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APPLICATION OF SUPERCRITICAL FLUIDS IN THE STUDY OF *TANACETUM PARTHENIUM* L.: FROM EXTRACTION TO ANALYSIS

PhD thesis

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LIST OF ABBREVIATIONS

^{1}H NMR	proton nuclear magnetic resonance
DXR	1-deoxy-d-xylulose-5-phosphate reductoisomerase
DXS	1-Deoxy-d-xylulose-5-phosphate synthase
DPPH	2,2-diphenyl-1-picrylhydrazyl
MEP	2-C-methyl-D-erythritol 4-phosphate
HMGR	3-hydroxy-3-methylglutaryl coenzyme-A reductase
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
5-HT	5-hydroxy-tryptamine, serotonin
AML	acute myeloid leukemia
BBB	blood brain barrier
CoA	coenzyme A
CID	collision-induced dissociation
COSY	correlation spectroscopy
COS	costunolide synthase
COX	cyclooxygenase
CD	dendritic cell
DNA	deoxyribonucleic acid
DOE	design of experiment
DMSO	dimethyl sulfoxide
DMAPP	dimethylallylpyrophosphate
DMAPT	dimethylamino parthenolide
DAD	diode array detector/detection
ESI	electrospray ionisation
BEH	ethylene-bridged hybrid
FPS	farnesyl diphosphate synthase
GC	gas chromatography
GC-MS	gas chromatography coupled with mass spectroscopy
GAO	germacrene A oxidase
GAS	germacrene A synthase
GSH	gluthation
HPLC-MS	high performance liquid chromatography coupled with mass spectrometry
HPLC	high-performance liquid chromatography
HNE	human neutrophil elastase
HSF	human skin fibroblast
iNOS	inducible nitric oxide synthase
ICAM	intercellular adhesion molecule
IL	interleukin
ICH	International Conference on Harmonisation

IκB	inhibitor of κB kinase
IPP	isopentenyl pyrophosphate
IKKB	IκB kinase b
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
MEP	2-C-methyl-D-erithritol-4-phosphate
MHC	major histocompatibility complex
MS	mass spectrometry
m/z	mass-to-charge ratio
NADPH	nicotinamide adenine dinucleotide phosphate
NP-HPLC	normal-phase high-performance liquid chromatography
NF-κB	nuclear factor-κB
PAMPA	parallel artificial membrane permeability assay
PTL	parthenolide
PTS	parthenolide synthase
PPAR	peroxisome proliferator-activated receptor
PBS	phosphate buffered saline
PBLE	polar brain lipid extract
PTFE	polytetrafluoroethylene
RCT	randomized controlled trial
RANK	receptor activator of nuclear factor κB
RSD	relative standard deviation
RP-HPLC	reversed-phase high-performance liquid chromatography
RA-FLS	rheumatoid arthritis fibroblast-like synoviocytes
SemiPrep- HPLC	semi-preparative high performance liquid chromatography
STL	sesquiterpene lactone
STAT	signal transducer and activator of transcription
SD	standard deviation
SC-CO_2	supercritical carbon dioxide
SFE	supercritical fluid extraction
SFC	supercritical fluid chromatography
MS/MS	tandem mass spectrometry
TOCSY	total correlated spectroscopy
TGF	transforming growth factor
TRPA	transient receptor potential ankyrin
TNF α	tumor necrosis factor α
UHPSFC	ultra high performance supercritical fluid chromatography
UPC^2	ultra performance convergence chromatography

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1 INTRODUCTION

Medicinal plants have been used to treat human diseases since ancient times. Nowadays an herbal renaissance is happening all over the world. In patients' point of view, the herbal products symbolize safety compared to synthetics. People are returning to natural products with the hope of safety, security and most importantly, less side-effects. The herbal medicine expertise was grounded in empirical knowledge that has been transferred from generation to generation. Phytochemical and pharmacological research can give a validated, evidence based background for the application. Plant extracts are complex matrices with numerous different compounds, the isolation of the putative biologically active components in most cases is not a viable option, because there is a synergistic effect between them or more than one constituents can contribute to the biological response. This makes the development of reproducible extraction and sophisticated and validated analytical methods necessary.

The application of novel solvents like supercritical fluids are highly desirable. In the last 17 years (2000-2017) more than 350 studies have been published about supercritical fluid extraction (SFE), concerning the extracts of plant species. The extraction with supercritical carbon dioxide was the first alternative for the widely used Soxhlet extraction, applying organochlorine solvents for sample preparation. SFE has many advantages compared to the traditional and popular extraction techniques, such as liquid-liquid extraction, solid phase extraction and purge/trap method. In the food, pharmaceutical, and fine chemical industries, supercritical fluid extraction is an often applied method. The other utilization of supercritical fluids is the area of chromatography. Although supercritical fluid chromatography (SFC) is an old chromatographic technique, the most commonly used chromatographic tools are still gas chromatography (GC) and liquid chromatography (LC). The first SFC systems which were specifically designed to manage supercritical fluids appeared in the recent years. This technological development has caused a dramatic uplift in the number of researches, publications and probable application of the method. The concurrent presence of semipolar and apolar components in a mixture, can be a good model system both for SFE and SFC to prove its effectiveness and selectivity.

Feverfews biologically active constituents fall mostly in the semipolar (sesquiterpene lactones, lipophilic flavonoids) and lipophilic (essential oil components, like camphor) cathegory, therefore traditional methods for the extraction and the qualitative and quantitative analyses of the compounds are less effective to study them in their complexicity. This makes the application of methods using supercritical fluids and feverfew an exceptional pairing.

Tanacetum parthenium L. has been used as medicinal plant for over 2000 years due to its pharmacological activities, particularly the migraine prophylactic effect. Feverfew contains a diverse range of compounds, including sesquiterpenoids, flavonoids and essential oil. Parthenolide is considered to be the major active constituent of the plant, however the pharmacological benefits of the lipophilic flavonoids and some of the volatile oil components cannot be neglected. Feverfew has COX-2, 5-HT releasing and NF-κB inhibitory effects besides other pharmacological activities. The research of the therapeutic effects of the plant is separated to two main directions, migraine therapy and cancer research. Migraine therapy protocol is mostly based on synthetic derivates. Some of the existing standard prophylactic treatments, such as propranolol, metoprolol, flunarizine, valproic acid and topiramate, which reduce attack frequency in some patients, are associated with side effects. Feverfew and other medicinal herbs can offer an effective, safe and well-tolerated alternative. The second, but not less important research area of feverfew, is the antitumoral activity of parthenolide, especially the half synthetic dimethylamino-parthenolide (DMAPT). The poor oral bioavailability of parthenolide made it necessary to derivatise to gain a more hydrophilic form. Although parthenolide and DMAPT cannot completely eradicate tumors, the application of them in combination with radiotherapy or with other drugs is advantageous, because their sensitizing effect to such therapies on cancer cells.

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2 LITERATURE OVERVIEW

2.1 *Tanacetum parthenium* L.

2.1.1 Taxonomic classification

Tanacetum parthenium L. is a hardy perennial herb belonging to the family Asteraceae (daisies) (Figure 1.).

Figure 1. *Tanacetum parthenium* L. (www.wikipedia.org, 2013)

The *Tanacetum* genus consists of 160 species native to the northern hemisphere, including *Tanacetum parthenium* L. which is endemic to the Balkan Peninsula but now it has a wide distribution in Europe, Asia, Australia and also in the United States. The common name derives from the latin word “febrifugia”, fever reducer, referring to the traditional use. The plant is also known as featherfew, because of its feathery leaves, bachelor’s button, wild chamomile, febrifuge plant, Mutterkraut, etc. *Tanacetum parthenium* is a long-day plant, in the vegetative period of growth needs sufficient light. The plant can be found along roadsides and in gardens [Pareek et al 2011, Pourianezhad et al 2016, Dános 1997, Hänsel and Sticher 2010, Bedoya 2008, Johnson 1984, Clark 2004, Majdi et al 2011, Brown et al 1996].

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2.1.2 Botanical description

Feverfew is an herbaceous, bushy perennial plant, which has a short direct root, growing a rhizome from the second year of cultivation (Figure 2).

Figure 2. *Tanacetum parthenium* L.
(www.wikipedia.org, 2013)

Depending on the climate and soil, *Tanacetum parthenium* L. grows 30-80 cm in height. Feverfew has a purple-green straight stem with longitudinal grooves. The yellow-green leaves have a long petiole in the lower half and are sessile or sub sessile in the upper half. They are usually less than 8 cm, divided and oval shaped, pinnate or bipinnate. The 2 cm diameter white flower heads are appearing from July to August in two genera and are self-fertile. The ray florets appear in a single layer. The daisy-like flower heads are arranged at the main stem in a dense cluster. The achene type seeds are grey-white colored [Pareek et al 2011, Pourianezhad et al 2016, Dános 1997, Hänsel and Sticher 2010].

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2.1.3 Phytochemistry

Tanacetum parthenium L. contains a diverse range of compounds. The most important, potentially biologically active components of feverfew are sesquiterpene lactones, flavonoids and monoterpenes in the essential oil. Some other principles such as coumarins and tannins are also present.

2.1.3.1 Sesquiterpene lactones

Sesquiterpenes belonging within the group of terpenes, the most numerous and structurally diverse class of secondary metabolites. They are built of three five-carbon isoprene units (5C). In most genus of Asteraceae, sesquiterpenes can be found containing a lactone group in the skeleton. These bitter tasting, non volatile, lipophilic molecules are present throughout the plants, but they are located most commonly in glandular thrichomes of leaves, flower heads and seeds.

Sesquiterpene lactones (STLs) can be synthetized by two pathways from the C5 isopentenyl diphosphate precursor: the mevalonate and the 2-C-methyl-D-erithritol-4-phosphat (MEP) pathway which are localized differently in the plant subcellulars; the previous one in the cytosol, the latter in the plastids [Vranová et al 2013, Liu 2013]. Figure 3. declares these two alternative biological production of isopentenyl-pyrophosphate (IPP). The localization of the STL synthesis was determined in feverfew based on the highest expression germacrene A synthase (GAS) by Majdi et al [Majdi et al 2011]. Although it was expressed in all aerial tissues, the highest expression was observed in the glandular thrichomes and in flower heads during the biosynthetically most active stages of flower head development. This high expression of GAS in glandular trichomes, which also contained the highest level of parthenolide, suggested that these tissues are not only the secretory tissues where parthenolide is synthetized but also the place where the accumulation occurs.

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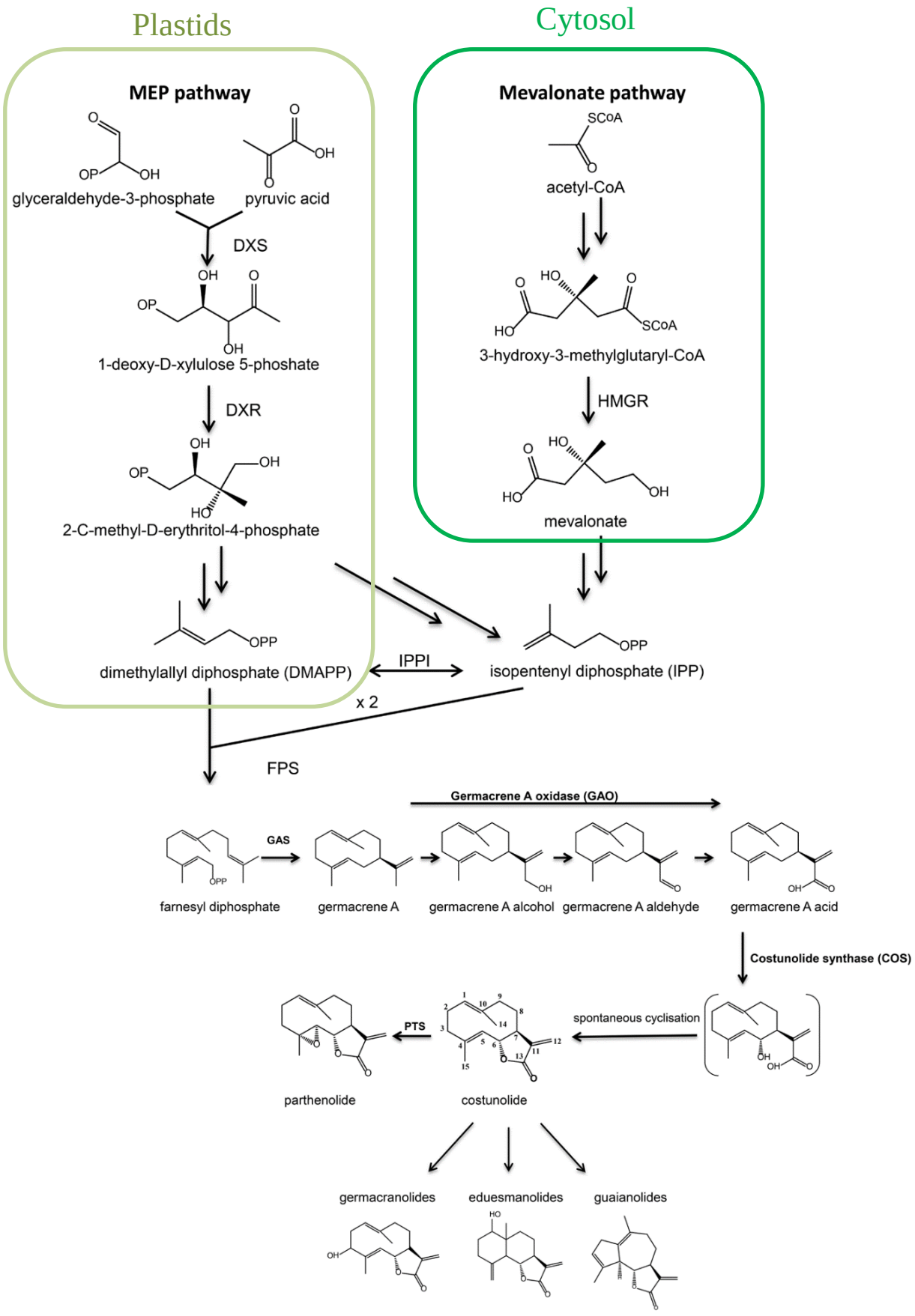

Figure 3. The two pathways of sesquiterpene lactone synthesis in plants: mevalonate and MEP pathway (Liu, 2013)

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Based on their skeleton, the main types of sesquiterpene lactones are germacranolides, eudesmanolides, elemanolides, guaianolides and pseudoguaianolides (Figure 4.). The skeleton of sesquiterpene lactones vary, but they all arise from the cyclization of the cyclodecadiene-type cation via the germacranolides.

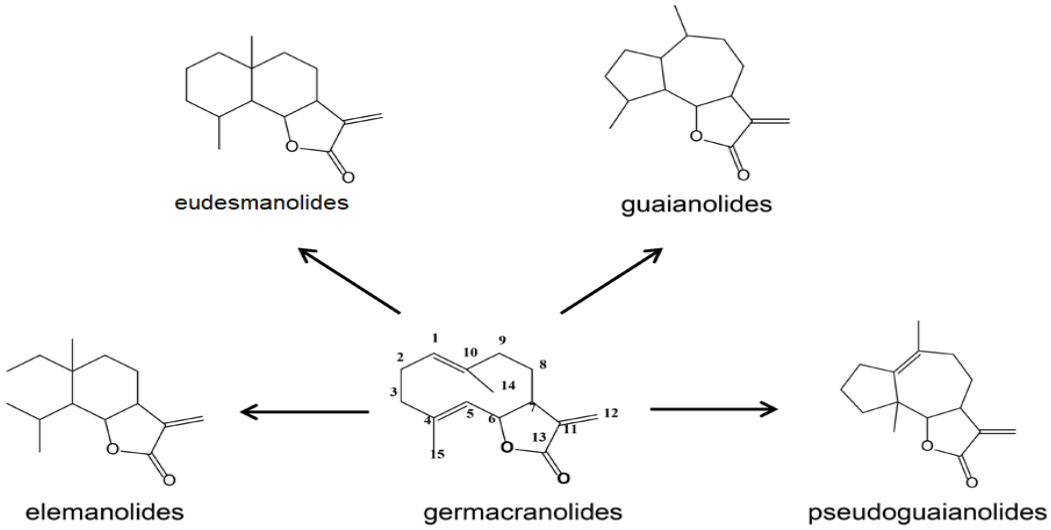
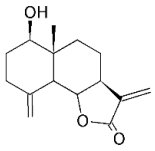
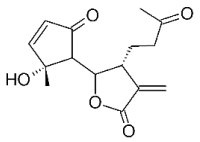
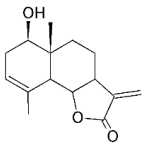
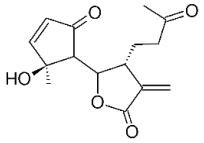
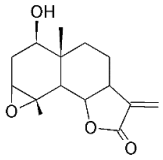
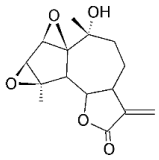
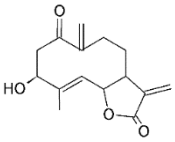
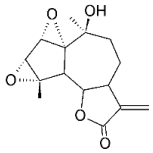
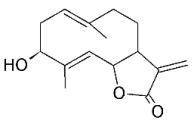
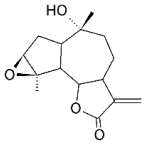
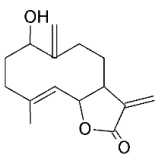
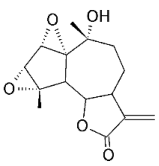


Figure 4. Five main types of sesquiterpene lactones: germacranolides, eudesmanolides, elemanolides, guaianolides and pseudoguaianolides

From these main types, feverfew contains eudesmanolide, germacranolide, guaianolide and pseudoguaianolide type sesquiterpene lactones. More than 30 STLs have been identified in *Tanacetum parthenium*, including parthenolide, costunolide, 3β-hydroxyparthenolide, 3β-hydroxycostunolide, artecanin, artemorin, balchanin, canin, 10-epicanin, epoxyartemorin, 1β-hydroxyarbusculin, 8β-hydroxyreynosin, manolialide, reynosin, santamarine, epoxysantamarine, seco-tanapartholide A, seco-tanapartholide B, tanaparthin-α-peroxide, tanaparthin-β-peroxide, 3,4β-epoxy-8-deoxycumambrin B, costic acid methyl ester, anhydroverlotorin-4α,5β-epoxide, 3β-hydroxyanhydroverlotorin, germacrene D, bicyclogermacrene, 8α-angeloyloxyestafiatin, 8α-hydroxyestafin, 8α-isobutyryloxyestafiatin [Pareek et al 2011, Gören et al 1993, Gören et al 2002, Fischedick et al 2012]. Parthenolide is considered to be the major active component of the plant [Mathema et al 2012, Merfort 2011], which can be 84% of the total STL quantity and 0 to 1.68% of the total extraction yield. The quantitative data on the sesquiterpene lactone content of feverfew is described in details in

the forthcoming supercritical fluid chapter. Besides parthenolide, epoxyartemorin is proved to be a potent inhibitor of prostaglandin synthesis [Sumner et al 1992, Knight 1995]. In plants of North American and Mexican origin, parthenolide can be absent, in return the eudesmanolide santamarine takes over its role. Some of the main STL constituents are described in Table 1 (The different STL types are color coded) [Gören et al 2002].

Table 1. The main sesquiterpene lactone constituents of feverfew, their LogP values and chemical structure [Gören et al 2002]

Sesquiterpene lactone	LogP	Chemical structure	Sesquiterpene lactone	LogP	Chemical structure
Reynosin	2.0		Seco-tanapartholide A	0.0	
Santamarine	2.0		Seco-tanapartholide B	0.0	
Epoxy-santamarine	2.0		Artecanin	0.2	
3β-hydroxy anhydro-verlotorin	1.4		10-epi-canin	0.2	
3β-hydroxy-costunolide	1.8		3,4-β-epoxy-8-deoxy-cumambrin B	0.8	
Artemorin	1.0		Canin	0.2	

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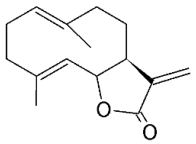
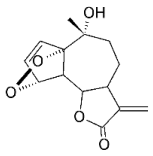
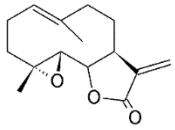
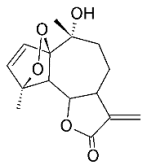
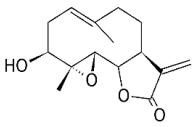
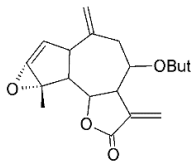
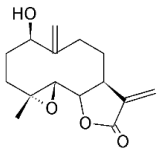
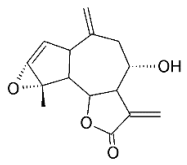
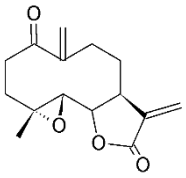
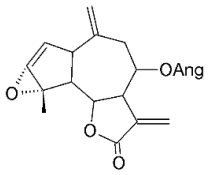
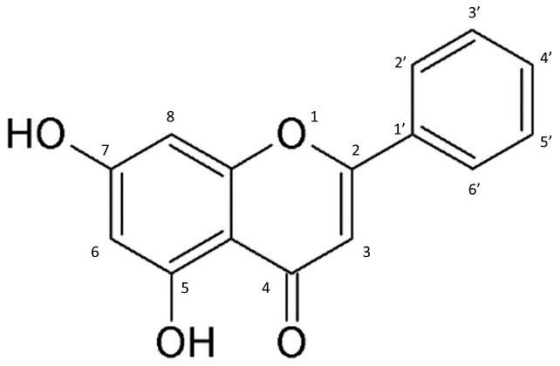
Sesquiterpene lactone	LogP	Chemical structure	Sesquiterpene lactone	LogP	Chemical structure
Costunolide	2.1		Tanaparthin-α-peroxide	0.9	
Parthenolide	2.3		Tanaparthin-β–peroxide	0.9	
3β-hydroxyl-parthenolide	1.2		Isobutyryloxy-estafiatin	1.9	
Epoxyartemorin	1.0		8α-hydroxy-estafiatin	1.2	
Anhydro-verlotorin-4α,5β-epoxide	1.4		8α-angeloyloxy-estafiatin	1.4	

(Eudesmanolides-blue, Germacranolides-green, Guaianolides-yellow, Seco-guaianolides-red)

2.1.3.2 Flavonoids

In the 1990's two type of flavonoid constituents were identified in *Tanacetum parthenium* L. The plant contains flavones and flavonols such as 6-hydroxykaempferol-3,6,4'-trimethylether (santin), 6-hydroxykaempferol-3,6-dimethylether, quercetagenin-3,6-dimethylether (axillarin) and quercetagenin-3,6,3'-trimethylether (jaceidin) accompanied by the isomeric quercetagenin-3,6,4'-trimethylether (centaureidin) (Figure 5,6.). These compounds were identified in dried leaves, flower heads and seeds by Williams [Williams et al 1995, Williams et al 1999, Williams et al 1999]. Apigenin, luteolin and chrysoeriol were also described in the plant. Williams et al. also noted that the lipophilic flavonoid constituents' structures are mainly related to 6-hydroxykaempferol-3,6,4'-trimethylether and quercetagenin-3,6,3'-trimethylether, but

up to 11 other flavonoids were present in the extract. The major constituents of the polar vacuolar flavonoids were apigenin-, chrysoeriol-, quercetin- and luteolin-7-glucuronides along with other minor apigenin and luteolin glycosides.

