11.38	11.07-11.69
14	60 (0)	25 (0)	35 (0)	30.45 ± 1.04	30.35	29.71-31.00	23.92 ± 0.22	24.06	23.15-24.97	11.37 ± 0.24	11.38	11.07-11.69
15	60 (0)	25 (0)	35 (0)	29.98 ± 1.26	30.35	29.71-31.00	23.82 ± 0.54	24.06	23.15-24.97	11.33 ± 0.43	11.38	11.07-11.69

I.T[a].- Irradiation time in min

[b]TPC- expressed as mg GAE g^{-1} DW.

[c] Σ phenolic compounds detected in HPLC-DAD-ESI-TOF-MS expressed as mg g^{-1} DW.

Table 3-26. ANOVA for response surface polynomial model of TPC, Σ phenolic compounds and oleuropein content.

Variables	Responses mg g^{-1} DW								
	TPC			∑ phenolic compounds			Oleuropein		
	Sum of squares	***p* value**	**Coefficient**	**Sum of squares**	***p* value**	**Coefficient**	**Sum of squares**	***p* value**	**Coefficient**
Model	241.95	<0.001***		150.80	<0.001***		19.38	<0.001***	
Intercept			30.35			24.06			11.38
X_1 (Temperature)	20.59	0.001***	1.60	48.46	<0.001***	2.46	3.55	<0.001**	0.67
X_2 (Extraction time)	0.05	0.629	-0.08	0.27	0.437	-0.18	0.33	0.040*	-0.20
X_3 (Water)	117.93	<0.001***	3.84	43.40	<0.001***	2.33	4.98	<0.001***	0.79
X_1X_1	0.63	0.127	-0.41	2.52	0.049*	0.83	0.32	0.043*	0.30
X_2X_2	17.36	<0.001***	-2.17	0.50	0.298	-0.37	0.43	0.027*	-0.34
X_3X_3	84.36	<0.001***	-4.78	46.58	<0.001***	-3.55	8.88	<0.001***	-1.55
X_1X_2	2.24	0.018*	-0.75	3.71	0.026*	-0.96	0.50	0.020*	-0.35
X_1X_3	3.75	0.007**	0.97	2.96	0.038*	0.86	0.12	0.158	-0.17
X_2X_3	0.96	0.073	0.49	0.81	0.201	0.45	0.15	0.127	0.19
R^2	**0.9961**			**0.9877**			**0.9887**		
Adjusted R^2	**0.9899**			**0.9656**			**0.9684**		
Predicted R^2	**0.9508**			**0.8238**			**0.8240**		
Adeq. Precision	**35.519**			**21.903**			**24.785**		

Level of significance: *p<0.05, **p<0.01; ***p<0.001

To study the interactive effects of the operational parameters on the extraction yields, the contour plots of multiple non-linear regression models were depicted (**Figure 3-22**). The contour plots (**Figure 3-22A-C**) showed the interaction percentage of water, temperature and irradiation time on the extraction yield of TPC. The TPC yield increased rapidly with water content in the DES, from 30% to 60%, and declined afterwards (**Figures 3-22A** and **3-22B).** TPC yield was increasing linearly with the temperature, and a maximum value was observed at 80 °C (**Figures 3-22A** and **3-22C**). The temperature exhibited a positive effect on the extraction efficiency as result of its influence on diffusion, viscosity, surface tension and solubility of the target compounds. In addition, the effect of irradiation time on the TPC was less noticeable than for the other parameters (**Figures 3-22B** and **3-22C**).

Similar trends were also observed for the Σ phenolic compounds (**Figure 3-22D-F**).

The increase in temperature (linear) and water content (up to 45%) increased the oleuropein yield (**Figures 3-22G** and **3-22I**). On the other hand, a simultaneous increase in the irradiation time and temperature led to a reduction in the oleuropein yield (**Figure 3-22G**), which was probably caused by its degradation.

To maximise the extraction yields of all the variables taken into account, the operational parameters were optimized by means of the model equation provided by the statistical program. Thus, the optimum conditions applied were 79.6 ºC, 43.3% of water and an irradiation time of 16.7 min. Under the selected conditions, the predicted values for TPC, Σ phenolic compounds and oleuropein were 32.12 mg g^{-1} DW, 28.28 mg g^{-1} DW and 10.56 mg g^{-1} DW, respectively.

Additionally, as part of this work, it was found that the extraction conditions tested were also statistically significant for the following individual PCs: Σ oleosido/secologanoside isomers (R^2=0.9849); Σ caffeoylglucoside (R^2=0.9913); Σ elenolic acid glucoside isomer 1 (R^2=0.9919); Σ phenethyl primeveroside isomer 2 (R^2=0.9864), Σ apigenin rutinoside (R^2=0.9865); and Σ 7-2-metoxyoleuropein isomer 1 (R^2=0.9792). Quadratic models based on second-order polynomial equations were built, and all statistically important parameters are listed in **Table 3-27**.