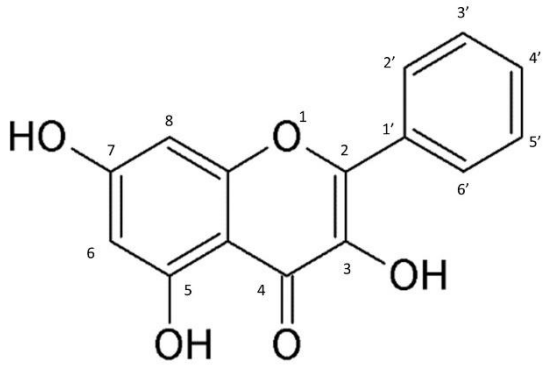


Name of the flavone	3	5	6	7	3'	4'	LogP
3-hydroxyflavone	OH	H	H	H	H	H	3.4
Apigenin	H	OH	H	OH	H	OH	1.7
Apigenin-7-diglucuronide	H	OH	H	O-di-gluc	H	OH	-1.3
Apigenin-7-glucosylglucuronide	H	OH	H	O-glugluc	H	OH	0.2
Apigenin-7-glucuronide	H	OH	H	O-gluc	H	OH	0.2
Chrysoeriol	H	OH	H	OH	OMe	OH	1.7
Chrysoeriol-7-glucuronide	H	OH	H	O-gluc	OMe	OH	1.1
Fisetin	OH	H	H	OH	OH	OH	2
Luteolin	H	OH	H	OH	OH	OH	1.4
Luteolin-7-glucoside	H	OH	H	O-glu	OH	OH	0.5
Luteolin-7-glucuronide	H	OH	H	O-gluc	OH	OH	0.8
Naringenin	H	OH	H	OH	H	OH	2.4

Figure 5. Flavone type flavonoids identified in *Tanacetum parthenium* L.

These results were revised in 2003 in a bio-guided fractionation of *Tanacetum parthenium* L. extract [Long et al 2003]. In 2008 the isolation and structure elucidation of three flavones (a 3-hydroxyflavone, kaempferol and fisetin) and one flavanone (naringenin) was reported [Shafaghat et al 2008]. Less attention has been given to the quantitative determination of the flavonoid constituents. Our workgroup has determined

in a non-published study, that the flavonoid amount is varying between 0.54±0.006g/100g and 0.92±0.025g/100g (in hyperoside) depending on the flowering stage of the plant. Other literature data estimates feverfews luteolin and apigenin content in 0.84% ± 0.10% and 0.68% ± 0.07%, respectively [Wu et al 2006].



Name of flavonols	3	6	7	3’	4’	LogP
Axillarin	OMe	OMe	OH	OH	OH	2.5
Centaureidin	OMe	OMe	OH	OH	OMe	2.8
Jaceidin	OMe	OMe	OH	OMe	OH	2.8
Kaempferol	OH	H	OH	H	OH	1.9
6-hydroxykaempferol-3,6-dimethylether	OMe	OMe	OH	H	OH	2.3
6-hydroxykaempferol-3,7-dimethylether	OMe	OH	OMe	H	OH	2.2
Quercetagenin-3,7,3’-trimethylether	OMe	OH	OMe	OMe	OH	3.5
Quercetin-7-glucuronide	OH	H	O-gluc	OH	OH	0.6
Santin	OMe	OMe	OH	H	OMe	3.1
Tanetin	OMe	OH	OMe	H	OMe	3.1
Tomentin	OMe	OH	OMe	OH	OH	1.5

Figure 6. Flavonol type flavonoids identified in *Tanacetum parthenium* L.

2.1.3.3 Essential oil

There are several reports on the composition of the essential oil in feverfew. The main components in most cases are camphor, camphene, p-cymene, bornyl-acetate and trans-chrysanthenyl acetate. Other components are identified as tricyclene, α-thujene, α-

pinene, β-pinene, α-phellandrene, α-terpinene, chrysantheone, pinocarvone, borneol, terpinen-4-ol, ρ-cymen-8-ol, α-terpineol, myrtenal, carvacrol, eugenol, trans-myrtenol acetate, isobornyl 2-methyl butanoate, and caryophyllene oxide [Mirjalili et al 2007].

The essential oil content of *T. parthenium* was determined based on the dry weight of wild and cultivated plants (0.1 and 0.4 w/w%) by Mirjalili et al [Mirjalili et al 2007]. 33 and 30 constituents were identified representing 95.7% and 97.5% of the total oil. In the oil of the wild sample, camphor (50.5%), germacrene-D (9.2%), camphene (7.7%), and (E)-sesquilavandulol (4.8%) were the main compounds, while camphor (57.6%), (E)-chrysanthenyl acetate (25.1%), camphene (4.6%), and bornyl angelate (2.2%) were detected as the major constituents in the oil of the cultivated sample. (E)-chrysanthenyl acetate and bornyl angelate were observed only in the oil of the cultivated sample, while (E)-sesquilavandulol (4.8%) and (E)-myrtanol (4.7%) were completely absent in the oil of this sample.

The composition of the essential oil of *Tanacetum parthenium* (L.) of various origins was investigated using GC-MS by Hendriks et al [Hendriks et al 1996]. Camphor and chrysanthenyl acetate were the main constituents of the samples originated from England and The Netherlands. No intraspecific variation in the composition of the oil was found. The essential oil content and composition of Dutch feverfew was studied during the vegetative period. The young herb, before the formation of the stems, yielded a relatively high percentage of oil (0.53%, v/w), calculated on the dry weight. The essential oil content increased until full bloom (0.83%, v/w). The percentage of camphor rose from 28% to 48% during the development of the plant, the amount of chrysanthenyl acetate decreased from 30% to 22%. Chrysanthenyl acetate may display an analgesic effect by inhibiting the enzyme prostaglandin synthetase. The potentially toxic monoterpenes α- or β-thujone were not detected in any of the oil samples investigated.

Banthrope analyzed the feverfew extracts of the flower heads by gas chromatography-mass spectrometry (GC-MS) [Banthrope et al 1990]. The components that could not be matched with the MS data banks were isolated and characterized by ^{1}H-NMR. Thirty-nine other compounds, mostly terpenoids were identified. The parthenolide content of the drugs were also investigated.

The aerial parts of *Tanacetum parthenium* L. were hydro-distilled and analyzed with GC-MS by Akpulat et al [Akpulat et al 2005]. A yield of 0.78 v/w% was produced in case of feverfew. Twenty-two components were identified representing 94.2% of the oil.

Haziri et al. described the chemical nature of essential oil from the aerial parts of *Tanacetum parthenium* L. from East part of Kosovo obtained by hydro-distillation [Haziri et al 2009]. The samples were analyzed by GC-MS. 22 components, which constitute 88% of the oil, were identified. The main compounds of *Tanacetum parthenium* L. were camphor (63%) and camphene (9.6%).

Izedi et al. extracted the essential oil from the aerial parts of feverfew [Izadi et al 2010]. They found that the highest amount of essential oil can be found in the leaves and the flower heads. The analysis of the extracted oil showed that the major constituent was camphor followed by chrysanthenyl acetate.

Although there are several studies about the major and minor components from the essential oil of *Tanacetum parthenium* L., none of them presents a validated quantitative method to determine the individual components. Only the percentage relative composition was studied in these reports. The chemical structures of the three main essential oil components, camphor, camphene and chrysanthenyl acetate are depicted in Figure 7.

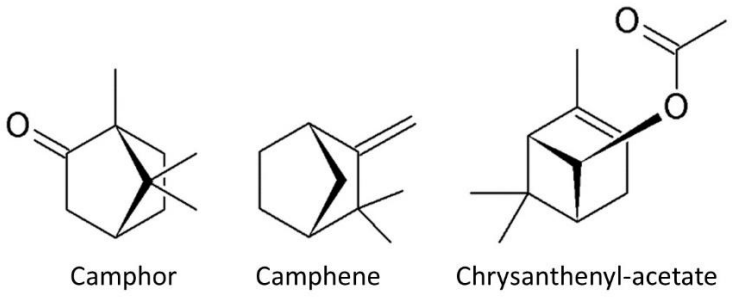


Figure 7. The three most significant monoterpene components: camphor, camphene and chrysanthenyl acetate in the essential oil of feverfew.

2.1.3.4 Other chemical components

The coumarins isofraxidin and an isofraxidin dimethyl ether (9-epipechtachol B) have been identified from the roots of feverfew [Stojakowska et al 1997, Kisiel et al 2002]. A coniferaldehyde derivative from the tissue culture of *Tanacetum parthenium* L. was

reported [Lai-King et al 1999]. Three antioxidant polyphenolic acids with potent 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities were isolated and characterized as 3,5-, 4,5- and 3,4-di-*O*-caffeoylquinic acids [Wu et al 2007].

2.1.4 Historical and traditional uses

Feverfew has already been referred to have medicinal properties since the ancient times. The first reference to its healing effect was in the Greek herbal 'Matera Medica' written by Dioscorides. The herb was described by Dodoens in 1619 (A new herbal, or historie of plants), by Gerard in 1636 (The herball, or generall historie of plantes) and Culpeper in 1650 (Culpeper's Complete Herbal and English Physician) [Chavez et al 1999, Jain et al 1999, Heptinstall et al 1992, Setty et al 2005, Pittler et al 2004, Sumner et al 1992].

Tanacetum parthenium has been used as treatment for fever, for a variety of inflammatory diseases, like toothache, rheumatism, insect bites and curing other health issues, like psoriasis, asthma, stomach ache, menstrual problems and miscarriage treatment. Feverfew was applied in two ways: freshly chewed or the dried herb was taken *per os* [Knight 1995].

Feverfew has been used to treat a variety of disorders in Central and South America: the Kallaway Indians of the Andes mountains use the herb for treating colic, kidney pain, morning sickness, and stomach ache. Costa Ricans propose a decoction of the herb to aid digestion, as a cardiotonic and as an enema for worms. Feverfew is also used as an antispasmodic and as a tonic to regulate menstruation in Mexico. In Venezuela, it is used for treating earaches [Pareek et al 2011, Chavez et al 1999].

The traditional use of feverfew as a medicinal plant, can be categorized in three groups:

- Migraine, headache and fever treatment
- Female problems
- Treatment of painful conditions, like stomach ache, toothache [Groenewegen et al 1992].

Tanacetum parthenium has a long history as a medicinal herb in the treatment of migraine and headache. In the 1970's there has been an uplift in the usage since there were studies carried out reporting that it was successfully used for treating and preventing both migraine and rheumatism. *In vitro* biological and phytochemical investigations were

carried out in order to prove its effectiveness and to find the chemical constituents responsible for these actions [Knight 1995].

2.1.5 Biological activities

In this chapter an overview about the biological activities of feverfew, sesquiterpene lactones, especially parthenolide and flavonoids is provided. Although *Tanacetum parthenium* L. is mostly used as herbal preparation, the main active compound is considered to be parthenolide, a sesquiterpene lactone. Therefore, the overview of pharmacological researches focuses on parthenolide beside feverfew extracts. Parthenolide, based on studies from the recent years, possesses several potent pharmacological effects, such as anti-inflammatory, antitumor and anti-microbial activities, effects contractile activity and possesses antimigraine properties.

Within the group of terpenes, the sesquiterpene lactones are in the focus of interest in the pharmaceutical research [Hänsel 2010]. Till now almost 5000 naturally occurring representatives of the class are known. The characteristic functional group of several STLs is an α-methylene-γ-lactone and many of them have an α, ß-unsaturated carbonyl– or epoxy group. These are reactive binding sites for nucleophiles, like thiols and amino groups. Because of that, a wide spectrum of biological and pharmacological effects can be expected. The antimicrobial, antiphlogistic, antitumoral and also the allergenic effects are based on the cytotoxicity or the alkylating potency of STLs. They can covalently bind to the nucleophile (especially thiol) groups of amino acids through a Michael-type addition performing a sesquiterpene-protein conjugate.

STLs from every structural class possess serotonin release inhibition activity [Marles 1995]. The previously mentioned α-methylene-γ-lactone functional group is essential but not sufficient for activity, and other functional groups contribute to the overall level of activity, particularly when substituted in an appropriate region of the molecule. Germacranolide type STLs are representing this enhanced contribution to the pharmacological effect; a lipophilic surface, the 1-10 double bound and the hydroxyl group at C15 are in the ideal position for the optimal biological activity. The bifacial lipophilic/hydrophilic character is vital; it ensures the bioavailability of the most

effective molecules. The increasing hydrophilicity is not only contributing to the better activity but also minimizing the cytotoxicity of the analogues.

Table 2. Medicinal plants with sesquiterpene lactone content: their application and biological activity [Hänsel 2010].

Plant	Main biologically active sesquiterpene lactones	Application, biological activity
***Inula helenium* L.**	Helenin	Induces apoptosis and cell cycle arrest in lung squamous cancer cells
***Arnica montana* L.**	Helenalin, Arnifolin	Anti-inflammatory effect, analgesic
***Artemisia vulgaris* L.**	Vulgarin	Analgesic, diuretic,
***Artemisia annua* L.**	Artemisinin, Arteannuin B	Antimalarial activity
***Matricaria recutita* (L.) Rauschert**	Matricin	Anti-inflammatory, anti-fungal, anti-bacterial, spasmolytic
***Cnicus benedictus* L.**	Cnicin, Artemisiifolin	Amarum aromaticum
***Taraxacum officinale* Weber**	Taraxacolid, Taraxin acid glucoside	Cholagogue, diuretic, anti-inflammatory, anti-tumoral
***Tanacetum vulgare* L.**		Antihelmintic
***Chamaemelum nobile* (L.) All.**	Nobilin	Amarum aromaticum
***Achillea millefolium* L.**	Achillicin, Achillin, Millefin	Anti-inflamatory, anti-fungal, anti-bacterial, spasmolytic
***Artemisia absinthium* L.**	Absinthin, Artabsin	Amarum aromaticum
***Petasites hybridus* (L.) Gaertn.**	Petasin, Petasol	Migraine prophylaxis
Thapsia garganica	Thapsigargin	Investigation of cellular calcium metabolism

The antimicrobial effect is due to the inhibition of key enzymes in the cytoplasmic membrane, while the antitumoral effect can be attributed to the selective alkylation of cell cycle regulating enzymes. The inhibition of HMG-CoA reductase and other enzymes of the lipid metabolism are considered to be responsible for the STLs antihyperlipidemic effect. A positive inotropic effect has also been observed. This increase in the force of

heart's contraction can be attributed to an indirect sympathomimetic action. Sesquiterpene lactones have several other effects, like helenalin and thapsigargin can influence the cellular calcium metabolism or the parasiticidal effect of artemisinin. In Table 2. medicinal plants are listed with biologically active sesquiterpene lactone content [Hänsel 2010].

2.1.5.1 Biological activities of sesquiterpene lactones in *Tanacetum parthenium* L.

The most well studied group of active compounds in feverfew are the sesquiterpene lactones. Since the first isolation of parthenolide in 1965, it is considered to be the main active ingredient of *Tanacetum parthenium* L. Figure 8. shows the phytotherapeutical and medical evolution of feverfew and parthenolide (PTL).

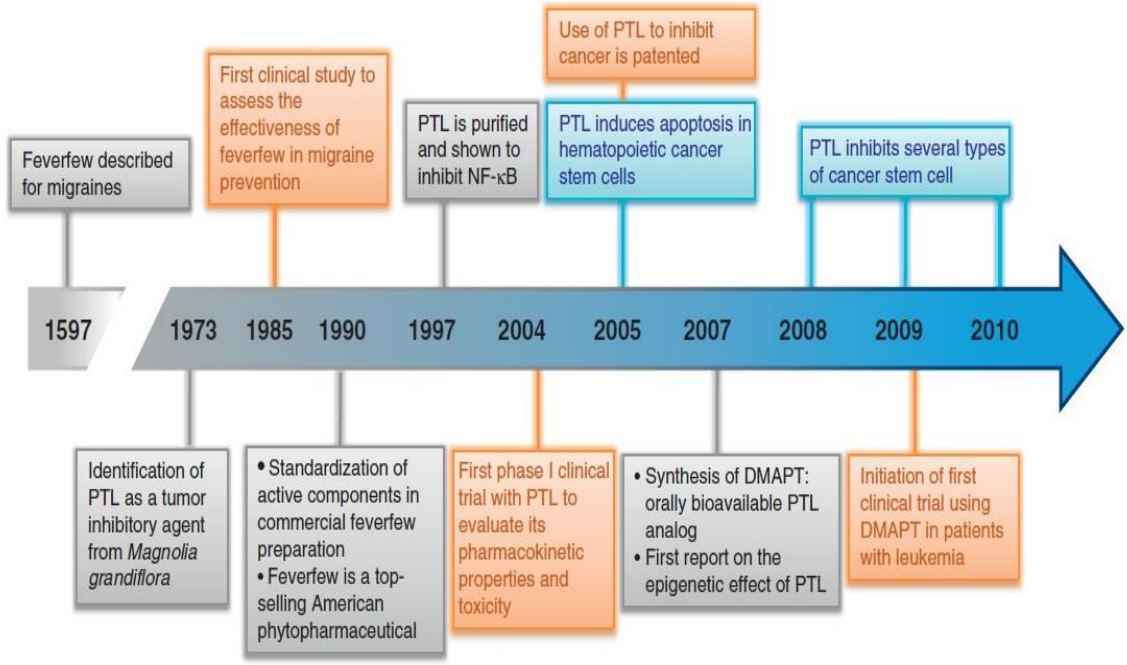


Figure 8. The phytotherapeutical and medical evolution of feverfew and parthenolide [Ghantous et al 2013]

After many researches, feverfew's biological action does not appear to be limited to a single mechanism. Feverfew extracts affect a wide variety of physiological pathways, including inhibition of prostaglandin synthesis, decrease of vascular smooth muscle

spasm, the inhibition of platelet aggregation and serotonin release from platelet granules. The researches are separated mainly in three directions: the anti-inflammatory effect, the migraine prophylactic effect and the antitumoral activity of a half synthetic parthenolide derivate. As it will be demonstrated, the wide range of biological activities of parthenolide, combined with its low toxicity, makes parthenolide and its analogues very promising therapeutic compounds with multitarget potential.

2.1.5.1.1 Anti-inflammatory activity

As previously mentioned, the STLs' several biological activities are primarily based on the reaction of their often conserved α, ß-unsaturated carbonyl structures (α-methylene-γ-lactone or α, β-unsubstituted cyclopentenone) with biological nucleophiles, especially cysteine sulfhydryl groups, by a Michael-type addition [Rodriguez et al 1976]. Inflammation is known to be caused by more than one factor; many signaling pathways in cells can mediate inflammation and there might be a crosstalk during the process [Wang et al 2014]. Parthenolide has been shown to attenuate inflammation interfering on multiple pathways. These suggested mechanisms for the anti-inflammatory effect of parthenolide and/or feverfew extracts are

- Inhibition of prostaglandin synthesis, platelet aggregation (inhibition of Phospholipase A_2, COX-2)
- Inhibition of histamine release from mast cells
- Inhibition of the vascular smooth muscle contraction through the selective blocking of voltage dependent potassium channels
- Reduction of the release of serotonin from thrombocytes and polymorphonuclear leukocytes (activation of protein kinase C, strong binding to 5-HT_{2A} receptor)
- Inhibition of the NF-kB transcription factor activation

A large number of studies have already been carried out using cell lines derived from mice, rats and humans. Most results of the experiments were positive, showing anti-inflammatory effects for parthenolide. These effects have been proved by *in vitro* and *in vivo* assays (Table 3.).

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Table 3. *In vitro* and *in vivo* assays over the anti-inflammatory effect of *Tanacetum parthenium* L. and parthenolide

Author		Cell line / animal model	Effects of parthenolide
In vitro	Hwang et al 1996	Murine macrophage cell line (RAW 264.7)	Suppression of LPS stimulated protein tyrosine phosphorylation
	Kazunori Fukuda et al. 2000	Human monocyte cell line THP-1	Suppression of i-NOS promoter activity
	Kwok et al. 2001	HeLa cell	Bonds directly to, and inhibits, I**k**B kinas b (IKKb)
	Sobota et al. 2000	Human hepatoma HepG2 cells	Inhibits IL-6-type cytokine signaling by blocking signal transduction through STATs
	Piela-Smith and Liu 2001	Human synovial FB cells	Inhibits the expression of ICAM-1
	Li-Weber et al. 2002	Jurkat T leukaemia cells	Suppresses IL-4 protein levels
	Uchi et al. 2002	Human monocyte-derived DCs	Up-regulates CD80, CD83, CD86, CD40, and MHC class II Down-regulates FITC-labelled dextran
	Siedle 2003	Human neutrophil	–
	Garcia-Pineres et al. 2004	Jurkat T Cells HeLa cells Raw 264.7 cells	Inhibits both NF-**k**B binding and I**k**B-kinase activity. Shows selectivity towards p65
	Herrera et al. 2005	Hippocampal HT22 cells	Total glutathione (GSH) is decreased
	Schorr et al 2005	Human neutrophil elastase (HNE)	Inhibits release of HNE
	Kurdi et al. 2007	Cardiac myocytes	Induces superoxide formation by mitochondrial and NADPH oxidase
	Lindenmeyer et al. 2007	Human epithelial cells (human 293 cell)	Constitutively expresses COX-2, TGF-b1, PPAR-a and RANK. Significantly inhibits COX-2
	Parada-Turska et al. 2008	Rabbit synoviocyte cell line HIG-82 Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) Human skin fibroblasts (HSF)	Inhibits the proliferation of human RA-FLS and HIG-82
	Rodriguez et al. 2013	Normal human keratinocytes Epidermoid cells (KB cells)	Induces Nrf2 nuclear translocation and increases antioxidant response element activity.
	Kang et al. 2001	RAW264.7	Inhibits IL-12 production and binds to the p40-kB site

Author		Cell line / animal model	Effects of parthenolide
In vitro	Lindenmeyer et al. 2007	293 cells	Inhibits NF-**k**B-driven gene expression
	Saadane et al. 2007	Cystic fibrosis cells	Inhibits IL-8 secretion Prevents NF-**k**B activation. Degrades I**k**Ba and I**k**B kinase
	Fonseca et al. 2010	Human peripheral blood mononuclear cells	Inhibits TNF-a and IL-6
	Juliana et al. 2010	Mouse bone marrow cells	Inhibits the ATPase activity of NLRP3
	Fonseca et al. 2011	Peripheral blood	Degrades IKK-b
	Sahler et al. 2011	Megakaryoblastic cell lines Meg-01 and MO7e cells	Inhibits NF-**k**B signaling
	Magni et al. 2012	BV-2 mouse microglia cells	Reduces LPS-stimulated IL-6 secretion and TNF-a secretion
	Materazzi et al. 2013	Human embryonic kidney (HEK293) cells	Inhibits CGRP
	Walshe-Roussel et al. 2013	THP-1 cells (human monocyteculture)	Moderates cytotoxic effects towards THP-1 monocytes
In vivo	Feltenstein et al. 2004	Carrageenan-induced paw oedema model	Significant attenuation of the oedema response and blockage of the hyperalgesic response
	Li et al. 2006	LPS challenged C57BL/6J mice	Early phase: decrease in the levels of NF-**k**B and 10 out of 13 other cytokines Late phase: increase in the levels of NF-**k**B and 11 out of 13 other cytokines
	Jain et al. 1999	acetic acid-induced writhing in mice carrageenan-induced paw edema in rats	Pain threshold increased as the number of writhing responses decreased
	Zhao et al. 2012	murine colitis model	Down-regulation of myeloperoxidase activity and phospho-NF-**k**B p65 expression Reduce in the production of TNF-a and IL-1b
	Materazzi et al. 2013	rat/mouse trigeminal neurons	Selective activation of TRPA1 and trigeminal nerve endings by targeting TRPA1
	Rummel et al. 2011	LPS challenged rat	Pretreatment with parthenolide attenuates the febrile response during LPS-induced systemic inflammation by reducing circulating IL-6 and TNFα and decreasing hypothalamic NF-**k**B/NF-IL6 activation, oxidative stress and expression of COX2.
	Xie et al. 2012	LPS challenged rat	Parthenolide, administered 6 h before LPS challenge, blocks NF-**k**B expression completely Only partial NF-**k**B expression blockage when administered before LPS challenge

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2.1.5.1.2 Migraine prophylaxis

Migraine is a common, incapacitating primary headache disorder. According to the International Headache Society it can be classified into two major sub-types: migraine without aura and migraine with aura [Headache Classification Committee of the International Headache Society 2013]. Migraine without aura manifests in attacks, lasting 4 to 72 hours; the headaches are characterized by their unilateral location, pulsating quality, moderate or severe intensity, aggravation by routine physical activity and association with nausea and/or photophobia and phonophobia. Migraine with aura that usually precede the headache, is primarily characterized by the focal neurological symptoms. Aura usually develops gradually over 5 to 20 minutes and lasts for less than 60 minutes. Headache may accompany or follow the aura symptoms within 60 minutes with or without the features of migraine. The Global Burden of Disease Study classified migraine as the third most common disorder to affect individuals globally in 2010, with a prevalence of 14.7% [Vos et al 2012]. Migraine was also ranked seventh highest among specific causes of disability globally [Steiner et al 2013].

Feverfew is considered to be migraine prophylactic, but the exact mechanisms of action remains unknown. The anti-migraine action is related to its principal bioactive ingredient, parthenolide. Parthenolide probably inhibits prostaglandin production, interferes with both contractile and relaxant mechanisms in blood vessels and perhaps inhibits the secretion of serotonin (5-HT) (Figure 9.) [ESCOP monograph 2003, Heptinstall et al 1985, Heptinstall et al 1987, Pugh et al 1988, Taylor 2011]. Despite the various positive applications, the clinical studies are controversial. According to a systematic analysis of five randomized, double-blind, placebo controlled studies (RCTs), Pittler and Ernst concluded that the therapeutic efficacy of feverfew preparations in migraine prophylaxis is not sufficiently proven and the effect is not better than placebo, but the safety is ensured [Pittler et al 2004]. Pfaffenrath et al. [2002] and Diener et al. [2005] came to a somewhat different conclusion. In two RCTs with a CO_2 extract (MIG-99) they found that the extract's effectiveness was superior to placebo.

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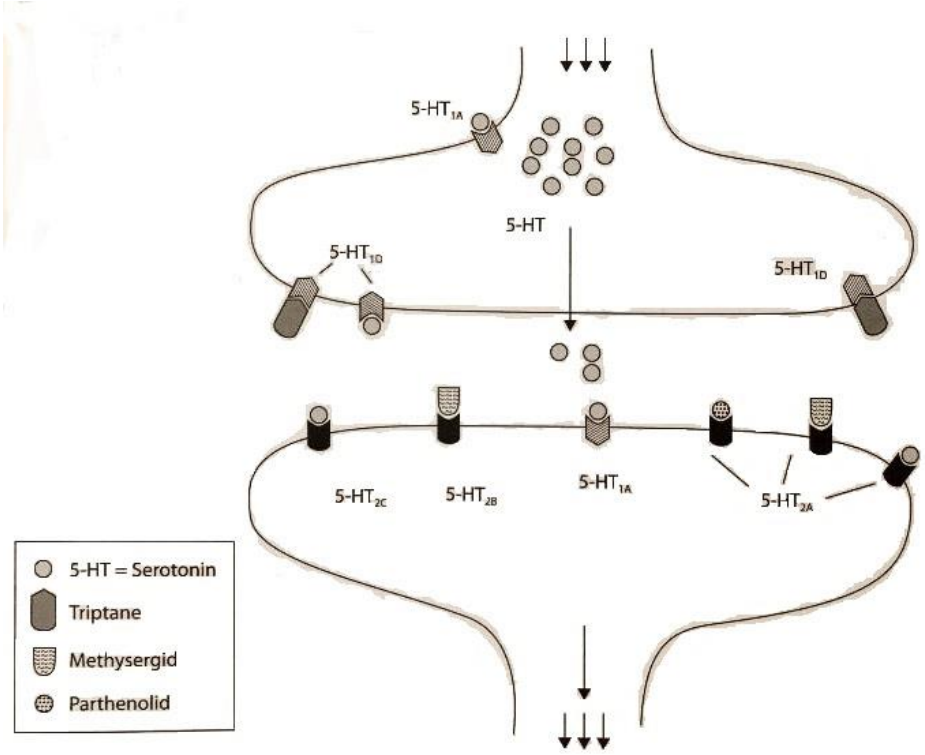

Figure 9. The mechanism of serotonin secretion inhibition by parthenolide, methysergid and triptane [Hänsel 2010].

The assumption, that not parthenolide as a monocomponent but the whole-leaf or flower head preparations are effective, has drawn attention to other components [Awang 1998]. A study of de Weerdt et al [1996] indicates, that the essential oil constituent of feverfew, chrysanthenyl acetate, might be important. This component inhibits prostaglandin synthetase *in vitro* and seems to possess analgesic properties. Other researchers agree that parthenolide is not the only pharmacologically active constituent in feverfew [Brown et al 1997, Hendriks et al 1996]. A link between the relatively high concentration of melatonin in different feverfew varieties [Murch et al 1997] and a decrease in melatonin excretion during migraine attacks has also been suggested [Brun et al 1995]. In conclusion, a definitive chemical link between parthenolide or any other constituents and the biology of migraine has still not been established [Kuritzky et al 1994].

2.1.5.1.3 Antitumoral activity

In 1973 parthenolide was isolated and identified from *Magnolia grandiflora* L. and has been proven to have antitumoral activities [Wiedhopf et al 1973]. Since then, many researches have been performed to determine the molecular mechanism of anticancer properties of parthenolide. The mechanisms of anticancer effect are associated with the highly reactive lactone ring, epoxide, and methylene groups of parthenolide. These active functional groups can cause a cytotoxic action associated with interruption of DNA replication, through the inhibition of the incorporation of thymidine into the DNA; oxidative stress, intracellular thiol depletion, endoplasmic reticulum stress, and mitochondrial dysfunction.

The lipophilic nature of parthenolide, which limits its oral bioavailability and solubility in blood plasma, made it necessary to derivatise parthenolide into a more hydrophilic form. The improved pharmacokinetic properties of dimethylamino-parthenolide (DMAPT) (Figure 10.) resulted in an increased oral bioavailability, high plasma concentrations following oral administration and acceptable toxicology profiles in animal studies [Guzman et al 2007]. Furthermore, the ability of DMAPT to eradicate acute myeloid leukemia (AML) stem cells selectively led to the initiation of an ongoing phase I clinical trial for its use in hematologic malignancies in the UK.

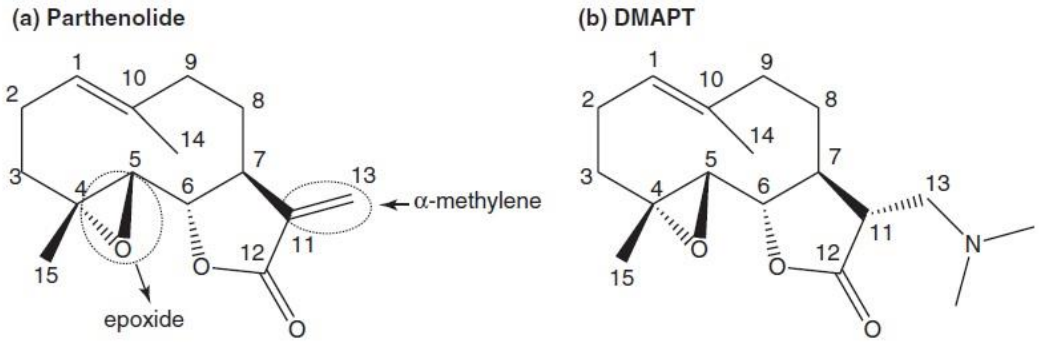

Figure 10. Parthenolide and dimethylamino-parthenolide (DMAPT) [Ghantous et al 2013]

However, it has been proven that parthenolide or its bioavailable analogs cannot completely eradicate tumors. Therefore, they should be used in combination with other drugs or radiotherapy, especially that parthenolide sensitizes cancer cells to such therapies [Won et al 2004, Zuch et al 2012]. Based on the structure of parthenolide,

chemical genomic approaches can identify molecules that maximize the eradication of heterogeneous tumor populations [Ghantous et al 2013].

2.1.5.2 Biologically active flavonoids in feverfew

Biologically active flavonoids in feverfew have exhibited functional activities that suggest they could play a remarkable role in the therapeutic effect of the plant.
The flower heads and leaves of feverfew showed significant analgesic, anti-inflammatory and antipyretic activities, particularly with reducing human neutrophil oxidative burst activity. These effects are attributed to the presence of both sesquiterpene lactones and flavonoids. Antioxidant activities of ethanolic feverfew extracts have also been shown. It was proven that the major flavonol and flavone methyl ethers inhibit the major pathways (cyclo-oxygenase and 5-lipoxygenase) of arachidonate metabolism in leukocytes [Williams et al 1999] and inhibit *in vitro* aggregation of platelets stimulated by adenosine 5″-diphosphate (ADP) or thrombin [Brown et al 1997]. Apigenin is one of the most widely distributed flavonoid in the plant kingdom, one of the most studied phenolics and a main flavonoid in feverfew [Johnson et al 1985]. Its properties as an antioxidant are well known, and it can also be a therapeutic agent to overcome diseases like inflammation, autoimmune, neurodegenerative diseases, and even several types of cancers. After absorption into the digestive tract, apigenin is able to reach the brain through the circulatory system, where it can cross the blood-brain barrier before exerting its affinity with the $GABA_A$-receptor and acting on the central nervous system. Tanetin, a lipophilic flavonoid found in the leaf, flower heads and seed of feverfew, blocks prostaglandin synthesis [Heptinstall et al 1985]. These bifacial molecules with hydrophilic and lipophilic properties are highly efficient in pharmaceutical application because of their high bioavailability. For a central nervous system activity, like migraine treatment, the compound has to cross the blood brain barrier. The lipophilic character of these flavonoids makes them an ideal candidate to have CNS activity.

2.1.5.3 Side effects and contraindications

In general, there is a low incidence of adverse reactions in individuals, so parthenolide

and feverfew appear to be well-tolerated. The most important side effect of feverfew is the contact dermatitis caused by the sesquiterpene lactones in the herb. This refers to an inflammation of the skin resulting from direct contact of a substance with the surface of the skin. The allergenic potency might be different for plants with sesquiterpene lactone content. *Tanacetum parthenium* is one of the drugs with strongly sensitizing effect. Patients, that chew feverfew leaves, sometimes report ulceration in the mouth, which can be avoided by taking dried herb containing capsules.

After a long-term use of feverfew followed by abrupt discontinuation may induce a withdrawal syndrome featuring rebound headaches, muscle and joint pains. In studies, patients who switched to placebo after taking feverfew for several years has experienced the postfeverfew syndrome, which is a cluster of nervous system reactions along with muscle and joint stiffness [Miller 1998, Agatonovic-Kustrin et al 2018].

Canker sores, dizziness and acidic eructation were also observed by patients taking feverfew [Pareek et al 2011].

Tanacetum parthenium contains high level of camphor, which is classified as very toxic and the probable human lethal dose has been estimated at 50–550 mg/kg [Opdyke 1978]. Toxic effects may follow a pattern of CNS stimulation (delirium, seizures) followed by depression (lack of coordination, respiratory depression, coma [Budavari 1989]. Neurologic symptoms can include anxiety, depression, confusion, headache, dizziness and hallucinations [Committee on Drugs 1994]. Initial symptoms of camphor toxicity have occurred in 5–15 minutes of ingestion. With mild poisoning, GI tract effects such as burning of the mouth, nausea, vomiting, and diarrhea are more frequently reported than neurologic effects. Seizures may be associated with apnea and asystole [Committee on Drugs 1994].

2.1.5.4 The significance of the bifacial lipophilic/hydrophilic character of the biologically active components in feverfew

The biologically active constituents of feverfew have lipophilic qualities. For a migraine therapeutic effect, the molecules have to cross the blood brain barrier, which can happen through passive or active transport. Moderately lipophilic compounds cross the BBB by

passive diffusion. High lipophilicity frequently leads to compounds with high rapid metabolic turnover, low solubility and poor absorption. As lipophilicity (LogP) increases, there is an increased probability of binding to hydrophobic protein targets other than the desired one, and therefore, there is more potential for toxicity. LogP is useful for prediction and optimization [Pajouhesh et al 2005]. Hansch and his colleagues developed regression analyses for these properties and initiated the field of quantitative structure activity relationships (QSAR). Hansch demonstrated in his initial work, that biological activity is almost entirely due to the LogP value and the rate of metabolism is linearly related to LogP. Furthermore, the optimal activity is observed at LogP=2 [Hansch et al 1967]. In the previous phytochemistry chapter, the LogP values of the identified compounds were given. The preselection of the compounds with a logP value of approximately 2, can be done by using special extraction methods, like supercritical fluid extraction. Through the modification of the fluids polarity, the degree of the lipophilic character can be regulated. The extracted lipophilic/hydrophilic parthenolide (LogP=2.3), its derivates and the lipophilic flavonoids have a higher plausibility to be bioavailable at the site of the effect, in the central nervous system. Their concurrent present in feverfew makes the plant an exceptional model for methods using supercritical fluids, like supercritical fluid extraction and supercritical fluid chromatography and for the blood-brain barrier specific artificial membrane permeability assay. In the next chapter the peculiarities and possible applications of the supercritical fluids are presented.

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2.2 Supercritical fluids in phytochemistry

2.2.1 Definition of supercritical fluids

Supercritical fluid can be any substance above the critical point, which is defined by the critical temperature and critical pressure. At this point, the substance is neither a gas nor a liquid, but possesses properties of both, including high diffusivity and low viscosity. The strict definition can be visualized by reference to the pressure/temperature diagram (carbon dioxide and water) shown in Figure 11. This phase diagram shows the relationship of the gas, liquid and solid state as a function of temperature and pressure, depicting the physical change of a substance from one state to another [Lee et al 1990].

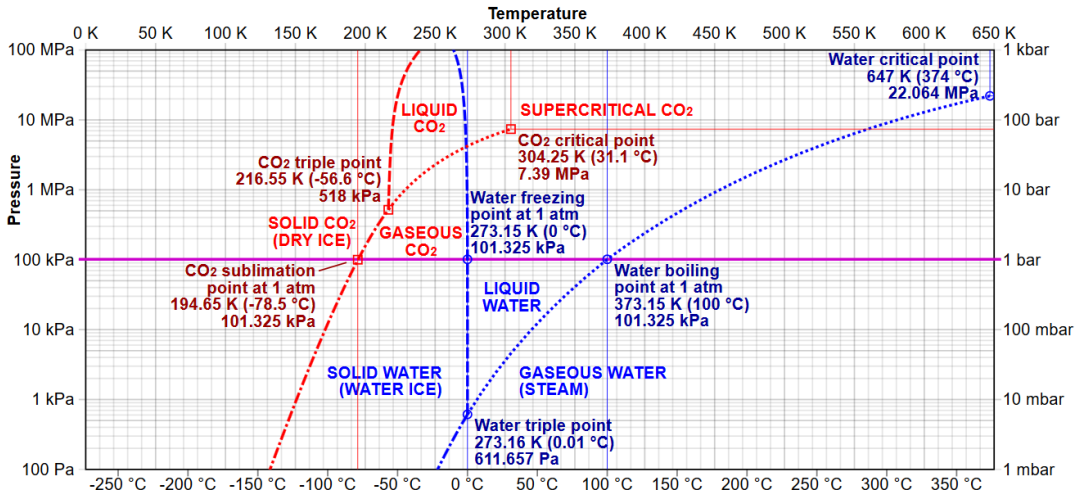

Figure 11. Pressure/temperature diagram of carbon dioxide and water (www.wikipedia.org, 2008)

Due to the control of pressure and temperature values, the physical properties (viscosity, diffusivity, density) are variable. The diffusion coefficients are substantially greater (10^{-4} -10^{-3} cm^2s^{-1}) in supercritical fluids, the viscosities mirror that, typically 10 to 100 times lower than for liquids. The density of fluids is higher (10^2-10^3 times) than a gas at the circumambient temperatures. The molecular interactions are increasing due to the smaller intermolecular distances. Table 4. introduces the physical parameters for some of the most commonly used substances [Lee et al 1990].