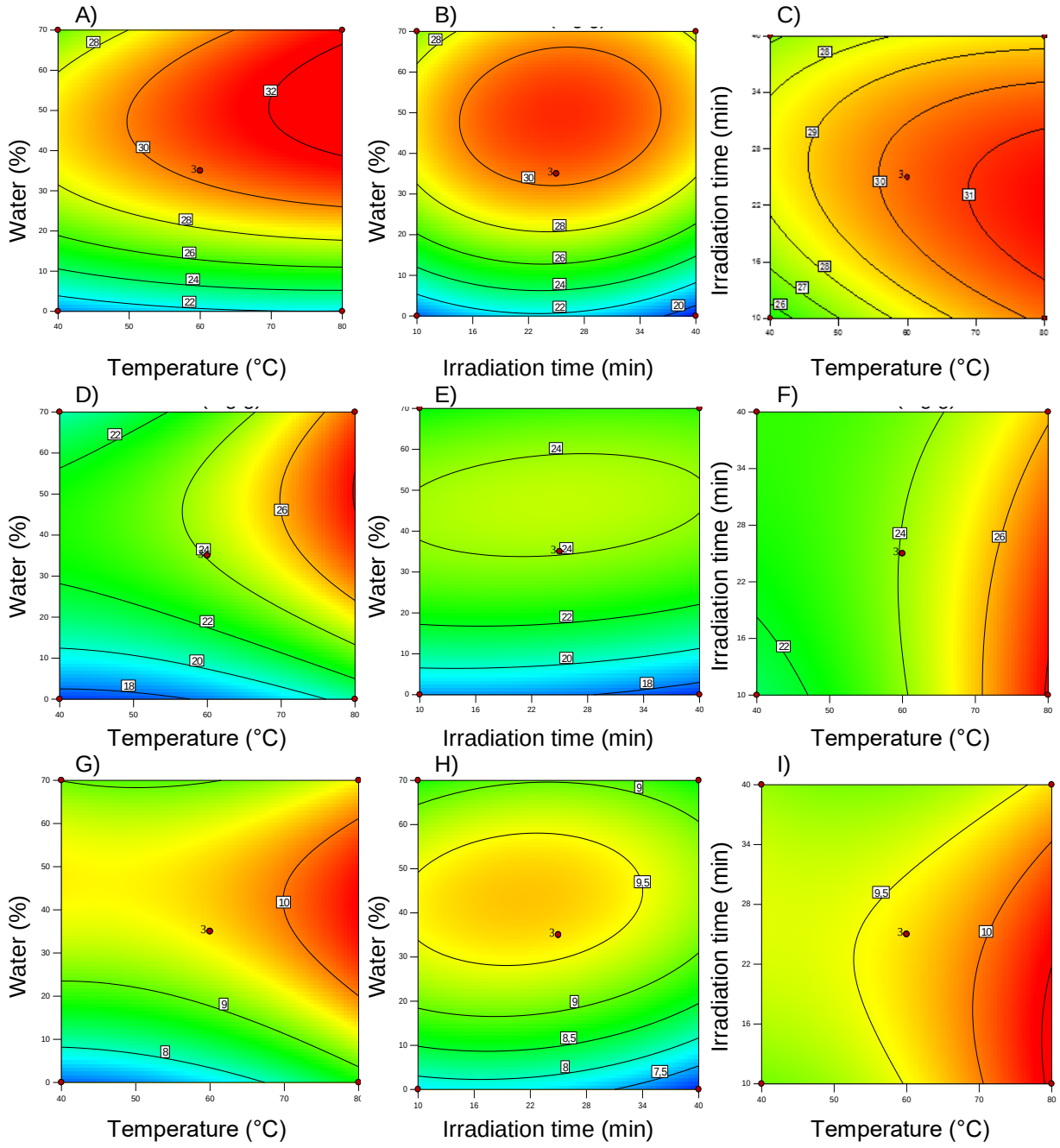

Figure 3-22. Contour plots for the interactions between the three extraction parameters on the:

2A-C) TPC; 2D-F) Σ phenolic compounds; 2G-I) oleuropein.

Table 3-27. ANOVA test of response surface polynomial model for PCs detected in olive leaves.