Table 4. The physical parameters of the most commonly used supercritical fluids

Substance	Critical temperature (°C)	Critical pressure (MPa)	Remarks
Carbon dioxide	31	7.4	Easily reachable conditions
Water	374	22.1	Extreme conditions
Methanol	240	8.0	High temperature
Ammonia	132	111	Corrosive
Nitrous oxide	37	73	Oxidizing agent
***n*-Butane**	152	3.8	High temperature

The most commonly utilized substance as a supercritical fluid is carbon dioxide (SC-CO_2) because of its easily reachable critical point. In case of the other substances extreme conditions are needed to reach the supercritical state or undesirable properties are appearing. Because of the limited solvating power of polar molecules, even at high densities, it is often necessary to mix the SC-CO_2 with modifiers and co-solvents. The term modifier is used for the addition of miscible compounds to the primary fluid. The most commonly used modifiers are listed in Table 5. Although the application of co-solvents has its significant positive effect on the physical properties of the supercritical fluid, the critical parameters must be considered carefully to avoid a vapor/liquid two phase carrier fluid [Hedrick et al 1992].

Table 5. Compounds used to modify the physical parameters of the supercritical fluids

Modifier	Critical temperature (°C)	Critical pressure (MPa)	Polarity index
Methanol	239.4	8.1	5.1
Ethanol	243.0	6.4	4.3
1-Propanol	263.5	5.2	4.0
2-Propanol	235.1	4.8	3.9
Acetonitrile	275	4.8	5.8
Chloroform	263.2	5.5	4.1
Water	374.1	22.0	10.2

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2.2.2 Supercritical Fluid Extraction (SFE)

Nowadays fast, environment friendly and selective methods like supercritical fluid extraction for the investigation of compounds in plant matrices are highly desirable. In 1879 Hannay and Hogarth demonstrated the solvating power of supercritical fluids for solids [1880]. They studied the solubility of cobalt(II) chloride, iron(III) chloride, potassium bromide, and potassium iodide in supercritical ethanol. They found that the concentrations of the metal chlorides in the solvent were much higher than the predicted values. They also found that increase in the pressure caused the dissolution of the substances.

During the first half of the twentieth century, extraction and separation of mixtures with supercritical fluids aroused very little interest. Zosel's patent presented a significant development in supercritical fluid extraction, in which he reported the decaffeination of green coffee with CO_2 [1971]. This process removes caffeine from green coffee by means of recirculating moist carbon dioxide in supercritical state. Currently, over a dozen of patents have been issued that concern the decaffeination of coffee by SFE.

In the last 17 years (2000-2017) more than 350 studies have been published about SFE concerning the extracts of plant species. The extraction with supercritical carbon dioxide was the first alternative for the widely used Soxhlet extraction, applying organochlorine solvents for sample preparation. SFE has many advantages compared to the traditional and popular extraction techniques, such as liquid-liquid extraction, solid phase extraction and purge/trap method [Hedrick et al 1992]. Supercritical fluid extraction is a commonly applied method in the food, pharmaceutical, and fine chemical industries [www.waters.com,2012]:

- Decaffeination of coffee and tea
- Extraction of essential oils (vegetable and fish oils)
- Extraction of flavors from natural resources (nutraceuticals)
- Extraction of ingredients from spices and red peppers
- Extraction of fat from food products
- Fractionation of polymeric materials

One of the most powerful and unique advantage of SFE is the capability to extract a lots of molecules of varying polarity and molecular size by mechanical compression. In addition, a fast extraction is possible from a variety of sample matrices because the binary diffusion coefficients are much greater in supercritical fluids than in liquid-liquid systems.

Supercritical fluid extraction can also provide other specific advantages. For example, thermosensible compounds can be extracted under conditions slightly above room temperature. SC-CO_2 is a nontoxic solvent, unlike many extraction media, and leaves a solvent free product after the extraction.

In practice, SFE is suitable for the separation of nonpolar to slightly polar compounds, however, the solubility of polar analytes can be enhanced by the addition of modifiers at low levels.

Several compounds have been examined as SFE solvents, however SC-CO_2 is the most widely used, because it is inexpensive, safe and environment friendly.

For the better understanding of the advantages, the comparison of physical properties of the SC-CO_2 to those associated with liquid solvents under the same operating conditions is useful (Table 6.) [Lee et al 1990]. The density of CO_2 is lower than that of methanol, but higher than *n*-hexane. This value suggests a liquid like nature in the intermolecular attraction. Otherwise, the kinetic based properties, like viscosity and diffusivity are more similar to gases than to liquids. These properties make a fast and rapid extraction possible.

Table 6. Comparison of the physical properties of SC-CO_2 to liquid solvents under the same operating conditions

Physical property	**SC-CO_2**	***n*-Hexane**	**Methanol**
Density (g/ml)	0.746	0.660	0.791
Kinematic viscosity ($m^2/s \times 10^7$)	1.00	4.45	6.91
Diffusivity of benzoic acid ($m^2/s \times 10^9$)	6.0	4.0	1.8

Another fundamental variable is the selectivity of the SFE. During the extraction, the product's composition can change depending on the operating conditions. The dissolution of the extractable compounds is a matter of both temperature and pressure.

Moreover, the molecular interactions and the modifiers are key factors in the separation. Pressure increases the density of the supercritical fluid, thus increasing its solvent power. With the increasing temperature of the fluid, density decreases (lower solubility), but the vapor pressure increases (higher solubility). These two opposing effects make the proper selection of temperature a necessity. The addition of a co-solvent to the supercritical fluid can increase the modified SC-CO_2's solvent power towards more polar compounds. The most commonly applied modifier is ethanol, due to its innocuous properties both on human health and environment. Beside these advantages, the addition of ethanol even in small quantities (usually max. 10%) may increase the polarity of the supercritical fluid significantly. The drawback of this method is that the larger solvent power could mean lower selectivity and the added co-solvent remains in the extract, so a follow-up solvent elimination process is required [Lee et al 1990].

Other crucial parameters are the solvent flow rate, the extraction time and the particle size of the matrix. The contact of the supercritical fluid solvent and the sample matrices has to be increased in order to enhance the effectiveness of SFE. If the extraction process is controlled by the mass transfer rate or equilibrium, the solvent flow rate is a crucial parameter, because in this case the amount of solvent fed to the vessel determines the extraction rate. Through a smaller particle size, the length of diffusion of the solvent can be reduced in processes controlled by internal mass transfer resistances. However, a channeling problem can occur in the extraction bed if the particle size is too small [Lee et al 1990].

An analytical scale off-line SFE system comprises a supercritical carbon dioxide pump, a modifier co-solvent pump, an extraction vessel charged with the raw material, and an automatic back pressure regulation valve (Figure 12.). The temperature of the extraction has to be controlled, usually by placing the extraction cell in a chromatographic oven or in a simple thermostated tube heater. SFE is normally performed at constant pressure, which is ensured by the back pressure regulation valve. The collection of the extracted analytes is dependent on the fluid flow and the depressurization method. At the exit, the supercritical fluid flows through a depressurization valve to a separator, in which the extracts are released from the medium due to the lower pressure and are collected. Another extraction scheme contains two or three separators to fractionate the extract of different composition due to the different temperature and pressure settings.

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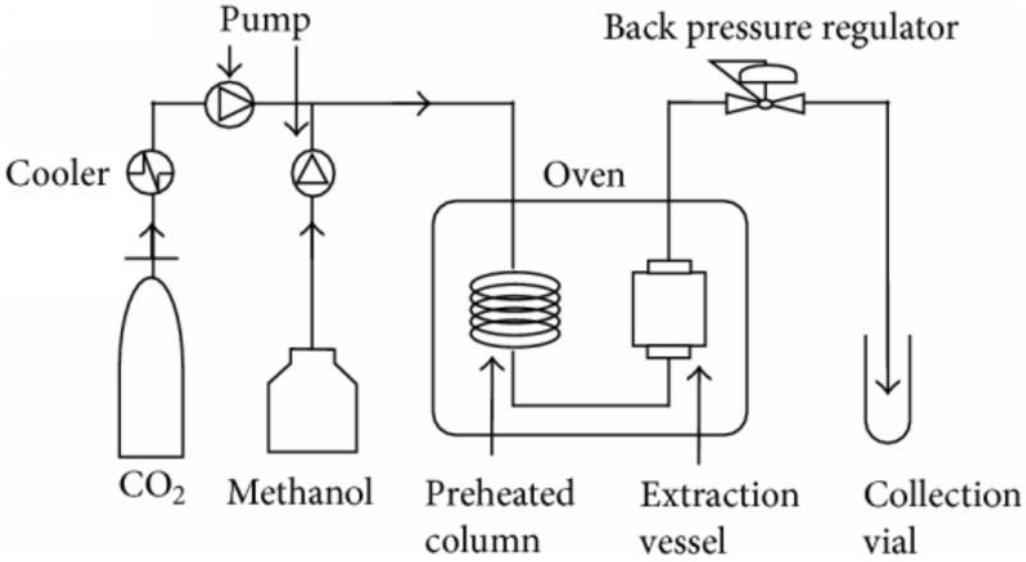

Figure 12. Scheme of an analytical scale off-line supercritical fluid extraction system [Juliasih et al. 2015].

For the effective and selective extraction of the compounds from the plants the experiments should be precisely planned. Design of experiment (DOE) is a commonly used statistical method for modeling supercritical fluid extraction. DOE allows for multiple input factors (pressure, temperature, modifier, flow rate, extraction time) to be manipulated determining their effect on the extraction. In other words, it is used to find cause-and-effect relationships. By manipulating multiple inputs at the same time, DOE can identify important interactions that might be missed when experimenting with only one factor at a time. All possible combinations can be investigated (full factorial) or only a portion of the possible combinations (fractional factorial). The results are usually evaluated by polynomial regression analysis using response surface methodology to determine the optimum point of the extraction and to evaluate the effect of the factors on the responses.

2.2.3 Supercritical Fluid Chromatography (SFC)

Although supercritical fluid chromatography (SFC) is an old chromatographic technique, the most commonly used chromatographic tools are still gas chromatography (GC) and liquid chromatography (LC). In GC the separation is achieved by varying the temperature

of the column during the analysis. Selectivity is determined only by the interaction of the analytes and the column chemistry, the carrier gas is inert. Gas chromatography is limited to a narrow polarity and volatile range [Balla 2006]. Liquid chromatography can separate a more diverse range of compounds in reversed phase mode. In LC the analytes have affinity for both the mobile and the stationary phases. The change in selectivity can be achieved by creating a competition between these two phases [Fekete 2006].

The discoverers of supercritical fluid chromatography are considered to be Klesper et al. [1962], although James Lovelock proposed the idea to use SF as chromatographic mobile phase first in 1958 [Lee et al 1990]. Despite the early debut, the major growth of the technic only occurred in the 1980's, when Hewlett Packard introduced their modified Model 1084 liquid chromatography instruments for packed column SFC. Supercritical fluid chromatography was considered as an odd form of HPLC or as an extension of GC for years. The instrumentation which was used for the separation were mostly repurposed HPLC or GC equipment. This caused some experimental issues:

- High operating temperatures
- Poor back pressure regulation
- Injection through a retention gap or full loop
- Lack of robustness
- No possibility for a reliable addition of co-solvent

Due to these problems, the method was plagued with lack of robustness, shifting retention times, shifting solvation power, poor accuracy and precision. The unreliable pumping systems, which were not designed to deliver supercritical fluids and the poor back pressure regulation caused problems in the sensivity and the robustness of the methods. The first SFC systems which were specifically designed to manage supercritical fluids (Waters Aquity UPC^2, Jasco systems, etc.) appeared only a few years ago. Packed column SFC is considered to be equivalent with normal phase LC, but the previous outperforms NP-HPLC and even reversed-phase HPLC in some applications. 80-85% of the compounds which can be analyzed by RP-HPLC, can be examined in SFC systems. In addition, SFC can also be applied for those compounds typically analyzed by NP-HPLC, chiral compounds, stereoisomers and diastereomers. This diverse range of applicability dramatically increased the utility of SFC (Figure 13.).

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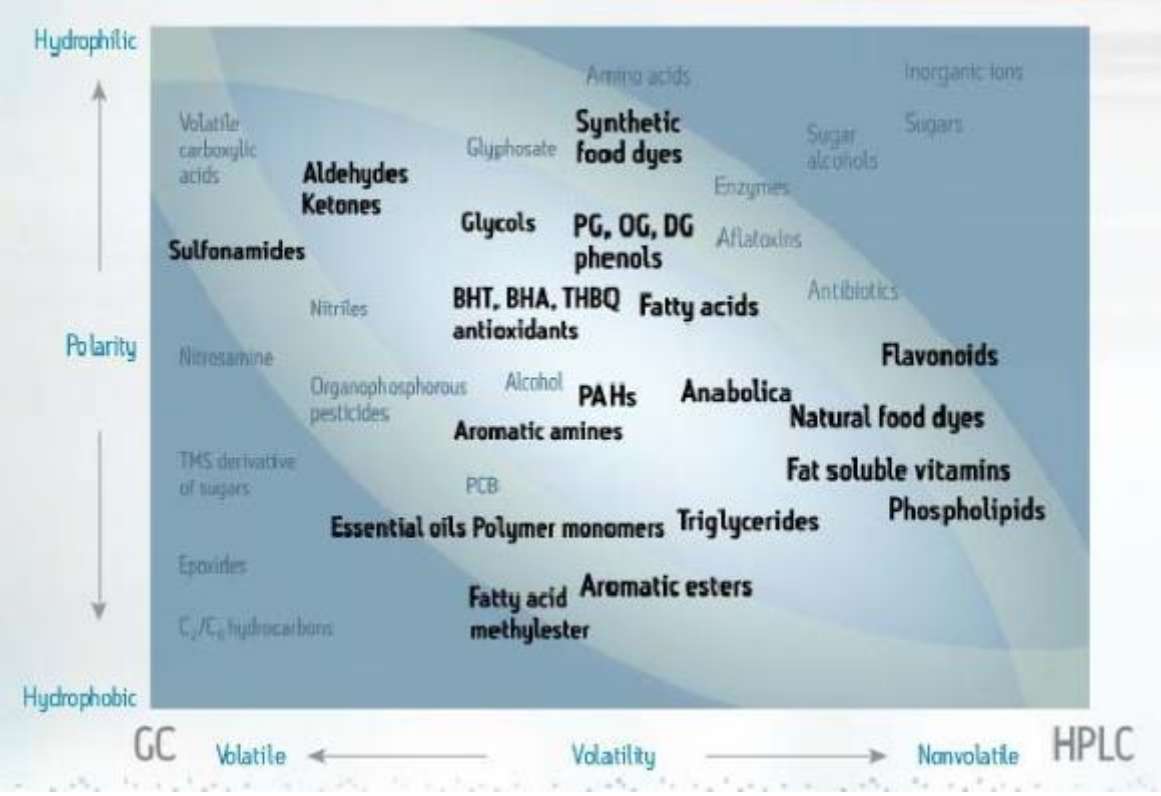

Figure 13. Potential applicability of supercritical fluid chromatography (compounds which can be analyzed by SFC are marked black) (www.waters.com,2012)

Some of the advantages of supercritical fluid chromatography [Taylor 2009]:

- Fast retention
- Extremely fast re-equilibration
- Allows steep gradients
- Very long columns can be applied
- Incorporates very small particles with moderate pressure drops
- Tolerates significant amount of water
- Peak efficiency is similar to those observed in GC
- High flow rates
- Wide range of polar and non-polar stationary phases and co-solvents can be employed (Figure 14.)

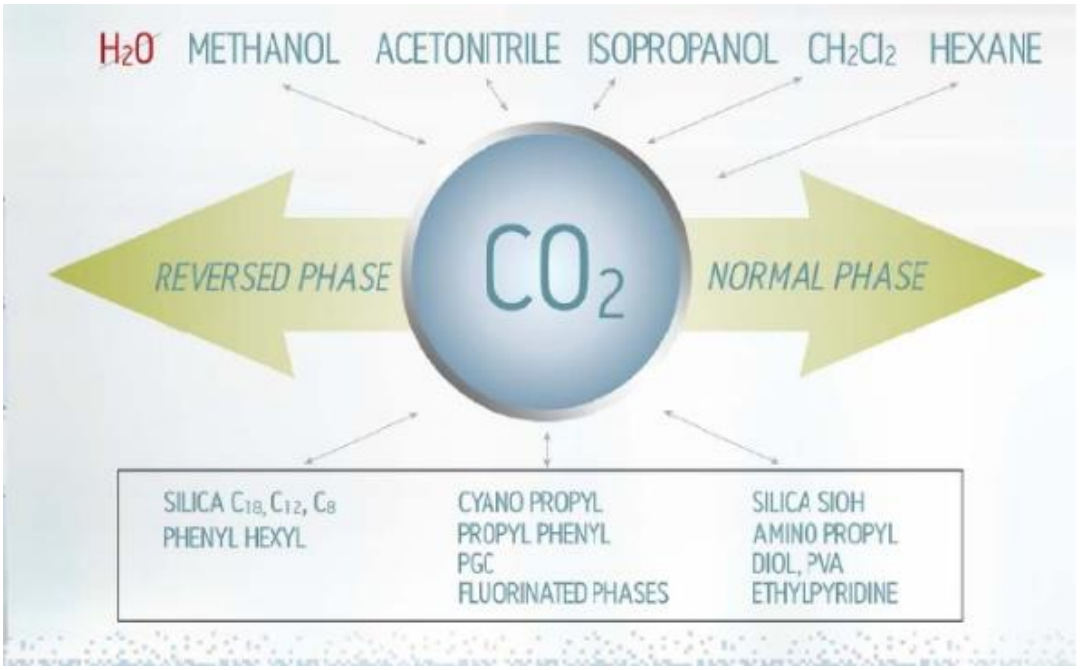

Figure 14. Polar and non-polar stationary phases and the wide range of co-solvents which can be applied in supercritical fluid chromatography (www.waters.com, 2012)

Although there is a significant rising in the number of publications, most of the analysts consider UHPSFC method as a chiral application, not as a quantitative method [Dispas et al 2014].

2.2.4 Supercritical fluids in the study of *Tanacetum parthenium* L.

There is a wide variation of conventional methods for the extraction of parthenolide from feverfew, using an initial organic or aqueous phase. Several solvent systems were examined applying bottle stirring or Soxhlet extraction [Zhou et al 1999, Rey et al 1992, Kery et al 1988]. Numerous studies have been performed on feverfew with SFE technique. The previously mentioned semipolar quality of several compounds in feverfew, makes the plant an exceptional subject for supercritical fluid extraction.

Smith and Burford [1992] compared the efficiency of supercritical extraction with three published, and a general extraction method, regarding the parthenolide content in dried feverfew. The French Pharmacopoeia version gave a yield of 0.04% parthenolide at 45°C

for 30 minutes extracting the dried plant with methanol. Another technique, in which after a methanol extraction, a lead acetate treatment is done, followed by re-extraction in chloroform, yielded 0.02% parthenolide [Bloszyk et al 1978]. The method of Marchand et al. is carried out at room temperature with chloroform for 30 minutes [Marchand et al 1983]. This produced 0.006% parthenolide. Supercritical fluid extraction was completed in 20 minutes at 250 bar and at 45°C and had a 0.07% yield of parthenolide. The addition of modifiers (4% of methanol or 4% of acetonitrile) to the fluid carbon dioxide doubled the extraction yield of parthenolide in both cases (0.16% and 0.14%) [Smith and Burford 1992].

Kaplan et al. [2002] compared the parthenolide yields obtained with CO_2 (400 bar, 100°C, 10 minutes) and with conventional solvents (chloroform, acetone). They applied a stirring method in refluxing for the extraction of parthenolide with chloroform for two hours. Fresh feverfew leaves, seeds and a powdered sample were investigated. Several conclusions were drawn from the results. They deduced that the seeds contained very high level of parthenolide (1.134%) and that there is a significant difference between the solvent extraction and the SFE results. The supercritical fluid carbon dioxide extracted 0.091% parthenolide from the plant powder, counter to the chloroform and acetone solvent extraction, which only yielded 0.062% and 0.04%, respectively. Although SFE contained more parthenolide, the fluid carbon dioxide has extracted a smaller number of sesquiterpene lactones based on the HPLC analyses.

Kery et al. [1999] optimized the extraction conditions on a larger scale equipment with respect to total extraction yield and the yield of parthenolide. They used a 3^2 factorial design in order to find the optimum point of the extraction. The pressures were 100, 250 and 400 bar and temperatures 40, 50 and 60°C. In this study the particle size was also considered because of its effect on the extraction yield. They found, that the optimum point for the yield of extract and for parthenolide are very similar and it is obtained at the border region of the extraction conditions (400 bar and 60°C).

Cretnik et al. [2005] performed supercritical fluid extraction from feverfew flower heads at the pressures from 200 to 800 bar and at the temperatures 40, 60 and 80°C. In order to concentrate the parthenolide in the obtained extract, a two-step separator was employed. Conventional solvent extraction was also performed for comparison. They found, that the best organic solvent was acetonitrile which extracted 350 mg parthenolide

from 100g raw material. They also tested the thermal stability of parthenolide. They found 13% degradation after one hour at 80°C. Using SFE, they came to the conclusion, that the parthenolide content increases with the decreasing temperature and pressure. Two separators were applied during the extraction. The extraction was carried out at 300 bar and 80°C and the conditions in the first separator were 100 bar and 40°C, so the parthenolide concentration was six time more in the second separator than in the first.

The insecticidal activity of feverfew extracts obtained by SFE and hydrodistillation was investigated by Pavela et al. [2010]. The study found, that the supercritical methods yields were 5-7 times higher than the standard distillation of the essential oil. The SFE method prevented the loss of terpene compounds with high biological activity. The mortality was strongly correlated with the terpenoid content of the samples and the extracts obtained with supercritical carbon dioxide pure or modified by acetone (280 bar, 50°C) were more efficient antifeedants and grow inhibitors than the essential oil alone, showing the combined effect of the essential oil and other non-volatile bioactive substances.