Varibales	Responses mg g^{-1} DW								
	Σ oleosido/secologanoside isomers			Σ caffeoylglucoside			Σ elenolic acid glucoside isomer 1		
	Sum of square	*p* value	Coefficient	Sum of square	p value	Coefficient	Sum of square	p value	Coefficient
Model	$4.52 \cdot 10^{-3}$	<0.001***		$8.90 \cdot 10^{-4}$	<0.001***		5.91	<0.001***	
X_1	$6.48 \cdot 10^{-4}$	0.001***	$9.00 \cdot 10^{-3}$	$1.63 \cdot 10^{-4}$	<0.001***	$4.51 \cdot 10^{-3}$	3.84	<0.001***	0.69
X_2	$1.03 \cdot 10^{-4}$	0.041*	$-3.59 \cdot 10^{-3}$	$6.64 \cdot 10^{-5}$	0.001**	$-2.88 \cdot 10^{-3}$	0.01	0.298	0.04
X_3	$2.50 \cdot 10^{-3}$	<0.001***	0.02	$3.64 \cdot 10^{-4}$	<0.001***	$6.75 \cdot 10^{-3}$	1.00	<0.001***	0.35
X_1X_2	$9.49 \cdot 10^{-5}$	0.047*	$5.07 \cdot 10^{-3}$	$3.16 \cdot 10^{-5}$	0.007**	$2.93 \cdot 10^{-3}$	0.52	<0.001***	0.38
X_1X_3	$3.76 \cdot 10^{-5}$	0.161	$-3.19 \cdot 10^{-3}$	$2.93 \cdot 10^{-5}$	0.008**	$-2.82 \cdot 10^{-3}$	0.02	0.278	0.06
X_2X_3	$7.10 \cdot 10^{-4}$	<0.001***	-0.01	$1.34 \cdot 10^{-4}$	<0.001***	$-6.02 \cdot 10^{-3}$	0.25	0.004**	-0.26
X_1X_1	$2.89 \cdot 10^{-4}$	<0.001***	$-8.50 \cdot 10^{-3}$	$8.80 \cdot 10^{-5}$	<0.001***	$-4.69 \cdot 10^{-3}$	$2.77 \cdot 10^{-3}$	0.619	-0.03
X_2X_2	$8.39 \cdot 10^{-5}$	0.057	$4.58 \cdot 10^{-3}$	$6.86 \cdot 10^{-6}$	0.092	$1.31 \cdot 10^{-3}$	0.20	0.007**	0.22
X_3X_3	$2.36 \cdot 10^{-5}$	0.249	$2.43 \cdot 10^{-3}$	$2.26 \cdot 10^{-7}$	0.723	$-2.38 \cdot 10^{-4}$	$4.87 \cdot 10^{-3}$	0.513	0.04
R^2	**0.9849**			**0.9912**			**0.9788**		
Adjusted R^2	**0.9577**			**0.9753**			**0.9407**		
Adeq. precision	**20.946**			**29.571**			**15.110**		

Table 3-27. Continued.

Varibales	Responses mg g^{-1} DW								
	Σ phenethyl primeveroside isomer			Σ apigenin rutinoside			Σ 7-2-metoxyoleuropein isomer 1		
	Sum of square	*p* value	Coefficient	Sum of square	p value	Coefficient	Sum of square	p value	Coefficient
Model	$2.59 \cdot 10^{-3}$	<0.001***		$4.39 \cdot 10^{-3}$	<0.001***		0.03	0.001**	
X_1	$6.62 \cdot 10^{-5}$	0.029*	$2.88 \cdot 10^{-3}$	$5.80 \cdot 10^{-4}$	<0.001***	$8.52 \cdot 10^{-3}$	$5.90 \cdot 10^{-3}$	<0.001***	0.03
X_2	$1.70 \cdot 10^{-4}$	<0.005**	$-4.60 \cdot 10^{-3}$	$1.26 \cdot 10^{-5}$	0.346	$-1.25 \cdot 10^{-3}$	$4.84 \cdot 10^{-6}$	0.840	$7.78 \cdot 10^{-4}$
X_3	$1.23 \cdot 10^{-3}$	<0.001***	0.01	$1.64 \cdot 10^{-3}$	<0.001***	0.01	$8.89 \cdot 10^{-3}$	<0.001***	0.03
X_1X_2	$6.73 \cdot 10^{-8}$	0.927	$-1.35 \cdot 10^{-4}$	$3.52 \cdot 10^{-5}$	0.142	$-3.09 \cdot 10^{-3}$	$3.03 \cdot 10^{-6}$	0.873	$9.06 \cdot 10^{-4}$
X_1X_3	$1.15 \cdot 10^{-4}$	0.010*	$-5.59 \cdot 10^{-3}$	$6.87 \cdot 10^{-5}$	0.059	$-4.31 \cdot 10^{-3}$	$9.18 \cdot 10^{-4}$	0.032*	-0.02
X_2X_3	$6.04 \cdot 10^{-4}$	<0.001***	-0.01	$1.70 \cdot 10^{-3}$	<0.001***	-0.02	$7.81 \cdot 10^{-3}$	<0.001***	-0.05
X_1X_1	$4.20 \cdot 10^{-4}$	<0.001***	-0.01	$3.73 \cdot 10^{-4}$	0.002**	$-9.66 \cdot 10^{-3}$	$5.72 \cdot 10^{-4}$	0.068	-0.01
X_2X_2	$2.09 \cdot 10^{-6}$	0.614	$7.23 \cdot 10^{-4}$	$2.97 \cdot 10^{-5}$	0.171	$2.73 \cdot 10^{-3}$	$7.21 \cdot 10^{-4}$	0.048*	0.01
X_3X_3	$1.68 \cdot 10^{-5}$	0.188	$2.05 \cdot 10^{-3}$	$1.82 \cdot 10^{-5}$	0.266	$2.13 \cdot 10^{-3}$	$7.20 \cdot 10^{-5}$	0.449	$4.24 \cdot 10^{-3}$
R^2	**0.9912**			**0.9870**			**0.9788**		
Adjusted R^2	**0.9753**			**0.9635**			**0.9407**		
Adeq. precision	**29.571**			**20.384**			**15.110**		