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3 OBJECTIVES

Feverfew is a well-known medicinal herb with large literature and research background. The primary aim of our work was to reevaluate and to complement the phytochemical and pharmaceutical information of the herb. Considering the fact, that nowadays the rapid and environmental friendly methods are desirable, and the semipolar nature of the simultaneously present molecules in the plant, we aimed to use techniques applying supercritical fluids.

1. The primary aim of our work was to optimize a supercritical fluid extraction method in order to maximize the extraction yield and parthenolide content of feverfew extracts. Our objective was to plan a detailed experimental program of the supercritical fluid extraction conditions (temperature, pressure, co-solvent content) in order to evaluate their individual and combined effects. We intended to compare the optimalised SFE method to other conventional extraction methods like Soxhlet and ultrasound assisted extraction.

2. We aimed to develop a validated qualitative and quantitative supercritical fluid chromatographic method for the determination of the parthenolide content of the feverfew extracts prepared by supercritical fluid extraction and conventional methods.

3. For the identification of simultaneously present co-extracts, mass spectrometry is acquired to gain structural information with particular attention on the lipophilic flavonoids.

4. Postulating that other components beside parthenolide take place in the bioactivity, our objective was to screen compounds possessing positive permeability rates through the blood-brain barrier (BBB+) in the supercritical fluid extracts of *T. parthenium*. We aimed to isolate these BBB+ components and identify them.

5. In order to gain information about the volatile oil of the herb, steam distillation was chosen to prepare extracts for the further analysis. An HS-SPME-GC/MS method was applied for the qualitative and quantitative analysis of the essential oil samples and supercritical fluid extracts. The main, potentially biologically active component of the essential oil is camphor beside camphene and *trans*-chrysanthenyl acetate based on literature data. Our objective was to develop and validate a supercritical fluid chromatographic method for the quantitative study of the camphor content of the volatile oil. As comparison, the camphor content of the supercritical fluid extracts is also aimed.

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4 MATERIALS AND METHODS

4.1 Plant material

Tanacetum parthenium L. seeds were collected from the medicinal plant collection of the

- National Botanical Garden in Vácrátót (Hungary, 2011),
- Botanical Garden of the University of Debrecen (Hungary, 2011)
- Botanical Garden of Bonn (Germany, 2011).

The seedlings were raised in Érd and collected before and during flowering. The blooming samples were manually separated to flower heads and leaves. The samples originated from the Vácrátót seedlings were investigated in experiments applying supercritical fluids. Only the essential oil and camphor content was determined from all three originations. After grinding, the size of the particles was between 40 and 50 Mesh. Voucher specimens are deposited in the Department of Pharmacognosy, Semmelweis University, Budapest.

4.2 Solvents and chemicals

Parthenolide, luteolin, apigenin, axillarin and casticin standards (≥98%, HPLC purity), camphor standard (≥96%, GC purity) and trifluoroacetic acid were purchased from Sigma-Aldrich (Germany).

Purity of the CO_2 employed in the supercritical fluid experiments was 99.5% and was supplied by Linde (Hungary). Reagent grade ethanol was purchased from Reanal-Ker (Hungary).

HPLC grade acetonitrile and isopropanol were supplied by Merck (Germany). Chemical solvents were reagent grade, HPLC grade. The HPLC grade water was prepared with a Millipore Direct Q5 water purification system (USA). All aqueous eluents for HPLC were filtered through MF-Millipore membrane filters (0.45 μm, mixed cellulose esters) (USA) and degassed in an ultrasonic bath before use.

4.3 Extraction and sample preparation

4.3.1 Analytical scale SFE

Supercritical extractions were carried out using a system consisting of a Jasco PU-2080 CO_2 pump with a Peltier cooling system for the delivery of carbon dioxide and a Jasco PU-2080 pump, linked to a Jasco MX-2080-32 Dynamic mixer for the addition of modifier. A 1 ml Jasco extraction vessel was mounted in the column position in a CO-2060 plus intelligent column thermostat. A Jasco BD-2080 back-pressure regulator was applied to adjust the pressure in the system. The 1 ml extraction vessel was packed with (ca. 0.5 g) plant material and the optimal parameters were investigated in regard to the amount and composition (parthenolide content) of the extract. The solute leaving the extractor was introduced through a pressure-reducing valve, where the product was collected. The extractions were carried out for over 1h, the solvent flow rate was 0.4 ml/min. The extracts were filtered through Phenex-RC 15 mm, 0.2 μm syringe filters (Gen-Lab Ltd, Budapest, Hungary).

4.3.2 Experimental design on the SFE

The relationship between the measured responses and the individual and combined effects was analyzed with response surface methodology. In the extraction process a factorial experiment using a full 3^3 design was followed. The influence of temperature (40, 60 and 80°C), pressure (at three levels between 10 and 30MPa in 10MPa increments) and modifier addition (0%, 5% and 10% ethanol) on the extraction yield and parthenolide content in the extracts of the different blooming stages and parts was studied. The plants raised from the seeds of the National Botanical Garden in Vácrátót were used for the extraction.

Two other independent variables, solvent flow rate (0.4 ml/min) and extraction time (1h) were kept constant.

27 experiments were performed for the Design of Experiment (DOE) and additional runs were performed to check the trueness and the reproducibility of the experiments and the model. Two additional runs were performed at the extraction optimum point, in case of the parthenolide content.

The mathematical relationship between the three significant independent variables was formulated by the general quadratic polynomial (Equation 1.).

$$Y=\beta_0+\beta_1X_P+\beta_2X_T+\beta_3X_{EtOH}+\beta_{11}X^2_P+\beta_{22}X^2_T+\beta_{33}X^2_{EtOH}+\beta_{12}X_P X_T+\beta_{13}X_PX_{EtOH}+\beta_{23}X_TX_{EtOH}, \qquad Eq.1$$

where Y is the response, β_0 is constant, β_1 (EtOH), β_2 (P) and β_3 (T) are the linear, β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the crossed coefficients. For the statistical analyses of the results Statistica software was used. The goodness of fit of the regression models was evaluated by using the coefficients of determination, R^2 and their adjusted values. To assess the significant factors and interactions, analyses of variance was utilized.

4.3.3 Conventional extraction methods

4.3.3.1 Soxhlet extraction

Soxhlet extraction was performed using laboratory-scale apparatus. The plants raised from the seeds of the National Botanical Garden in Vácrátót were used for the extraction. Dried and milled plant samples (10.0 g each) were extracted with chloroform at 60ºC and methanol at 90ºC, each for 4 hours. The extracts were evaporated to dryness under reduced pressure at 50ºC, then dissolved in supergradient grade acetonitrile (3.0 ml) and filtered through a Phenex RC 15 mm, 0.2 μm syringe filter (Gen-Lab Ltd., Budapest, Hungary).

4.3.3.2 Ultrasonic extraction

Ultrasound assisted extraction was carried out on the fresh and dried leaves collected before flowering, leaves collected during flowering and flower heads (10.0 g each). The plants raised from the seeds of the National Botanical Garden in Vácrátót were used for the extraction.

The plant samples were extracted with chloroform at room temperature for 30 minutes. The extracts were evaporated to dryness under reduced pressure at 50ºC, then dissolved

in supergradient grade acetonitrile (3.0 ml) and filtered through a Phenex RC 15 mm, 0.2 μm syringe filter (Gen-Lab Ltd., Budapest, Hungary).

4.3.3.3 Steam distillation of *Tanacetum parthenium* L.

The determination of essential oil in *Tanacetum parthenium* L. was carried out by steam distillation in a special apparatus described in the Pharmacopoeia Hungarica VII. (Ph. Hg. VII.) [1987]. The essential oils were collected in a graduated tube and the aqueous phase was returned to the distillation flask. The apparatus comprises of a 500 ml round-bottom flask, a condenser assembly which closely fits the flask and a heating device. 40.0 g drugs and 250 ml of water and a few pieces of porous pumice stone were introduced to the flask. Water was introduced through the filling tunnel. The mixture of the drug and water in the flask was heated till boiling and the distillation rate was adjusted to 2-3 ml/min. The quantity of the essential oil was determined after 3 hours of distillation.
The essential oil was collected in a previously calibrated vessel. Both volumetric and gravimetric methods were applied to determine the quantity.
The density was calculated from the weight of the volume in the calibrated vessel according to Equation 2.,

$$\rho = \frac{m}{V}, \qquad Eq.2.$$

,where ρ is the density, m is the weight in grams and V is the volume in ml of the essential oil.

4.4 Chromatographic methods

4.4.1 Quantitative analyses by Ultra Performance Convergence Chromatography

Quantification of parthenolide and camphor in the feverfew supercritical fluid extracts was performed by the external standard method. The chromatographic analysis was performed using a Waters ACQUITY Ultra Performance Convergence Chromatography (UPC²) system, equipped with a binary solvent delivery pump, an auto-sampler, a column oven, a diode array detector and a back-pressure regulator.

4.4.1.1 Determination of parthenolide

The quantitative analysis of parthenolide was performed using an Acquity UPC² BEH C18 column (100mmx3mm, 1.7 µm, Waters, MA, USA). The eluents were supercritical carbon dioxide (A) and acetonitrile (B). An isocratic program was applied: 0-5 min. 18% B with a flow rate of 2 ml/min. The column temperature was 45°C and the detection wavelength was 210 nm during the analysis, UV spectra were recorded from 200 nm to 400 nm. The back pressure was set to 2000 psi. Empower 3 software was used for the data processing.

The UPC^2 method was in accordance with International Conference on Harmonisation guidelines [ICH Expert Working Group 1996]. The parthenolide standard was dissolved in acetonitrile to yield stock solutions containing 0.0313-0.5000 mg/ml reference substance. Standard solutions at six concentrations were analyzed in triplicate to establish calibration plots. Linearity was determined by linear regression analysis of mean peak area against the corresponding concentration. Limit of detection (LOD) and limit of quantitation (LOQ) values of the method were determined as the ratio between the parthenolide peak height and the baseline noise. The repeatability was calculated according to 9 determinations (3 concentrations/3 replicates) covering the range of the procedure. The intermediate precision was determined in inter-day evaluations (low, mid and high concentration of the standard in three parallel runs on three successive days).

4.4.1.2 Determination of camphor content

The quantitative determination of camphor in the extracts was performed using an Acquity UPC² BEH-2EP column (100mmx3mm, 1.7 µm, Waters, MA, USA). The eluents were supercritical carbon dioxide (A) and isopropanol (B). A gradient program was applied 0-10 min 0% →10% B with a flow rate of 2 ml/min. The column temperature was 50°C and the detection wavelength was 290 nm during the analysis, UV spectra were recorded from 200 nm to 400 nm with a resolution of 2.4 nm, the sampling rate was 20 points/min The back pressure was set to 2000 psi. Empower 3 software was used for the data processing.

A detailed, statistically evaluated method validation was performed on the camphor content of the extracts. The UPC^2 method was validated in accordance with the ICH for the undermentioned parameters:

- specificity
- accuracy
- precision
    - repeatability
    - intermediate precision
- linearity
- range
- limit of detection (LOD),
- limit of quantification (LOQ),
- robustness

Selectivity

Selectivity is the ability to assess the analyte in the presence of components that may be expected to be present. During the procedure a blind sample, a standard solution and a fortified essential oil sample of *Tanacetum parthenium* L. was injected. Each solution was injected once. The UV spectra from 210 to 400 nm were recorded in order to confirm peak purity. The threshold of the peak purity factor was 95.0%.

Accuracy

The accuracy was established covering the specified range for the procedure. Three concentration levels (0.125, 0.500, 1.000 mg/ml) were injected three times.

The accuracy was reported as percent recovery which is calculated as described in Equation 3.:

$$Recovery\ (\%) = \frac{c_{measured}}{c_{real}} \cdot 100, \quad Eq.3.$$

where $c_{measured}$ is the measured concentration and c_{real} is the calculated concentration. The measured concentrations were calculated from the linear regression equation calculated during the linearity investigation.

Precision

The precision tests for the method included the determination of the repeatability and intermediate precision. During the repeatability determinations the precision of the

system and the method was investigated. The measurements were done in short time interval (one day) under the same operating conditions. The system precision was determined by assaying aliquots of one concentration in nine replicates. For the standard solution 1.000 mg of camphor standard was dissolved in 2.000 ml isopropanol. System repeatability was expressed as the standard deviation and relative standard deviation of the standard peak area with the confidence intervals. The method precision was assessed with five concentrations (0.125, 0.250, 0.500, 0.750, 1.000 mg/ml). Each sample was injected in three replicates. All five concentrations were weighted separately. Method repeatability was expressed as the relative standard deviation of the measured concentration. Inter-day precision of the method was evaluated by measuring freshly prepared standard solutions in triplicate on three consecutive days. One concentration (0.500 mg/ml) was injected in six replicates. The intermediate precision was expressed as the relative standard deviation of the measured concentration.

Linearity

Linearity was evaluated across the range of the analytical procedure. Standard solutions were analyzed in the range of 0.125 mg/ml and 1.000 mg/ml. Camphor standard solutions were prepared in five concentrations (0.125, 0.250, 0.500, 0.750, 1.000 mg/ml). All five concentrations were prepared separately.

The relationship between the peak areas and the concentrations was evaluated with linear regression analysis by the method of least squares. The correlation coefficient, y-intercept, slope of the regression line and a plot of the data was submitted.

Detection limit

The determination of the limit of detection (LoD) was based on the calibration curve and was expressed as:

$$LoD = \frac{3.3 \cdot \sigma}{S}, \qquad Eq.4,$$

where σ is the standard deviation of y-intercepts of the regression line and S is the slope of the calibration curve.

Quantitation limit

The determination of the limit of quantitation (LoQ) was based on the calibration curve and was expressed as:

$$LoQ = \frac{10 \cdot \sigma}{S}, \qquad Eq.5,$$

where σ is the standard deviation of y-intercepts of the regression line and S is the slope of the calibration curve

Robustness

In convergence chromatography the most crucial parameters are the system pressure, the system temperature and the flow rate because these ensure the supercritical conditions of the carbon dioxide. The variations of the parameters are described in Table 7. The robustness was determined by assaying aliquots of one concentration (0.500 mg/ml) in three replicates. The robustness was expressed as the relative standard deviation of the measured concentration and the retention time of the standard peak.

Table 7.: Variation of the parameters for the determination of robustness

Chromatographic parameters	Default value of method parameter	Variation in method parameter	New value of method parameter
System pressure	2000 psi	-50 psi	1950 psi
		+50 psi	2050 psi
System temperature	50°C	-1°C	49°C
		+1°C	51°C
Flow rate	2.50 ml/min	-0.02 ml/min	2.48 ml/min
		+0.02 ml/min	2.52 l/min

For the statistical analysis of the results the Statistica software [StatSoft, Inc. 2014] was used.

4.4.2 SemiPrep-HPLC

SemiPrep-HPLC was carried out on a Hanbon AS20005 semi-preparative HPLC system consisting of a NP7005C preparative pump, a NU3000C UV detector, a preparative injector and a high pressure gradient mixer for the isolation of flavonoids. The samples were separated on a Phenomenex Luna C18 column (150 x 10.0 mm, 5µm), the UV detection occurred at 254 nm. The following gradient elution program was applied,

where eluent A was 0.1 % (v/v) trifluoroacetic acid in water, eluent B was 0.1 % (v/v) trifluoroacetic acid in (acetonitrile: water = 95: 5):

- 0 min: 30 % (v/v) B;
- 30 min: 100 % (v/v) B;
- 35 min: 100 % (v/v) B.

4.4.3 Characterization of phenolics in the *Tanacetum parthenium L.* supercritical fluid extracts by HPLC-DAD-MS/MS

The HPLC-DAD-MS/MS experiments were performed on an Agilent 1200 HPLC system (G1379B degasser, G1312B binary gradient pump, G1367C autosampler, G1316B column thermostat and G1315C diode array detector) coupled with an Agilent 6410 triple quadrupole mass spectrometer equipped with an ESI ion source (Agilent Technologies, Waldbronn, Germany). The Masshunter B.04.01 software was used for qualitative analyses.

The samples were separated on a Kinetex-XB C18 column (150×4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) maintained at 45°C. The injection volume was 30 μL. The following gradient elution program was applied at a flow rate of 1.0 mL/min; where eluent A was 0.1 % (v/v) trifluoroacetic acid in water, eluent B was 0.1 % (v/v) trifluoroacetic acid in (acetonitrile: water = 95: 5):

- 0 min: 5 % (v/v) B;
- 20 min: 28 % (v/v) B;
- 40 min: 80 % (v/v) B;
- 55 min: 100 % (v/v) B; 65 min: 100 % (v/v) B.

Chromatograms were acquired at 210±5 nm and 254±5 nm.

Triple quadrupole mass spectrometric parameters were as follows:

- Ion source: ESI, positive and negative ionization mode,
- Drying gas (N_2) temperature: 350 °C;
- Drying gas (N_2) flow rate: 13 l/min;
- Nebulizer gas (N_2) pressure: 40 psi;
- Fragmentor voltage: 135 V;
- Capillary voltage: 4000 V.

Full-scan mass spectra were acquired over the *m/z* range of 50-1000 (1 scan/sec). Collision energy was changed between 10–50 eV, according to differences in molecule structures (high purity N_2 was used as collision gas).

For structural characterization of the compounds retention times, molecular masses and calculated molecular formulas, UV and mass spectral data were compared to literature data and to those of authentic standards, where available.

4.4.4 Solid Phase Micro Extraction (SPME)

Air-dried plant material (0.5 g) or 50 µl essential oil was put into vials (20 ml headspace) sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 µM StableFlex polydimethyl siloxane/divinyl benzene (PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100ºC, extraction was performed by exposing the fiber to the headspace of the 20 mL vial containing the sample for 10 min at 100ºC. The fiber was then immediately transferred to the injector part of the GC/MS, and desorbed for 1 min at 250ºC. The SPME fiber was cleaned and conditioned in a Fiber Bakeout Station in pure nitrogen atmosphere at 250ºC for 15 min after desorption.

4.4.5 Gas Chromatography coupled with Mass Spectrometry (GC-MS)

The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent HP-5MS capillary column (30mx250µmx0.25µm). The GC oven temperature was programmed to increase from 60ºC (3 min isothermal) to 200ºC at 8ºC/min (2 min isothermal), from 200–230ºC at 10ºC/min (5 min isothermal) and finally from 230–250ºC at 10 ºC/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. The injector temperature was 250°C and the split ratio was 1:50. The analyses were conducted using a mass selective detector operated in electron

ionization mode at 70 eV, full scan mode coupled with a quadrupole mass analyzer (41–500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the NIST 05 library.

4.5 Nuclear Magnetic Resonance (NMR) spectroscopy

All NMR experiments were carried out on a 600 MHz Varian DDR NMR spectrometer equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in VnmrJ 3.2 C/Chempack 5.1 were used for structure identifications. The complete resonance assignments were established from direct $^{1}H–^{13}C$, long-range $^{1}H–^{13}C$, and scalar spin–spin connectivities derived from 1D ^{1}H, ^{13}C, 1D TOCSY, $^{1}H–^{1}H$ gCOSY, zTOCSY experiments, respectively. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. The ^{1}H chemical shifts were referenced to the applied NMR solvents CD_3OD (δ (CD_2HOD) = 3.310 ppm) and ^{13}C chemical shifts were referenced to 49.00 ppm while in $CDCl_3$ the ^{1}H chemical shifts were referenced to (δ H = 7.260 ppm) and ^{13}C chemical shifts to 77.160 ppm, respectively.

4.6 Blood-Brain Barrier Specific Artificial Membrane Permeability Assay (PAMPA-BBB)

The solutions of the *Tanacetum* extract and the individual compounds were prepared with dimethyl sulfoxide (DMSO) at concentrations of 50.0 mg/mL and 10.0 mM, respectively. These were diluted with PBS (Phosphate Buffered Saline; pH = 7.4) to obtain the donor solutions (295.0 μL buffer+5.0 μL DMSO solution), then shaken for an hour at room temperature in a 96-well polypropylene plate (Agilent, Waldbronn, Germany), and filtrated after (Vacuum Manifold, Millipore). A parallel artificial membrane permeability assay (PAMPA) system was used to determine the effective permeability (P_e) for the compounds of interest (Figure 15.).