Level of significance: $^{*}p<0.05$, $^{**}p<0.01$; $^{***}p<0.001$

4 Conclusions

PCs, as the secondary metabolites of plants, have a wide range of pharmaceutical and biological effects and are widely used in different industries. Consequently, considering the importance of PCs as natural antioxidants, this work aimed to present the development of different extraction methods, ranging from conventional to modern techniques, as well as improving existing methods and developing new instrumental methods used in their identification and quantification.

The first segment of the work described stability studies of the most common naturally occurring hydroxycinnamic acids, namely *trans*-CA and *trans*-FA. Stability studies of bioactive compounds are necessary to determine substance degradation paths and establish suitable analytical methods for their determinations. The first part of the work involved a long-term stability study (up to 1 month) of the selected PAs. For this purpose, pure standard compounds were dissolved in two different solvents (THF and MeOH) and stored under various conditions (RT, deep freeze and daylight). The second part focused on the stability of *trans*-CA and *trans*-FA under the influence of UV light. Conventionally, HPLC with UV-VS detector is the most commonly used technique for the determination of PCs. Alternatively, the determination can be performed using GC-MS, which has several advantages, such as simultaneous, complete and high-resolution separation of compounds and their isomers, sensitive detection and unambiguous identification and quantitation. For this reason, the degradation products formed in the present study were separated and identified by GC-MS in the form of trimethylsilyl derivates, prepared using MSTFA as the derivatization agent.

In the present study, *cis*-isomers of the investigated PAs were confirmed as the only conversion or degradation products in all experiments. Geometrical isomers were successfully separated by GC and determined by MS throughout the fragmentation patterns in their mass spectra's. It was shown that in both solvents, the isomerization was minimal in the first 24 h when the solutions were stored at RT in darkness or in the refrigerator (from 3–10%). The highest rate of isomerization of *trans*-CA and *trans*-FA was achieved after 1 month of storing in the MeOH solutions at RT and daylight (34.6% and 42.8%, respectively). From these series of experiments, it was determined that UV radiation promotes the isomerization of *trans*-hydroxycinnamic acids to their *cis*-forms. The maximum isomerization of *trans*-CA and *trans*-FA (31.1% and 33.6%, respectively) were determined in the THF solutions exposed to UV

radiation at 366 nm, for 6 h. These results showed that for the correct quantification of the selected PAs in real samples, the peak areas of both *cis*- and *trans*-isomers should should be considered.

PAs should be handled with caution in the laboratory, and stringent precautions should be maintained when considering lighting, usage of protic organic solvents; higher temperatures and long-term analysis should also be avoided to minimise the isomerization or degradation of the compounds. These results may be further used for investigations where the optimal conditions for efficient extraction, isolation and quantification of PAs from different materials should be established. Furthermore, knowledge of the stability of compounds helps provide proper packaging and storage conditions for the raw materials.

The main objective of the second segment of the research was the optimization of a simple and fast SPE technique for the isolation of PAs from red wine samples. Additionally, a GC-MS method for the identification and quantitative determination of the five selected PAs, together with their geometrical isomers, was developed. The method was validated for linearity, precision as repeatability, LOD and LOQ. Wine is a widely consumed beverage and one of the most important products of the Slovenian economy. The chemical composition of wine is highly dependent on several oenological factors, such as origin, vine variety, winemaking practices, vintage and region, which enables the confirmation of a wine's authenticity. Consequently, the objective of this second segment of the research was the establishment of a statistical-mathematical model for the classification of Slovenian wines according to these important factors, wine-growing region and vine variety.