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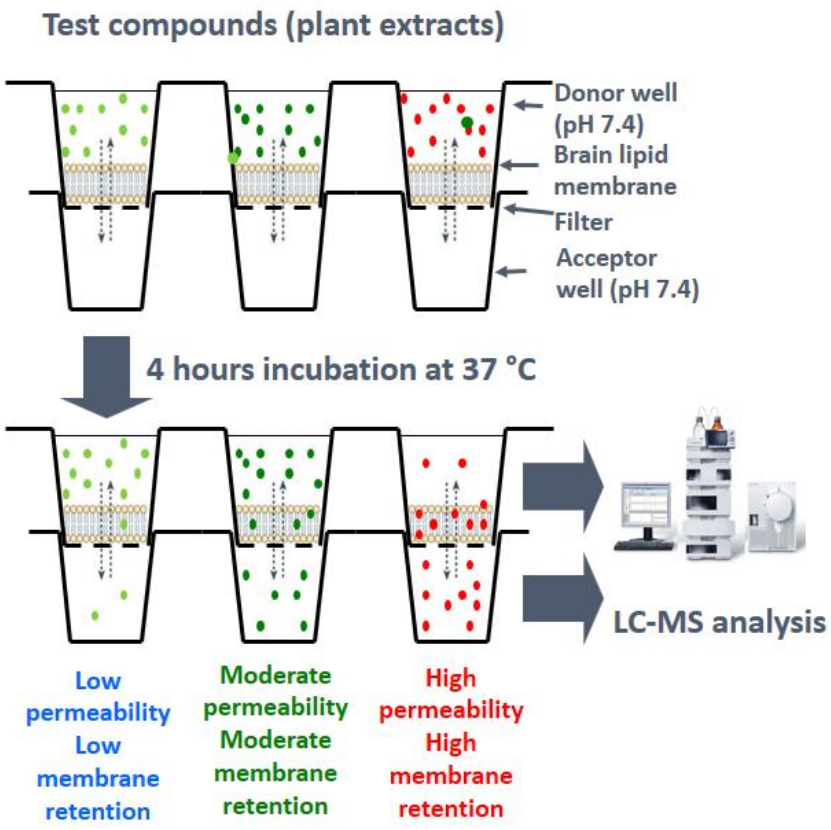

Figure 15. The PAMPA system and the principles of operation (Könczöl 2014)

Each well of the top plate (96-well polycarbonate based filter donor plates (Multiscreen™-IP, MAIPN4510, pore size 0.45 μm; Millipore)) was coated with 5 μL of porcine polar brain lipid extract (PBLE) solution (16.0 mg PBLE + 8.0 mg cholesterol dissolved in 600.0 μL *n*-dodecane), then 150.0 μL of the filtrate was placed on the membrane. The bottom plate (96-well polytetrafluoroethylene (PTFE) acceptor plates (Multiscreen Acceptor Plate, MSSACCEPTOR; Millipore)), was filled with 300.0 μL buffer solution (0.01 M PBS buffer, pH= 7.4). The donor and acceptor plates were fit, and then the sandwich system was incubated at 37 °C for 4 hours in a Heidolph Titramax 1000. After the incubation the PAMPA plates were separated and the compound concentrations in the donor ($C_{D(t)}$) and acceptor ($C_{A(t)}$) solutions, as well as in the donor solution at zero time point ($C_{D(0)}$) were determined by HPLC-DAD. At iso-pH conditions, the effective permeability and the membrane retention of drugs were calculated by the following equation [Avdeef et al 2012]:

$$P_e = \frac{-2.303}{A(t-\tau_{SS})} \cdot \left(\frac{V_A \cdot V_D}{V_A + V_D}\right) \cdot lg\left[1 - \left(\frac{V_A + V_D}{(1-MR)\cdot V_D}\right) \times \left(\frac{C_A(t)}{C_D(0)}\right)\right], \quad Eq.6$$

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where P_e is the effective permeability coefficient (cm/s), A is the filter area (0.24 cm^2), V_D and V_A are the volumes in the donor (0.15 cm^3) and acceptor phases (0.30 cm^3), t is the incubation time (s), τ_{SS} is the time (s) to reach steady-state (240s), $C_{D(t)}$ is the concentration of the compound in the donor phase at time t, $C_{D(0)}$ is the concentration of the compound in the donor phase at time 0, and MR is the estimated membrane retention factor (the estimated mole fraction of solute lost to the membrane) (Equation 7.):

$$MR = 1 - \frac{C_D(t)}{C_D(0)} - \frac{V_A}{V_D}\frac{C_A(t)}{C_D(0)}\ , \qquad Eq.7$$

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5 RESULTS

5.1 Supercritical fluid and conventional extraction methods. Quantitative determination of parthenolide in the extracts

5.1.1 Design of experiment for SFE

The aim of our work was to develop a supercritical fluid extraction method in order to maximize the parthenolide content of *T. parthenium* extracts. Leaves collected before flowering, leaves collected during flowering and flower heads were studied based on a full factorial 3^3 experimental design. The total extraction yield and parthenolide content of the 3^3 SFE experiments of the three samples are shown in Table 8. Accordingly, there are 27 experiments for the DOE and 6 for the validation of the model. These additional runs showed, that the extraction was accurate and reproducible. The total extraction yield in the case of all three samples reached the lowest value in run 1 (10MPa, 40°C, 0% EtOH) and the highest value in run 31 (30MPa, 80°C, 10% EtOH), while the parthenolide content of samples led to a minimum value in run 14 (30MPa, 40°C, 5%EtOH) and a maximum value in run 5 (20MPa, 60°C, 0%EtOH).

Polynomial regressions were fitted to investigate the main factors influencing the extraction yield and parthenolide content, and to evaluate their trends. The influencing effects coded and un-coded and the coefficients of the responses are presented in Table 9. In case of the extraction yield, the coefficients of determination have shown a value from 0.973 to 0.996, indicating good fitting of the model. The R^2 coefficients of the parthenolide content showed a lower value (0.931-0.934).

The effects which were not significant at 95% confidence level were removed from the equations. The final, reduced equations and the regression coefficients are shown in Table 10. After the removal, the adjustable R^2 values raised, consequently the model didn't include any non-significant effect. All the individual factors (ethanol content, pressure, temperature) have a statistically significant positive or negative impact on the responses. The effect of each factor can be evaluated from the regression coefficients of the reduced experimental models.

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Table 8. Results of the SFE experimental design of *Tanacetum parthenium* L. samples

			Leaves before flowering			Leaves during flowering			Flower heads		
EtOH	**Pressure**	**Temperature**	**Exp.**	**Extraction yield (g/100g)**	**Parthenolide (g/100g)**	**Exp.**	**Extraction yield (g/100g)**	**Parthenolide (g/100g)**	**Exp.**	**Extraction yield (g/100g)**	**Parthenolide (g/100g)**
0%	10MPa	40°C	1	1.926	0.433	1	1.292	0.407	1	1.889	0.476
0%	20MPa	40°C	2	2.039	0.444	2	1.624	0.416	2	1.996	0.489
0%	30MPa	40°C	3	2.121	0.415	3	2.286	0.389	3	2.142	0.456
0%	10MPa	60°C	4	2.119	0.448	4	1.844	0.421	4	1.985	0.494
0%	20MPa	60°C	5	2.379	0.549	5	2.022	0.515	5	2.235	0.604
0%	30MPa	60°C	6	2.724	0.504	6	2.488	0.473	6	2.278	0.554
0%	30MPa	80°C	7	2.515	0.442	7	2.284	0.414	7	2.138	0.485
0%	20MPa	80°C	8	3.226	0.462	8	2.384	0.433	8	2.412	0.509
0%	30MPa	80°C	9	3.412	0.475	9	2.724	0.445	9	2.497	0.523
0%	20MPa	60°C	10 valid	2.402	0.541	10 valid	2.050	0.508	10 valid	2.231	0.596
0%	20MPa	60°C	11 valid	2.368	0.553	11 valid	2.006	0.519	11 valid	2.240	0.603
5%	30MPa	40°C	12	2.174	0.344	12	1.504	0.323	12	2.142	0.376
5%	20MPa	40°C	13	2.291	0.352	13	1.782	0.330	13	2.260	0.388
5%	30MPa	40°C	14	2.402	0.334	14	2.554	0.317	14	2.343	0.373
5%	30MPa	60°C	15	2.390	0.359	15	2.082	0.340	15	2.246	0.398
5%	20MPa	60°C	16	2.710	0.412	16	2.310	0.387	16	2.518	0.455
5%	30MPa	60°C	17	3.089	0.391	17	2.804	0.367	17	2.579	0.431
5%	30MPa	80°C	18	2.712	0.352	18	2.578	0.330	18	2.415	0.388
5%	20MPa	80°C	19	3.652	0.375	19	2.750	0.351	19	2.701	0.413
5%	30MPa	80°C	20	3.751	0.382	20	3.084	0.359	20	2.811	0.422
5%	20MPa	60°C	21 valid	2.694	0.404	21 valid	2.344	0.379	21 valid	2.501	0.445
5%	20MPa	60°C	22 valid	2.726	0.421	22 valid	2.302	0.396	22 valid	2.529	0.464
10%	30MPa	40°C	23	2.370	0.354	23	1.590	0.332	23	2.325	0.389
10%	20MPa	40°C	24	2.505	0.365	24	1.950	0.341	24	2.460	0.397
10%	30MPa	40°C	25	2.610	0.345	25	2.820	0.325	25	2.580	0.381
10%	30MPa	60°C	26	2.610	0.368	26	2.280	0.346	26	2.445	0.407
10%	20MPa	60°C	27	2.940	0.423	27	2.490	0.397	27	2.745	0.465
10%	30MPa	60°C	28	3.360	0.397	28	3.060	0.371	28	2.805	0.435
10%	30MPa	80°C	29	2.970	0.371	29	2.820	0.347	29	2.625	0.408
10%	20MPa	80°C	30	3.975	0.391	30	2.940	0.367	30	2.940	0.431
10%	30MPa	80°C	31	4.080	0.395	31	3.360	0.370	31	3.075	0.435
10%	20MPa	60°C	32 valid	3.010	0.411	32 valid	2.502	0.385	32 valid	2.751	0.451
10%	20MPa	60°C	33 valid	2.890	0.421	33 valid	2.486	0.396	33 valid	2.739	0.464

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Table 9. Regression coefficients of the quadratic model, their significance at 95% confidence level, and the determination coefficients for the full model

	Leaves before flowering				Leaves during flowering				Flower heads			
	Extraction yield		**Parthenolide**		**Extraction yield**		**Parthenolide**		**Extraction yield**		**Parthenolide**	
	Effect	**p**	**Effect**	**p**	**Effect**	**p**	**Effect**	**p**	**Effect**	**p**	**Effect**	**p**
β_0	2.769345	<0.001	0.404526	<0.001	2.361099	<0.001	0.379719	<0.001	2.432023	<0.001	0.445351	<0.001
(β_1) EtOH (%)(L)	0.553545	<0.001	-0.093273	<0.001	0.481273	<0.001	-0.087545	<0.001	0.495182	<0.001	-0.102364	<0.001
(β_{11}) EtOH (%)(Q)	0.028682	0.083	-0.057000	<0.001	0.040273	0.001	-0.052682	<0.001	0.025318	<0.001	-0.061182	<0.001
(β_2) Pressure (MPa)(L)	0.640333	<0.001	0.018611	0.001	0.767333	<0.001	0.017333	0.002	0.322222	<0.001	0.021000	0.001
(β_{22}) Pressure (MPa)(Q)	0.085535	<0.001	0.028829	<0.001	-0.159035	<0.001	0.026491	<0.001	0.076070	<0.001	0.030886	<0.001
(β_3) Temp. (°C)(L)	1.095000	<0.001	0.028833	<0.001	0.835778	<0.001	0.026222	<0.001	0.386333	<0.001	0.032111	<0.001
(β_{33}) Temp. (°C)(Q)	-0.147132	<0.001	0.041829	<0.001	0.028965	0.005	0.039825	<0.001	0.004237	0.252	0.046219	<0.001
(β_{12}) 1L by 2L	0.067167	0.020	-0.004420	0.322	0.078667	<0.001	-0.004000	0.361	0.026667	0.002	-0.005167	0.279
(β_{13}) 1L by 3L	0.078833	0.011	0.001083	0.800	0.095000	<0.001	0.001000	0.813	0.042500	<0.001	0.001833	0.688
(β_{23}) 2L by 3L	0.397167	<0.001	0.020667	0.002	-0.298000	<0.001	0.019000	0.003	0.082667	<0.001	0.021667	0.003
R^2	0.973		0.932		0.996		0.931		0.993		0.934	
$R^2_{adj.}$	0.962		0.905		0.994		0.904		0.989		0.909	

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Table 10. The reduced equation fitted on the results of the experimental models

	Flowering period and response	Reduced equation	R^2	$R^2_{adj.}$
Leaves before flowering	Extraction yield (g/100g)	$Y = 2.518075 + 0.029744EtOH + 0.003297P - 0.038594T - 0.000855P^2 + 0.000368\ T^2 + 0.000672P*EtOH + 0.000394T*EtOH + 0.000993P*T$	0.972	0.963
	Parthenolide (g/100g)	$Y = 0.025631 – 0.032127EtOH + 0.009362P + 0.012236T + 0.002280EtOH^2 – 0.000288P^2 – 0.000105T^2 + 0.000052P*T$	0.931	0.912
Leaves during flowering	Extraction yield (g/100g)	$Y = -0.298052 + 0.020003EtOH + 0.015519P + 0.042109T – 0.001611EtOH^2 + 0.001590P^2 – 0.000072T^2 + 0.000787P*EtOH + 0.000475T*EtOH – 0.000745P*T$	0.996	0.994
	Parthenolide (g/100g)	$Y = 0.021211 – 0.029827EtOH +0.008613P + 0.011653T + 0.002107EtOH^2 - 0.000265P^2 – 0.000100T^2 + 0.000047P*T$	0.930	0.911
Flower heads	Extraction yield (g/100g)	$Y = 1.355731 + 0.041562EtOH + 0.033258P + 0.004463T – 0.001013EtOH^2 – 0.000772P^2 + 0.000267P*EtOH + 0.000212T*EtOH – 0.000207P*T$	0.993	0.990
	Parthenolide (g/100g)	$Y = 0.024646 – 0.034709EtOH + 0.010154P + 0.013585T + 0.002447EtOH^2 - 0.000309P^2 – 0.000116T^2 + 0.000054P*T$	0.934	0.916

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5.1.2 Soxhlet extraction

Soxhlet extraction was carried out on leaves collected before flowering, leaves collected during flowering and flower heads using chloroform and methanol as medium. The total extraction yield and the parthenolide content of the samples are shown in Table 11.

Table 11. Total extraction yield and parthenolide content of the feverfew Soxhlet extracts

	Flowering period and response	Chloroform extract	Methanolic extract
Leaves before flowering	Extraction yield (g/100g)	9.41	7.32
	Parthenolide (g/100g)	0.321	0.251
Leaves during flowering	Extraction yield (g/100g)	6.02	4.23
	Parthenolide (g/100g)	0.298	0.234
Flower heads	Extraction yield (g/100g)	7.42	5.39
	Parthenolide (g/100g)	0.348	0.306

5.1.3 Ultrasound assisted extraction of the fresh and dried samples of feverfew

Fresh and dried leaves collected before flowering, leaves collected during flowering and flower heads were extracted with chloroform at room temperature with ultrasonic bath. The samples were not grinded considering the fact, that based on previous literature data, most of the parthenolide content of the plant is synthetized and localized in the glandular

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thrichomes [Majdi et al 2011]. The total extraction yield and parthenolide content of the samples are presented in Table 12.

Table 12. Total extraction yield and parthenolide content of the fresh and dried feverfew sample extracted with ultrasound assisted extraction

	Flowering period and response	Fresh samples	Dried samples
Leaves before flowering	Extraction yield (g/100g)	4.097	5.057
	Parthenolide (g/100g)	0.358	0.262
Leaves during flowering	Extraction yield (g/100g)	3.294	4.087
	Parthenolide (g/100g)	0.267	0.221
Flower heads	Extraction yield (g/100g)	3.574	3.602
	Parthenolide (g/100g)	0.367	0.309

5.1.4 Quantitative analyses of parthenolide by Ultra Performance Convergence Chromatography

A convergence chromatographic method was developed to determine the parthenolide content of the supercritical fluid and the Soxhlet extracts of *Tanacetum parthenium* L. The target compound was identified by comparison of its retention time and UV spectrum to authentic standard.

Calibration and linearity

Parthenolide standard solutions were prepared as described earlier to determine the linearity of UPC2 response. Standard solutions were analyzed between the range of 0.0313 mg/ml and 0.5000 mg/ml. The relationship between the peak areas and the concentrations was evaluated with linear regression analysis (y=271.08x+0.917) for parthenolide. The obtained correlation coefficient of the determination (R^2=0.99997) indicated high linearity over the investigated concentration range. The statistical significance of the intercept was calculated by Student's t-test. The calculated test

statistic for the intercept was 0.302, which was not greater than the $t_{0.05}$ value (3.038), thus the absence of interferences was confirmed. The correlation coefficient, slope and y-intercept of the calibration curve are shown in Table 13.

Sensitivity

The limit of detection and the limit of quantitation were determined as LOD= 3*S/N and LOQ= 10*S/N. In case of the LOD this value was 3.677 µg/ml and it was found to be 11.142 µg/ml for LOQ, which values are given in Table 13.

Table 13. Calibration, linearity and sensitivity of the developed convergence chromatographic method

Regression equation (y=ax+b)		**R^2**	**Linear range (µg/ml)**	**R_t repeatability n=6**	**LOD (µg/ml)**	**LOQ (µg/ml)**
a (slope)	**b (intercept)**					
271.080	0.917	0.99997	31.25-500	RSD%=0.075	3.677	11.142

Precision

Inter-day precision of the method was evaluated by measuring freshly prepared standard solutions in triplicate on three consecutive days. Intra-day precision was evaluated at three different concentrations of the freshly prepared standard solutions in triplicate during the same day. Results were expressed as relative standard deviation (RSD%) (Table 14.). The RSD% for the repeatability ranged from 0.044% to 0.062% and the intermediate precision ranged from 0.052% to 0.063%.

Table 14. Precision of the developed convergence chromatographic method

Nominal concentration (mg/ml)	**Intra-day**		**Inter-day**	
	Accuracy (%)	**RSD (%)**	**Accuracy (%)**	**RSD (%)**
0.5000	99.97	0.047	98.87	0.054
0.2500	101.22	0.044	100.89	0.052
0.1250	101.03	0.062	100.96	0.063

Recovery

The recovery of the method was determined by adding known amounts of parthenolide standard in a known *T. parthenium* extract at three concentrations. The recovery ranged from 94.77% to 100.72% and RSD% values were less than 0.20 % (Table 15.).

Table 15. Recovery of the developed convergence chromatographic method

Parthenolide content (mg/ml)	Added amount (mg/ml)	Theoretical amount (mg/ml)	Recorded amount (mg/ml)	Recovery (%)	RSD (%)
0.147	0.0625	0.2095	0.2110	100.72	0.121
0.147	0.1250	0.2720	0.2650	97.43	0.175
0.147	0.1875	0.3345	0.3170	94.77	0.142

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5.2 Characterization of sesquiterpene lactones and phenolics in the supercritical fluid extracts of feverfew

5.2.1 HPLC-DAD-MS/MS

The characteristic UV and MS spectra indicated thirteen compounds with flavonoid structure in the *Tanacetum* extract beside other constituents. For the characterization of these, UV spectral data obtained by LC-DAD and fragmentation pattern acquired by collision-induced dissociation (CID) in ESI-MS/MS analyses were compared to those of authentic standards and to literature data [Ablajan et al 2013, Cuyckens et al 2004, Fabre et al 2001]. In the case of the compounds with matching standards, identification was performed also by spiking the sample solutions with the standards in two different chromatographic systems. Although, the applied MS/MS method was not suitable for the accurate identification of the molecules where no matching standards were available, conclusions about the hydroxylation and methylation level of the flavonoid aglycones could have been drawn and some other constituents could have been tentatively identified. The LC-DAD experiments were performed on 254±5 nm (Figure 16.). During the LC-MS/MS product ion analyses of the flavonoid and other constituents ESI was operated in negative and positive ion mode. The identified compounds are listed in Table 17.

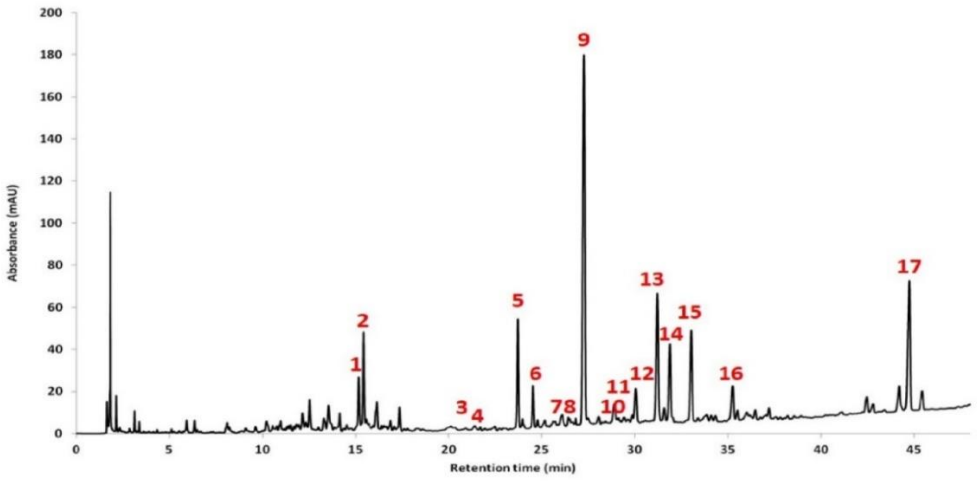

Figure 16. HPLC-DAD chromatogram of the supercritical fluid extract of *Tanacetum parthenium* L. leaves at 254 nm

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Table 16. Sesquiterpene lactones and flavonoids detected by LC-ESI-MS/MS in supercritical fluid extract of *Tanacetum parthenium* L.

	Rt	Molecular ion	Fragment ions	Tentative identification	Ion mode
1	15.2	285	241, 201, 175, 151, 133, 121	**Luteolin**	negative
2	15.4	269	225, 201, 181, 151, 117, 107	**Apigenin**	negative
3	22.3	315	300, 283, 271, 255, 227, 151, 135, 107	**Methylquercetin**	negative
4	23.1	315	300, 271, 255, 243, 227, 201, 199, 163, 137	**Methylquercetin**	negative
5	23.7	345	330, 315, 287, 243, 175, 149	**Axillarin**	negative
6	24.5	299	284, 163, 151, 107	**Trihydroxy-methoxyflavone**	negative
7	24.8	249	231, 213, 203, 185, 175, 161, 145, 133, 119, 105, 95, 85	**Costunolide**	positive
8	25.6	307	289, 235, 211, 185, 121, 97, 65	**Dihydro-β-cyclopyrethrosin**	negative
9	26.4	329	314, 299, 271, 243, 227, 215	**Dimethylquercetin**	negative
10	26.9	359	334, 329, 314, 301, 286, 270, 258, 230, 202	**Trihydroxy-trimethoxyflavone**	negative
11/12	27.3	359	334, 329, 314, 301, 286, 270, 258, 230, 202	**Sudachitin/Acerosin**	negative
13	28.2	293	275, 235, 211, 183, 171	**Tanacetol A isomer**	negative
14	28.8	265 ($M+H_2O-H$)	249, 231, 213, 203, 195	**Parthenolide**	positive
15	30.1	373	358, 343, 328, 315, 300, 285, 272, 257, 179	**Casticin**	negative
16	31.2	313	298, 283, 269, 255	**Dihydroxy-dimethoxyflavone**	negative
17	31.2	343	328, 313, 298, 285, 270, 242	**Nevadensin**	negative
18	31.9	277	233	**Tanaphillin**	negative
19	33	279 (M+H2O-H)	261, 243, 207, 195, 173, 159, 149, 137, 123, 109, 95, 81, 67	**3-β-Hydroxyanhydroverlotorin**	negative
20	35.2	277	259, 221, 149, 135, 121, 107, 93, 81	**Seco-tanapartholide A**	positive
21	44.2	277	259, 221, 149, 135, 121, 107, 93, 81	**Seco-tanapartholide B**	positive
22	44.7	301	239, 227, 165, 133, 107	**Hispidulin**	positive
23	45.5	339	163	Unknown	negative

The flavonoid type components are marked with red, the terpenoid type components with green

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5.2.2 SemiPrep-HPLC of the supercritical fluid extracts and the NMR analysis of the fractions

During the semi-preparative HPLC eight major fractions were collected from the supercritical fluid extract of the *Tanacetum parthenium* L. leaves with SemiPrep-HPLC, which were separated into fifty-three minor fractions.

Compound 11/12 was found in the 4.3, compound 17 in the 7.5 minor fraction, respectively. Fraction 4.3 contained two isomeric compounds at low concentration (approx. 0.2 mg/600 uL each) in nearly equal amount. The NMR data confirmed the components as sudachitin, acerosin and nevadensin.

5.3 Blood-Brain Barrier Specific Parallel Artificial Membrane Permeability Assay (PAMPA-BBB)

5.3.1 PAMPA-BBB of the supercritical fluid extract

The preliminary results of the LC-MS analyses that indicated the presence of methylated flavonoid aglycones in the *T. parthenium* extract raised the question whether these compounds would contribute to the overall CNS activity of the plant. In the first instance, their capability of crossing the blood−brain barrier (BBB) should have been tested. The PAMPA-BBB model was chosen for this purpose, based on previous studies [Könczöl et al 2013] that had proven its feasibility for the investigation of the membrane permeability potential of compounds in complex plant matrices. The semipolar nature of the molecules in the extract of *Tanacetum parthenium* L. was guaranteed by the modified supercritical fluid. This lipophilic/hydrophilic quality of the compounds assured the possible penetration through the BBB with passive transport.

The model has been validated for a set of natural compounds in our previous study. We found that the arbitrary cutoff value of −6.0 for $\log P_e$ discriminated effectively between compounds possessing log BB (BB: brain/plasma ratio) values greater (considered as BBB+) and less than −0.5 (considered as BBB−) [Könczöl et al 2013].

The whole *T. parthenium* extract was subjected to the PAMPA-BBB analysis without purification or prefractionation. The results are listed in Table 17.

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5.3.2 PAMPA-BBB of the purified compounds

The identified and purified compounds were subjected to the PAMPA-BBB analysis also as individual compounds. The results are listed in Table 17.

Table 17. The identified components in *Tanacetum parthenium* supercritical fluid extract and their $logP_e$ values measured in the extract and as individual compounds (n=3)

Compound		LogP	$LogP_e$	
			in the extract	as individual cpd.
1	**luteolin**	1.4	-	-4.51±0.19
2	**apigenin**	1.7	-	-4.54±0.14
5	**axillarin**	2.5	-	-
11/12	**sudachitin/acerosin**	2.6/2.6	-4.56±0.12	-4.56±0.10
14	**parthenolide**	2.3	-4.27±0.17	-4.30±0.14
15	**casticin**	3.1	-	-4.60±0.13
17	**nevadensin**	2.9	-4.40±0.19	-4.92±0.04

5.4 Qualitative and quantitative analysis of the essential oil and supercritical fluid extracts of feverfew

5.4.1 Quantitative determination of essential oil in feverfew samples

Percentages of the essential oils extracted from *Tanacetum parthenium* L. samples are summarized in the Table 18. The density of the volatile oil was 0.93 g/ml.

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Table 18. Essential oil content of *Tanacetum parthenium* L. samples

Place of origin	Part of plant	Essential oil (g/100g drug)	Essential oil (ml/100g drug)
Botanical Garden of the University of Debrecen	Leaf	0.60	0.65
	Flower heads	0.35	0.37
Botanical Garden of Bonn	Leaf	0.59	0.63
	Flower heads	0.32	0.35
National Botanical Garden in Vácrátót	Leaf	0.69	0.75
	Flower heads	0.39	0.41

5.4.2 Qualitative analysis of the essential oil and supercritical fluid extracts of *Tanacetum parthenium* L.

The compounds in the essential oil samples and the supercritical fluid extract of the plant sample from the Botanical Garden in Vácrátót were analyzed with HS-SPME-GC/MS method (Table 19.). The identity of the constituents was confirmed by retention time and recorded spectra with the data of NIST 05 library. The percentage distribution was calculated as relative proportion of total peak area from the GC-FID chromatogram.

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Table 19. Identified compounds detected by GC-MS in the essential oil and the supercritical fluid extract of *Tanacetum parthenium* L. samples and their relative contents in %

Peak no.	Compound	Essential oil						SFE		
		Botanical Garden of the University of Debrecen		**Botanical Garden of Bonn**		**National Botanical Garden in Vácrátót**		**National Botanical Garden in Vácrátót**		
		Leaves	**Flower heads**	**Leaves**	**Flower heads**	**Leaves**	**Flower heads**	**Leaves before flowering**	**Leaves during flowering**	**Flower heads**
1	**α-Pinene**	1.32	1.421	1.46	1.392	1.452	1.421	-	-	-
2	**Camphene**	9.32	8.658	9.115	8.946	8.384	8.427	9.28	9.098	9.127
3	**p-Cymene**	2.432	2.076	2.187	2.038	2.197	2.156	6.298	6.372	6.47
4	**Limonene**	2.145	2.145	2.207	2.145	2.234	2.295	2.687	2.561	2.614
5	**γ-Terpinene**	1.976	2.104	2.112	2.087	2.145	2.543	3.197	3.284	3.182
6	**Camphor**	42.329	42.72	42.952	43.14	45.576	45.108	45.294	44.549	44.722
7	**Chrysanthenyl acetate**	34.178	32.769	33.498	32.587	30.29	30.128	31.301	32.153	32.028
8	**Bornyl acetate**	1.526	1.891	1.126	1.934	1.893	1.883	-	-	-
9	**β-Caryophyllene**	1.963	2.018	2.111	2.001	2.143	2.113	-	-	-
10	**β-Farnesene**	1.389	2.54	1.59	2.132	2.012	2.052	-	-	-
11	**Germacrene D**	1.422	1.658	1.642	1.598	1.674	1.874	1.943	1.983	1.857

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5.4.3 Quantitative analyses of camphor by Ultra Performance Convergence Chromatography

For the quantitative analysis of camphor in the essential oil and supercritical fluid extracts of feverfew an Ultra Performance Convergence Chromatographic method was developed and validated. The high toxicity of camphor made a detailed, precise validation of the method a necessity.

Selectivity

Selectivity was determined by examining the chromatograms of the pure solvent, the standard solution of camphor and a fortified sample (Figure 17.). In case of the pure solvent the complex peaks of the solvent were detected at the column dead time (t_0~0.200 min). The retention time of the standard peak was t_R ~0.785 min. On the sample chromatogram a peak was detected with the same retention time as the peak of the camphor standard. Between the camphor and the other constituents, a baseline separation was obtained. The spectrum from 210 to 400 nm was recorded and the peak purity was established. Based on the results above, the selectivity of the method was acceptable.

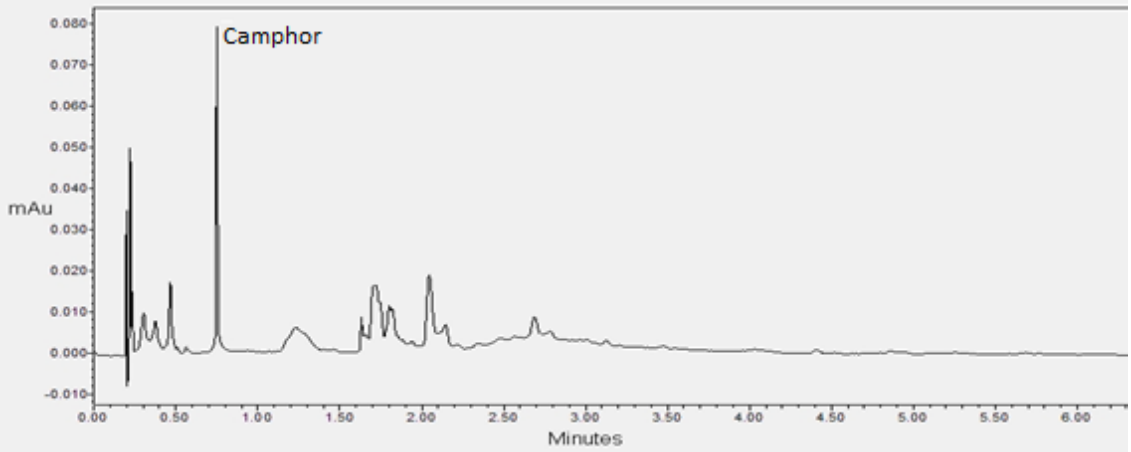

Figure 17. Chromatogram of the fortified *Tanacetum parthenium* L. essential oil sample

Accuracy

The accuracy of the method was studied at three concentration levels. The accuracy was between 95.0% and 105.0%. The average recovery was 100.2%. To evaluate the results

of the accuracy the measured concentration was plotted against the real calculated concentration. The obtained correlation coefficient was 1.000, consequently the two concentrations have a strong positive linear relationship, thus the accuracy of the method was established.

Precision

During the precision studies the system and method repeatability and the intermediate precision were studied.

System repeatability

The system repeatability was studied under the same operating conditions. The standard solution (0.5 mg/ml) was injected 9 times over a short interval of time. The variance between the theoretical peak area and the measured peak area was between -0.2 and 0.8. The relative standard deviation of the peak area was 0.28% and 0.34% for the retention time. Based on the results of the statistical tests the system precision was acceptable.

Method repeatability

During the method repeatability tests five concentrations (0.125, 0.250, 0.500, 0.750, 1.000 mg/ml) of the standard solution were injected three times. The measured concentrations were calculated from the equation of the calibration curve obtained during the linearity studies. The relative standard deviation (RSD%) of the measured concentrations was between 0.2% and 1.0%. The highest value was in the case of the 0.250 mg/ml standard solution. Based on the results of the statistical tests the method precision can be established.

Intermediate precision

Inter-day precision of the method was evaluated by measuring freshly prepared standard solutions six times on three consecutive days. The average recovery was 99.5% on day 1., 99.7% on day 2. and 99.6% on day 3. The highest RSD% values were observed in case of the measured concentration and recovery on day 2 (0.373% and 0.374%) and of the retention time on day 3 (0.852). Student's t-tests were performed to test the null hypothesis ($\boldsymbol{H_0}$) which says that there was no significant difference between the results obtained on the three different days. The null hypothesis could not be rejected because the *p* value was more than 0.05 in all three cases. Because of these results the intermediate precision of the method can be accepted.

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Linearity

The method's linearity was tested at five different concentrations in five replicates. The standard solutions were analyzed between the range of 0.125 mg/ml and 1.000 mg/ml. The relationship between the peak areas and the concentrations was evaluated with linear regression analysis. The equation of the fitted line is shown in Figure 18.

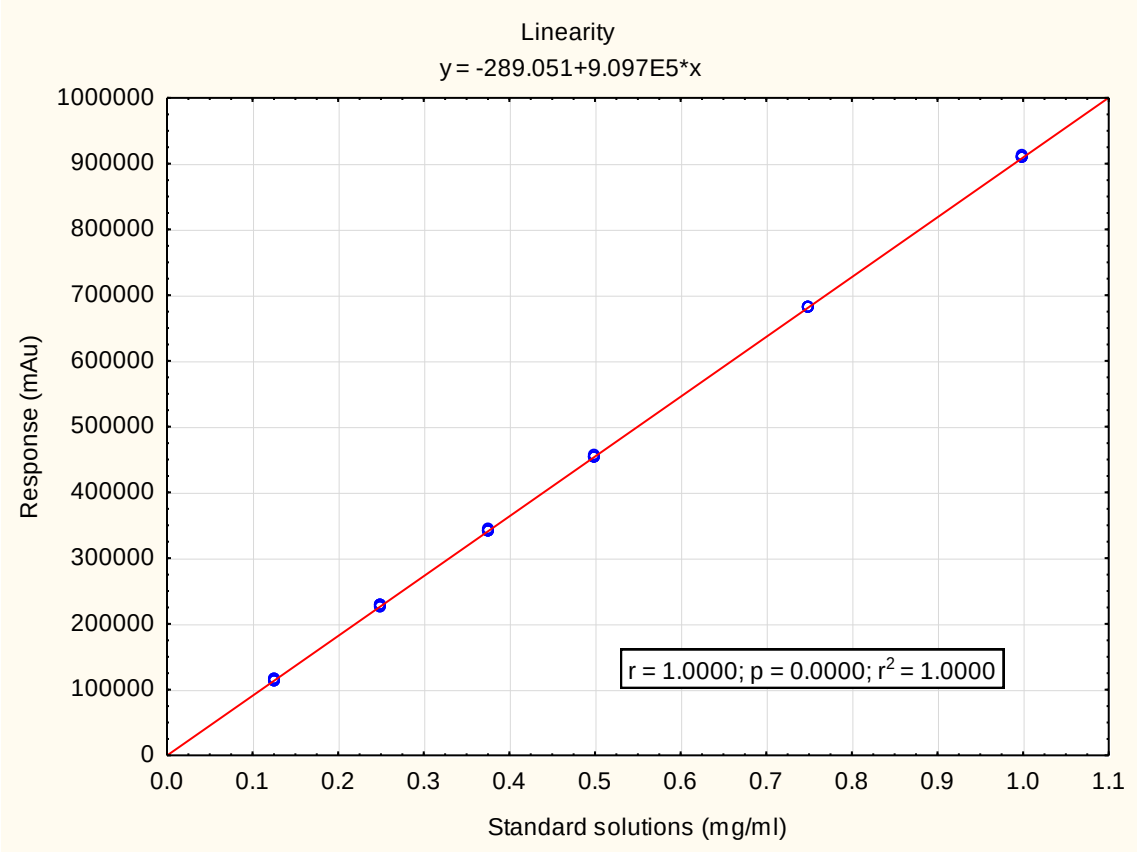

Figure 18. Fitted line, the equation of the line, and the value of r^2 in the linearity studies

The presentation of residuals against the concentration showed, that the distribution was random around the x axis and no trend could be observed. The regression analysis of the repeated measurements and the lack of fit were investigated with five different methods. Table 20. shows the estimated parameters, the y- intercept (-289.1) and the slope (909695.4), so the regression equation was obtained to be $y = 909695.4 * x - 289.1$.

The $\boldsymbol{H_0}$ in case of the Student's t-test was that the parameter value is 0. The $\boldsymbol{p}$ value shows the probability of type I error. The intercepts t-value was -0.954 and the p=0.348368. Because the *p* value was more than 0.05 and the confidence interval (-909.9

– 331.8) contained 0, it can be established that the intercept does not differ significantly from 0. The slopes t-value was 1746.488 and the p=0.0000. Because the p value was less than 0.05, the $\boldsymbol{H_0}$ (the real value of the slope is 0) could be rejected. From the results above it can be ascertained that there is a linear relationship between the response and the concentration.

Table 20. Estimated parameters in the linearity studies of camphor

Parameter Estimates Sigma-restricted parameterization						
Effect	**Response (mAu) Parameters**	**Response (mAu) Standard Error**	**Response (mAu) time**	**Response (mAu) pressure**	**-95% confidence limit**	**+95% confidence limit**
Intercept	-289.1	303.1	-0.954	0.348	-909.9	331.8
Standard solutions (mg/ml)	909695.4	520.8	1746.4	0.000	908628.5	910762.4

The correlation coefficient was 0.999 and the coefficient of determination was 0.999, so the regression line approximates the real data points well. The F statistic says with a p-value less than 0.05 that the $\boldsymbol{H_0}$ could be rejected – there is a significant relation between the dependent and independent variables in case of the fit of the statistical model.

The F statistic was also performed on the pure error. The p-value was less than 0.05 so the $\boldsymbol{H_0}$ can be rejected – there is a significant relation between the dependent and independent variables.

The F-statistics was performed on the variance of the lack of fit and the pure error and it was established that the fitted function is adequate so the lack of fit contained only the random error in addition to the repeated measurements.

A variance analysis of the significance for the model parameters (intercept, slope and error) was performed. The $\boldsymbol{H_0}$ says that the examined parameter does not have influence, so does not have to be included in the model. The p-value showed that the intercept had no significant influence on the model.

The regression assumptions were tested (independent ε_i errors, homogeneity of the variances) and it was proven that the residuals follow normal distribution. The residuals were plotted in order of the measurements and it was established that the ε_i errors were independent.

The homogeneity of the variances was tested with the Bartlett-test. The p-value was more than 0.05 so the H_0 could not be rejected – the variances are equal across the samples so the σ^2 = constant criteria verifies.

Detection limit

The limit of detection was determined as *LOD= 3.3*S/N*. The result was 2.055µg/ml so the UPC^2 method allows the detection of relatively small quantities of camphor.

Quantitation limit

The limit of quantitation was determined as *LOQ= 10*S/N*. The result was 6.228µg/ml so the UPC^2 method allows the quantitation of very small quantities of camphor.

Robustness

The robustness of the method was tested on the three critical parameters: the system pressure, system temperature and flow rate. Only one parameter was changed at a time.

Effect of pressure

The effect of the pressure was tested on two levels (1950psi, 2050psi). The relative standard deviations of the measured concentrations were 0.306% on 1950 psi and 0.232% on 2050 psi. A Student's t-tests were performed to test the null hypothesis (H_0) and it was established that there is no significant difference between the two levels, so the ±50 psi fluctuation of pressure does not redeem a significant variation in the measured concentration.

Effect of temperature

The effect of the temperature was tested on two levels. The relative standard deviations of the measured concentration were 0.232% on 49°C and 0.115% on 51°C.

The results of the Student's t-tests proved that there is no significant difference between the two levels, so the ±1°C fluctuation of temperature does not redeem a significant variation in the measured concentration.

Effect of flow rate

The effect of the flow rate was tested on two levels. The relative standard deviations in this case were 0.232% and 0.306% at a flow rate of 2.48 ml/min and 2.52 ml/min, respectively. The Student's t-test showed that ±0.02 ml/min fluctuation of the flow rate does not redeem a significant variation.

5.4.4 Camphor content of the essential oils and the supercritical fluid extracts from *Tanacetum parthenium* samples

The camphor content of the essential oils and the supercritical fluid extracts from *Tanacetum parthenium* L. leafs and flowers are given in Table 21.

Table 21. Essential oil and camphor content of *Tanacetum parthenium* L. samples

Place of origin	Part of plant	Camphor content (mg/100g drug)
Botanical Garden of the University of Debrecen	Leaf	178.20
	Flower heads	59.05
Botanical Garden of Bonn	Leaf	146.08
	Flower heads	47.49
National Botanical Garden in Vácrátót	Leaf	224.11
	Flower heads	73.16
Supercritical fluid extract	Leaves before flowering	137.23
	Leaves during flowering	95.38
	Flower head	36.82

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6 DISCUSSION

6.1 Comparison of supercritical fluid and conventional extraction methods of *Tanacetum parthenium* L.

Supercritical fluid extraction is a highly desirable method, because it is fast, environmental friendly and can be customized with the variation of the extraction parameters for the dissolution of specific extractable compounds. The plausibly bioavailable constituents of feverfew, like the amphiphilic parthenolide, its derivates and the lipophilic flavonoids have semipolar qualities. The preselection and enrichment of these foreseeably biologically active compounds can be done by using special extraction methods, like supercritical fluid extraction. Through the modification of the fluids polarity, the degree of the lipophilic character can be regulated. The variation in the operation parameters can influent the solvent power, enhance or decrease the selectivity. For the optimization of the extracting conditions, a systematic experimental plan was needed. Design of experiment was used to determine the effect of pressure, temperature and modifier rate on the extraction and to find cause-and-effect relationships between the extracting factors and the parthenolide content of the extracts. The results were evaluated with polynomial regression using a surface respond methodology. With these results the optimum point of the extraction and the effect of the factors could have been evaluated. In our work we developed a supercritical fluid extraction method, where *Tanacetum parthenium* L. leaves collected before flowering, leaves collected during flowering and flower heads were investigated. A full factorial 3^3 experimental design was followed to maximize the total extraction yield and the parthenolide content of the supercritical fluid extracts. Based on the statistical results, the models represented the experimental data sufficiently for all three samples. It was confirmed that all the individual factors had significant positive or negative effect, influencing the extraction. One of the main effecting component was the ethanol content of the fluid which is consistent with literature data [Marchand et al 1983]. The modification in the fluids polarity caused an increase in the extraction yield, enhancing the extraction of more polar components, however the larger solvent power also meant an increase for the parthenolide content of the extracts, decreasing the selectivity and the efficiency. So during the method optimization this drawback had to be considered, so the optimum point was not

determined on the dry plant material, but on the total extraction yield. It was confirmed, that the highest extraction yield was not reached in the examined range, but the parthenolide content achieved its maximum at 7% EtOH, 22MPa, 64°C as it is described in the following chapters.