The developed GC-MS method was validated, and the linear concentration range for all the analytes ranged from 1–100 mg L^{-1} with correlation coefficients above 0.9990. The proposed method was repeatable (RSD<2%), with recoveries above 96% and acceptable values of LOD and LOQ. For the isolation of the PAs from wine samples, SPE using HLB cartridges was used. Included in this step, pigments, sugars and other interferences were successfully removed without losing PAs. The bound PAs were extracted after alkaline hydrolysis using NaOH in the presence of L-ascorbic acid and EDTA as the stabilizers.

From the results obtained, caffeic acid and p-coumaric acid are the most important compounds of total PAs in Slovenian red wines, with contents varying between 17.10 and 71.98 mg L^{-1}. The highest contents of the investigated PAs were found in wine samples from

the Posavje wine region. The concentrations of free and bound PAs were between 0.03–11.80 mg L^{-1} and 3.80–71.90 mg L^{-1}, respectively. In accordance with the data obtained using statistical analysis and chemometric methods (PCA and CLU), it can be concluded that vine variety has more of an influence on the PA content of Slovenian red wines, but the influence of the wine region cannot be completely ignored. Additionally, LDA was performed and resulted in the satisfactory classification of samples by both vine variety and region.

In conclusion, the classification of wine regarding the different parameters is important for establishing wine authenticity and confirmation of its quality. This study demonstrated the usefulness of the variables selected and also indicated the application of a developed model that could be used for wine classification.

Aromatic plants and their extracts are also recognised as a rich source of the functional bioactive compounds with the potential for the application in different industries. Consequently, the third part of this work represented a contribution to the understanding of the *Coriandrum sativum L.* polyphenol's composition. First, the TPC and TFC in methanolic extracts, depending on the different extraction conditions, were determined. The second aim of this part was the determination of the individual PAs in coriander fruits. Bonded PCs are often unwanted products because, their identification and quantification is much more difficult and expensive compared to their free forms. Additionally, the physical and chemical properties, especially antioxidant activity, of bonded PCs are different compared to their free forms. Therefore, the goal was to produce free PAs in the final extracts. Furthermore, the target PAs were determined after alkaline hydrolysis following the procedure established in the previous segment. Ultrasound-assisted extraction was used to improve the PAs' extraction yield, which were subsequently determined using GC-MS. For the optimization of the most influencing extraction factors (temperature, sonication time and NaOH concentration), BBD combined with the RSM was applied.

The maximum measured values for the TPC and TFC in the coriander fruits were 2 900 mg GAE g^{-1} DW and 850 mg RUT g^{-1} DW, respectively. Satisfactory statistical parameters (R^2 and CV) and ANOVA indicated that the developed second-order polynomial models provided an adequate mathematical description of the UAE extraction yields. Furthermore, according to the results obtained, all tested parameters had a significant impact on the extraction yields of PAs, but sonication time was the most critical factor. With the aim of maximizing the extraction yields of all independent variables, the following extraction

conditions were determined to be the most optimal: sonication time of 17.4 min at 35.3 °C and an NaOH concentration of 2.02 M.

It can be concluded that coriander fruit is rich in PCs, including PAs, and can represent a potential natural source of antioxidants for the food and pharmaceutical industries.

The last segment of this PhD thesis proposed the application of deep eutectic solvents for the isolation of PCs from different plant materials. DES, as the new generation of ionic liquids, present good properties, such as being liquid at RT, having a viscosity that can be adjusted easily and being sustainable and safe. The first part of this chapter was based on the optimization of the UAE method for the isolation of PCs from dried Aronia, followed by HPLC-UV determination. Four kinds of DESs were synthesised based on choline chloride-ChCl as the hydrogen bond acceptor in combination with different hydrogen bond donors (sugars, organic acids and urea) and tested. In the second part, the isolation of PCs was carried out by means of MAE, and the obtained extracts were subsequently analysed by HPLC-DAD-ESI-TOF-MS. In this study, nine different DESs were first tested, and the most appropriate one was selected for further optimization. Additionally, other factors, such as temperature, water content in the DES solvent and extraction time were studied systematically by applying BBD in combination with RSM.

With the aim to maximise the extraction of the bioactive PCs in both experiments, alternative isolation techniques using DES were evaluated in comparison with conventional solvents. However, it should be noted that the phenolic contents in plant materials are highly affected by geographical origin, seasonal variations, time of sample collection and other factors. Consequently, the differences observed in the results reported in the literature should not be generalised.

Results for the dried Aronia revealed that the highest values of the TPC and TFC were found in the extract obtained with ChCl-fructose DES (36.15±3.39 mg GAE g^{-1} DW and 4.71±0.33 mg RUT g^{-1} DW, respectively) while methanolic extraction still showed the highest capacity for the extraction of total anthocyanins (1.246±0.058 mg Cya-3-Glu g^{-1} DW). The extraction recoveries obtained for the individual PCs were, in most cases, much higher compared to 80% MeOH (up to 33% higher). The extraction recoveries were highly dependent on the PC's structure, with relative mean values ranging from 36–119%.

In the second study, a total of 48 PCs were quantitatively determined in all olive leave extracts by using HPLC-DAD-ESI-TOF-MS. Among the nine DESs preliminary tested, ChCl-ethylene glycol was selected as the most promising, providing to be more effective in the extraction of PCs when compared to conventionally used organic solvent (80% MeOH). The second part of the work focused on the optimization of MAE, according to which the three most important extraction parameters are water content in the DES solvent, temperature and microwave irradiation time. Considering the maximum amounts of the selected dependent variables (TPC, Σ phenolic compounds and oleuropein were 32.12 mg g^{-1}, 28.28 mg g^{-1} and 10.56 mg g^{-1}, respectively), the optimal extraction conditions were 43% aqueous solution of ChCl-ethylene glycol, irradiation time of 16.7 min and temperature of 79.6 °C.

Generally, we can conclude that DESs are a good alternative to the conventionally used hazard organic solvents, and it is not surprising that their application in different fields of chemistry is rapidly growing. The good physical and chemical properties of DESs probably can classify them as the solvents that will be used in the 21st century. The main problem that still limits the application of DESs in large-scale processes is their low evaporator pressure. Furthermore, understanding the extraction processes involving DES solvents is still in its infancy, and additional studies should be performed. Some future research goals should be stability studies of PCs in the DESs, separation of target compounds from the obtained extracts and the possibility for recycling of DESs.

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6 Biographic informations about the candidate

PERSONAL INFORMATION

Milena Ivanović

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milena.ivanovic@hotmail.com

Sex Female | Date of birth 11/02/1987 | Nationality BiH

WORK EXPERIENCE

October 2013 to present

Senior assistant, Department of Analytical Chemistry , MSc

Faculty of Technology, University of Tuzla (BiH)

- Planing and implementation of experimental laboratory work with students
- Assisting to the professor in the teaching process and preparation of lectures
- Organization and planning of examinations
- Colabration and assistance to students
- Personal development

Business or sector Education

November 2009 - October 2013

Assistant, Department of Analytical Chemistry , BSc

Faculty of Science, University of Tuzla (Bosnia and Herzegovina)

- Planning and implementation of experimental laboratory work with students
- Assisting a professor in the teaching process
- Organization and planning of examinations
- Personal development

Business or sector Education

EDUCATION AND TRAINING

February 2017 to May 2017

PhD student exchange

Faculty of Science, University of Granada (Spain) and Research and Development Centre for Functional Food-CIDAF (Granada, Spain). Financial support by Erasmus Mundus JoinEU See Penta project.

- Development of new extraction methodologies based on the use of deep eutectic solvents
- Training on HPLC-DAD-ESI-TOF-MS chromatograhic system.

October 2014 to present **PhD study**

Faculty of Chemistry and Chemical Engineering, University of Maribor (Slovenia**).**

Financial support by Erasmus Mundus JoinEu See Penta project.

- Planning and performing laboratory experiments.
- Simplification of challenging laboratory procedures based on experience.
- The ability to analyze the samples for various purposes.
- Use of a range of analytical techniques, instrumentation and software.
- Organization of independent scientific research.
- Creation of team oriented atmospher.

November 2011-01.07.2013 **Master of Chemical Technology/Engineering Chemistry programme**

Faculty of Technology, University of Tuzla (BiH)

- Planning and performing laboratory experiments.
- Use of a range of analytical techniques, instrumentation and software.
- Organization of independent scientific research.
- Creation of team oriented atmosphere.

MSc thesis: „The effects of surfactant structures and concentrations in mixed surfactant solutions on the precipitation of Ca(II) ions“.

October 2005-15.09.2009 **Bechelor of Science**

Faculty of Science, University of Tuzla (Bosnia and Herzegovina)

- Individual and group laboratory work under the supervision of assistant
- Processing and presentation of obtained laboratory results
- Participation in teamwork

PERSONAL SKILLS

Mother tongue(s)	Serbian				
Other language(s)	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	B2	B2	B2	B2	B2
Slovenian	C1	C1	B1	B1	B2
Spanish	B1	A2	A1	A1	A2
	Levels: A1/A2: Basic user - B1/B2: Independent user - C1/C2 Proficient user Common European Framework of Reference for Languages				

Computer skills

- Microsoft Office (Word, Excel, Power Point, Publisher), ChemOffice, ACD labs, Spectrum Database for Organic Comunds (SDBS), SPSS chemometric software, Softweres for Experimental optimization (Desigh Expert, DOE)

Driving licence

B-car

ADDITIONAL INFORMATION

PhD study (2014-2017)

Publications

COBISS

Projects

- From February to July 2015, I participated actively, as the student; in the realization of the project entitled "Development of Automated and Coupled Methods in Analytical Chemistry", as a part of "PKP2-Creative path to practical knowledge", partially funded by the EU funds.
- September 2016 - December 2017. Bilaterally project between Slovenia (University of Maribor) and Bosnia and Herzegovina (University of Tuzla) (member).

Conferences

COBISS

Seminars

- Dani za kromatografiju, organized on 15 Octobar, 2015 in Maibor, Slovenia.
- GC and GC-MS seminar, organized on 12 May, 2015 in Zgornji Brnik, Slovenia.
- LC-MS seminar, organized on 13 May, 2015 in Zgornji Brnik, Slovenia.
- HPLC Troubleshooting Seminar, organized on 8 Octobar, 2014 in Maribor, Slovenia.

Honours and awards

- Grantee of Erasmus Mundus scholarship for doctoral study in the framework of JoinEU-SEE<Penta project.
- Silver Medal of the University of Tuzla for outstanding success in the fourth year of undergraduate study , GPA 9.67.
- Silver Medal of the University of Tuzla for outstanding success in the third year of undergraduate study, GPA 9.45.

7 Scientific bibliography of the candidate

Orginal scientific articles

1. ALAÑÓN, M. E., IVANOVIĆ, Milena, GÓMEZ-CARAVACA, A. M., ARRÁEZ-ROMÁN, David, SEGURA-CARRETERO, Antonio. Choline chloride derivative-based deep eutectic liquids as novel green alternative solvents for extraction of phenolic compounds from olive leaf. *Arabian journal of chemistry*, ISSN 1878-5352, Available online 31 January 2018, str. 1-18, doi: 10.1016/j.arabjc.2018.01.003. [COBISS.SI-ID 21143574].

2. IVANOVIĆ, Milena, PETEK, Anja, ISLAMČEVIĆ RAZBORŠEK, Maša, KOLAR, Mitja. Chemometric characterization of Slovenian red wines. *Acta chimica slovenica*, ISSN 1318-0207. [Tiskana izd.], str. 537-542, ilustr. [COBISS.SI-ID20730646].

3. IVANOVIĆ, Milena, ISLAMČEVIĆ RAZBORŠEK, Maša, KOLAR, Mitja. Simultaneous GC-MS determination of free and bound phenolic acids in Slovenian red wines and chemometric characterization. *Acta chimica slovenica*, ISSN 1318-0207. [Tiskana izd.], 2016, vol. 63, no. 3, str. 661-669. https://journals.matheo.si/index.php/ACSi/article/view/2534, doi: 10.17344/acsi.2016.2534. [COBISS.SI-ID 19728662].

4. ISLAMČEVIĆ RAZBORŠEK, Maša, IVANOVIĆ, Milena. Stability studies and determination of carnosic acid and its oxidative degradation products by gas chromatography-mass spectrometry. *International Journal of Mass Spectrometry*, ISSN 1387-3806. [Print ed.], 2016, vol. 407, str. 29-39, doi: 10.1016/j.ijms.2016.07.002. [COBISS.SI-ID 19669526].

5. ISLAMČEVIĆ RAZBORŠEK, Maša, IVANOVIĆ, Milena, KERMC, Domen, KOLAR, Mitja. Monosaccharides determination in Lepidium meyenii capsules by GC-MS. *Technologica acta*, ISSN 1840-0426, june 2016, vol. 9, no. 1, str. 33-38, ilustr. [COBISS.SI-ID 19789846].

Professional paper

6. PLOHL, Klavdija, ISLAMČEVIĆ RAZBORŠEK, Maša, IVANOVIĆ, Milena, GRADIŠNIK, Lidija, MAVER, Uroš, LIPOVŠEK DELAKORDA, Saška. Matični mleček - možnosti za zunanjo uporabo. *Slovenski čebelar : glasilo čebelarskih organizacij Slovenije*, ISSN 0350-4697, 2018, letn. 120, št. 1, str. 9-11, ilustr. [COBISS.SI-ID 23651848].

Published scientific conference contribution

7. ISLAMČEVIĆ RAZBORŠEK, Maša, IVANOVIĆ, Milena, PETEK, Anja. Kemometrijska karakterizacija slovenskih rdečih vin Podravja, Posavja in Primorja = Chemometric characterization of Slovenian red wines from Podravje, Posavje and Primorje. V: KAUČIČ, Venčeslav (ur.), BEŠTER-ROGAČ, Marija (ur.), GANTAR, Marjana (ur.). *Zbornik referatov in povzetkov*, 22. Slovenski kemijski dnevi, Portorož, 28.-30. september 2016 = 22. Slovenian Chemical Days Portorož, September 28-30, 2016. Ljubljana: Slovensko kemijsko društvo. 2016, str. 1-6, ilustr. [COBISS.SI-ID 19919894].

8. IVANOVIĆ, Milena, ISLAMČEVIĆ RAZBORŠEK, Maša, KOLAR, Mitja. Optimization of extraction of phenolic acids from coriander using Box-Behnken design with response surface methodology. V: KAUČIČ, Venčeslav (ur.), BEŠTER-ROGAČ, Marija (ur.), GANTAR, Marjana (ur.). *Zbornik referatov in povzetkov*, 22. Slovenski kemijski dnevi, Portorož, 28.-30. september 2016

= 22. Slovenian Chemical Days Portorož, September 28-30, 2016. Ljubljana: Slovensko kemijsko društvo. 2016, str. 1-6, ilustr. [COBISS.SI-ID 19922198].

9. ISLAMČEVIĆ RAZBORŠEK, Maša, IVANOVIĆ, Milena, KOLAR, Mitja. Stabilnostna študija kafeinske in ferulične kisline z GC-MS = Stability studies on caffeic and ferulic acid by GC-MS. V: KAUČIČ, Venčeslav (ur.), et al. *Zbornik referatov in povzetkov*, Slovenski kemijski dnevi 2015, Ljubljana, 24. - 25. september 2015 = Slovenian Chemical Days 2015, Ljubljana, September 24 - 25, 2015. Ljubljana: Slovensko kemijsko društvo. 2015, str. [1-7]. [COBISS.SI-ID 18975510].

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10. ALAÑÓN, Elena, IVANOVIĆ, Milena, ARRÁEZ-ROMÁN, David, SEGURA-CARRETERO, Antonio. Optimization of extraction phenolic compounds from olive leaves by means of alternative solvents: deep eutectic solvents. V: *Abstract book*, 19th International Symposium on Advances in Extraction Technologies, Santiago de Compostela, Spain, June 27th-30th, 2017. Santiago de Compostela: INGACAL-CIAM. 2017, str. 319. [COBISS.SI-ID 20680982].

11. IVANOVIĆ, Milena, ALAÑÓN, Elena, ARRÁEZ-ROMÁN, David, SEGURA-CARRETERO, Antonio. Deep eutectic solvents: a "green" alternative for the extraction of phenolic compunds from Lippia Citriodora. V: *Abstract book*, 19th International Symposium on Advances in Extraction Technologies, Santiago de Compostela, Spain, June 27th-30th, 2017. Santiago de Compostela: INGACAL-CIAM. 2017, str. 320. [COBISS.SI-ID 20681238].

12. IVANOVIĆ, Milena, ISLAMČEVIĆ RAZBORŠEK, Maša, KOLAR, Mitja. Deep eutectic solvents (DES): green extraction of phenolic compound from chokeberries (Aronia melanocarpa). V: *Book of abstracts*, 24th Young Investigators Seminar on Analytical Chemistry, Venice, 28th-30th June 2017, Italy. [Venice: s. n. 2017], str. 19. [COBISS.SI-ID 20681494].

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UNIVERZA V MARIBORU

FAKULTETA ZA KEMIJO IN KEMIJSKO TEHNOLOGIJO

Izjava doktorskega kandidata

Podpisani-a **MILENA IVANOVIĆ**, vpisna številka **K3000755**

izjavljam,

da je doktorska disertacija z naslovom

RAZVOJ ANALIZNIH METOD ZA SOČASNO IDENTIFIKACIJO IN DOLOČEVANJE FENOLNIH SPOJIN

(Development of analytical methods for simultaneous identification and determination of phenolic compounds)

- rezultat lastnega raziskovalnega dela,
- da predložena disertacija v celoti ali v delih ni bila predložena za pridobitev kakršnekoli izobrazbe po študijskih programih drugih fakultet ali univerz,
- da so rezultati korektno navedeni in
- da nisem kršil-a avtorskih pravic in intelektualne lastnine drugih.

Podpis doktorskega kandidata