6.1.1 Total extraction yield of the supercritical fluid extracts

The fitted response surfaces of the total extraction yield of the leaves collected before flowering, the leaves collected during flowering and the flower heads are shown in Figure 19., for 5% ethanol content, based upon the equations represented in Table 10. The determination coefficients were 0.972; 0.996 and 0.993, respectively, proving that the models represented the experimental data well. This ascertainment is also confirmed by the diagrams, where the predicted values are depicted against the observed values (Figure 19.). The points are in all cases near to the diagonal, which means that the equation describing the surface fits well. The R^2_{adj} did not differ significantly.

The Pareto chart of the effects showed, that all the individual factors have a significant effect on the extraction yield (Figure 19.). Regarding the leaves collected before and during flowering, the temperature had a higher influence on the extraction, than the pressure. Positive effect of pressure on the extraction is consistent with the increasing solubility of compounds by the increasing extraction pressure at constant temperature. At high pressures, the solubility of compounds increases by increasing temperature. This crossover effect, usually occurring from 20 to 35 MPa, is caused by the competing effects of the reduction in solvent density and the increase of the vapor pressure. The ethanol content of the supercritical fluid is also among the three most significant factors. The interaction between pressure and temperature and the quadratic effect of these two variables are also notable.

The flower heads differed from the other two samples, because the most significant factor was the ethanol content of the supercritical fluid. It is well known that the modifier changes the polarity of the supercritical solvent, thus solubility of both valuable and non-valuable co-extracts may increase [Smith et al 1992]. Pressure and temperature also have a significant positive effect. The positive effect of pressure and temperature interaction on the extraction was also noted on a larger scale equipment by Kery et al. [1999]. The

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optimum values of the models were also investigated. The extraction yield did not stabilize within the studied ranges, but reached a maximum value at 30 MPa, 80°C and 10% ethanol content.

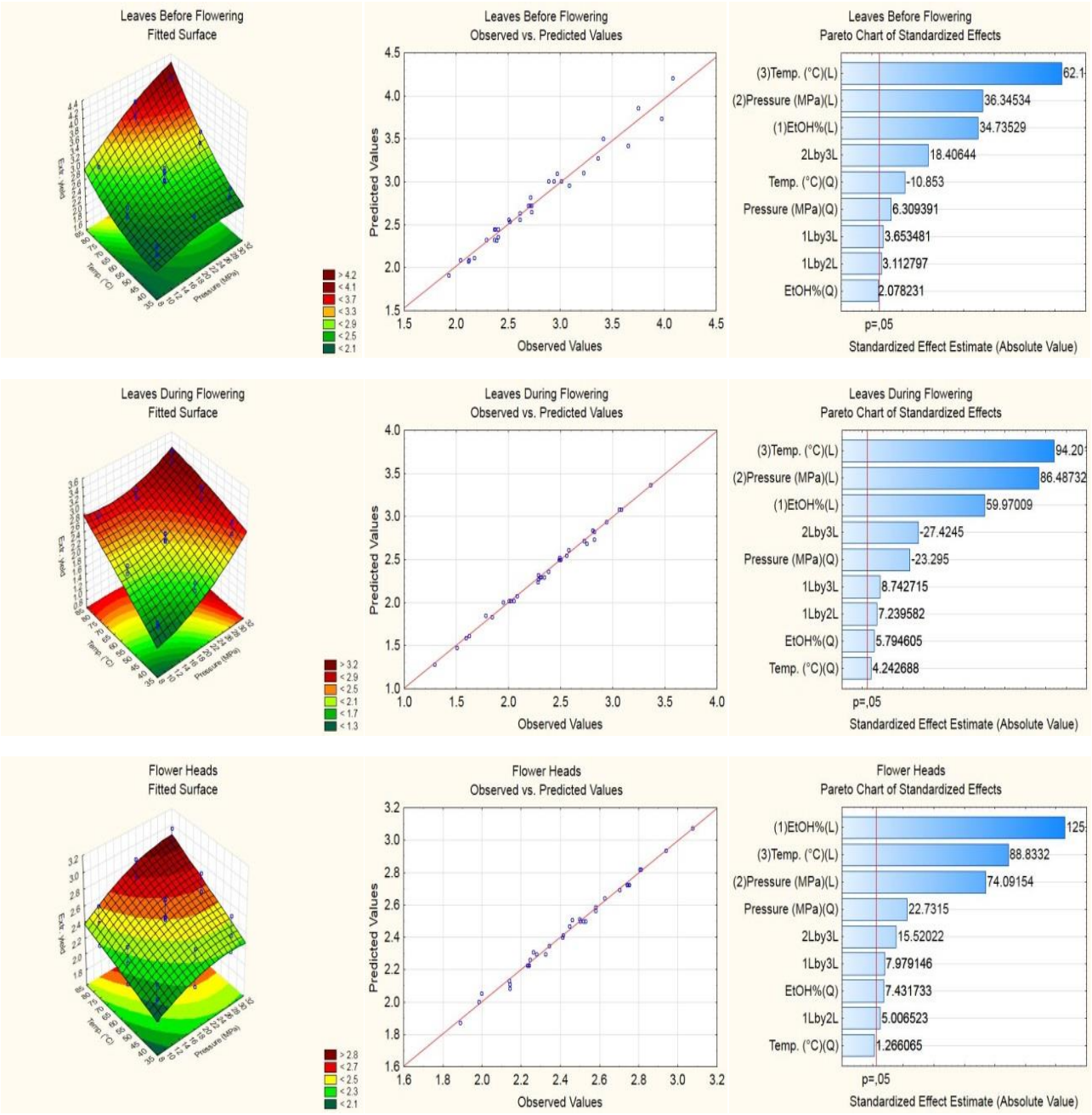

Figure 19. Response surfaces, observed and predicted values and Pareto charts over the extraction yield of the three *Tanacetum parthenium* L. samples

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6.1.2 Parthenolide content of the supercritical fluid extracts

Figure 20. shows the response surface of the parthenolide content in leaves collected before and during flowering and flower heads, where the modifier content was 5% ethanol. The determination coefficients of the equation were lower (0.931; 0.930; 0.934, respectively), than in the case of the total extraction yield. The observed and predicted values were near to diagonal, they show a god fit of the models. The R^2_{adj} did not differ significantly, indicating that the model didn't include non-significant terms (Table 10.).
The Pareto charts have shown that the ethanol content of the fluid individually and also in quadratic form had the most significant, however negative effect on the extraction of parthenolide. This effect was explained earlier. The co-solvent enhanced the solubility of both valuable and non-valuable components. The increasing polarity, caused by the addition of ethanol, also enhanced the co-extraction of the more polar sesquiterpene lactones and flavonoids. All the other linear and quadratic impacts had a statistically significant positive effect. In all three cases the temperature had more significant influence on the extraction, than the pressure, also in quadratic form. The observed results on these factors influencing the extraction correlate with previous study [Čretnik et al 2005]. Only one linear interaction, the pressure-temperature was significant, with a positive effect. It could be observed, that the parthenolide content rose until 60°C, then decreased at 80°C. This regressive tendency can be attributed either to the solubility of the compound, which is decreasing with increasing temperature close to the critical pressure, or to the thermo sensibility of parthenolide [Kaplan et al 2002]. Since no degradation products were found during the chromatographic analyzes in any of the samples, the protective effect of the complex sample matrix may occur. The flower heads contained the highest amount of parthenolide (0.604%), which is consistent with previous studies [Smith et al 1992]. Higher parthenolide content was observed in the leaves collected before flowering than in the leaves collected during flowering.
The critical values of the extraction of the leaves collected before and during flowering and the flower heads are the same (7% EtOH, 22MPa, 64°C). The optimum point of the temperature was 64°C, which showed, that a higher rate was necessary to achieve the optimum point than in previous studies [Čretnik et al 2005]. Control extractions for all three samples were made to survey the model's trueness. These extractions resulted in

the following outcomes: the parthenolide content of the leaves collected before flowering under the critical circumstances was 0.399%, the leaves collected during flowering resulted 0.375% and the flower heads 0.453%. The predicted values were 0.406%; 0.381% and 0.447%, respectively, which were not significantly different from the observed ones, thus the equations of the fitted surfaces were acceptable.

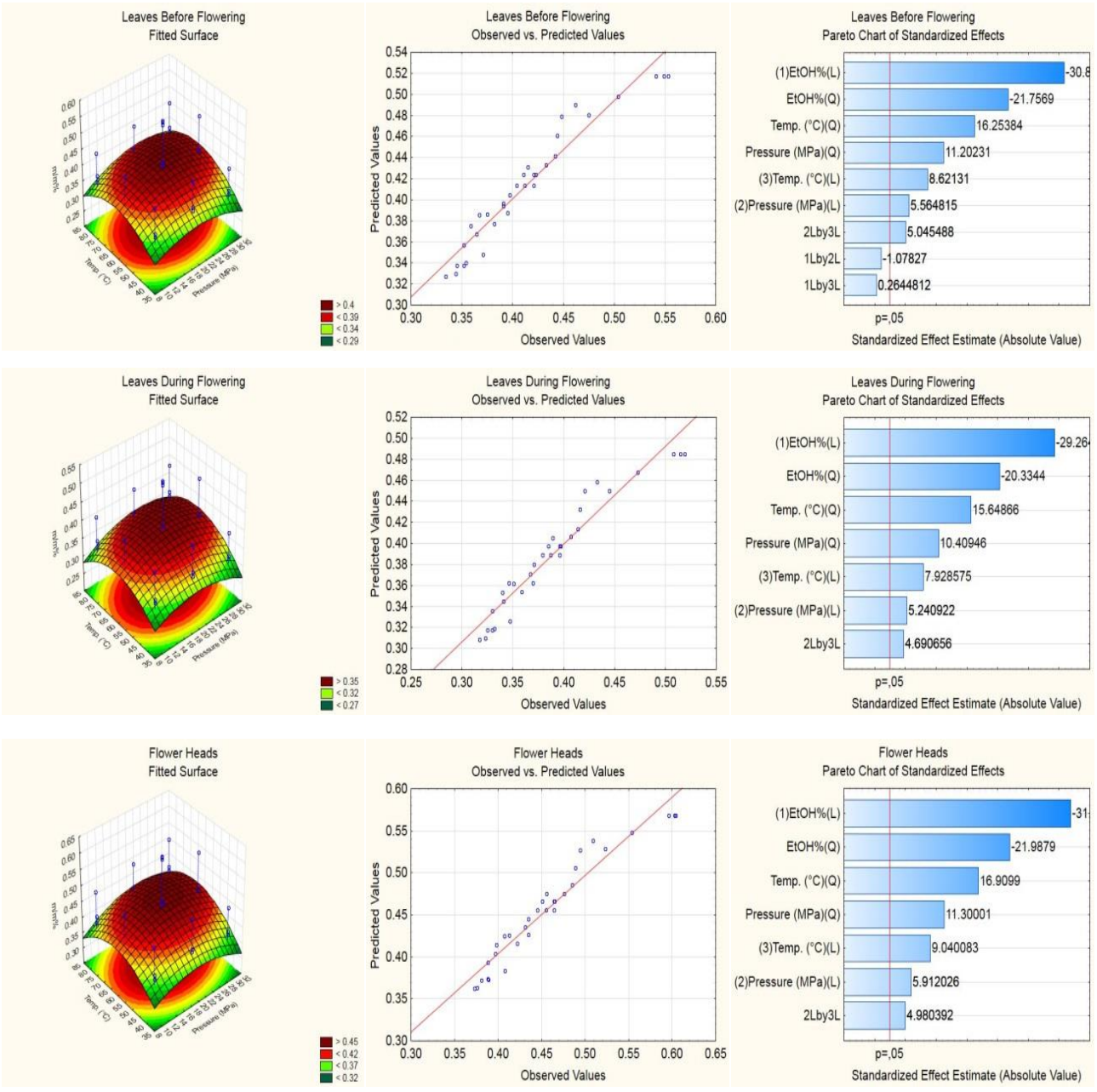

Figure 20. Response surfaces, observed and predicted values and Pareto charts over the parthenolide content of the three *Tanacetum parthenium* L. samples

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6.1.3 Conventional extraction methods

6.1.3.1 Soxhlet extraction

Conventional methods such as Soxhlet extraction are the most commonly applied technics for the recovery of parthenolide and other constituents from *Tanacetum parthenium* L. The highest total extraction yield was reached with the leaves before flowering, the maximum parthenolide content was observed in the flower heads. When methanol was used as organic phase, the results were only approximately 60-70% as of the results using chloroform in both of the observed cases in all three samples (Figure 21).

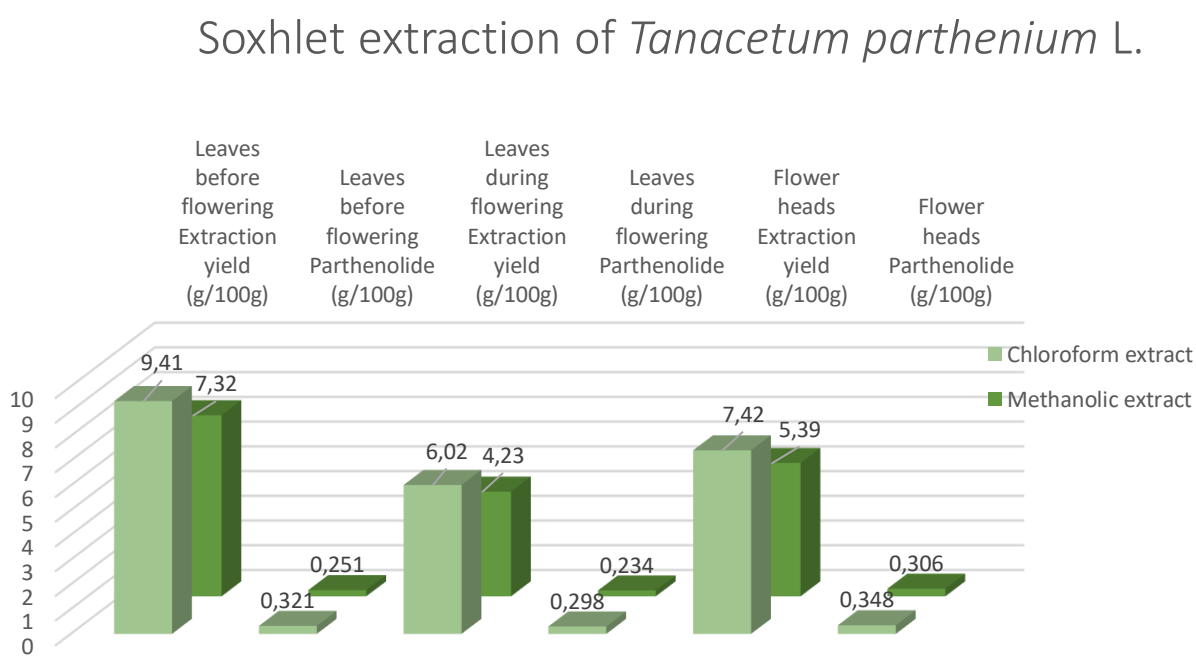

Figure 21. Results of the Soxhlet extraction method from *Tanacetum parthenium* L. samples

6.1.3.2 Ultrasound assisted extraction

A bottle stirring method in ultrasonic bath was carried out for the determination of the total extraction yield and parthenolide content in fresh and dried samples of feverfew.

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Chloroform was used, as organic phase, because of the semipolar quality of parthenolide. The previously presented literature data [Bloszyk et al 1978, Kaplan et al 2002, Majdi et al 2011] and the results of the performed Soxhlet extraction were also confirming the application of chloroform in the extraction. The results were aligned with the foundings of Majdi et al. [2011], the fresh samples contained more parthenolid then the dried ones. The extracts made from fresh plant material, are modeling the parthenolide content of the glandular thrichomes, since no grinding was applied, so the deeper cell structure of the plant was not erupted. These plant structures can be found on both the leaves and the flower heads of feverfew. The results are supporting the theory that parthenolide is accumulated and enriched in the glandular hairs of the plant (Figure 22.).

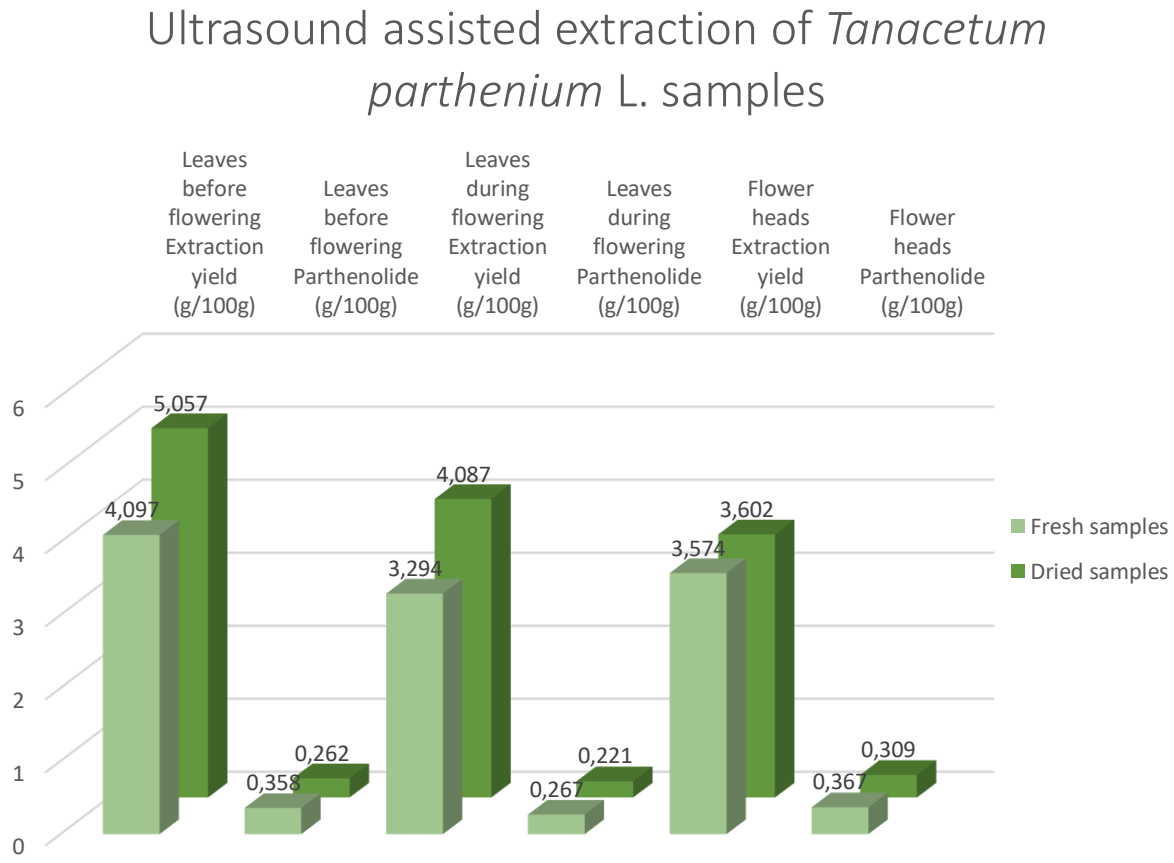

Figure 22. Results of the ultrasound assisted bottle stirring method from *Tanacetum parthenium* L. samples

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6.1.4 Comparison of supercritical fluid extraction and conventional methods

Conventional methods, like Soxhlet and ultrasound assisted extraction are still the first choice for the separation of compounds from plant matrices. Supercritical fluid extraction is a fast, environment friendly, selective and effective alternative for the widely applied sample preparation processes using organochlorine solvents. As it can be seen from the results, the supercritical fluid extraction method produced the highest amount of parthenolide, in the highest concentration, which confirms its effectiveness and selectivity of the method (Figure 23.). With the fine tuning of the process, the optimal rates for the parthenolide/extraction yield was reached. Only the ultrasound assisted bottle stirring method of the fresh leaves produced similar outcome, which is persistent with the fact that parthenolid is placed in the glandular thrichomes. Since the deeper cell structures were not damaged, the quantity of the coextracted compounds, like chlorophyll, are lower.

6.1.5 Quantitative analyses of parthenolide by Ultra Performance Convergence Chromatography

Ultra Performance Convergence Chromatography is one of the new supercritical fluid chromatography systems, which are specially designed for handling supercritical fluids. The predecessors of UPC^2 could not fulfill all the criteria, like accuracy, precision, robustness. The parthenolide content of feverfew extracts was quantitavely analyzed. In our work a rapid, fast and selective UPC^2 method was developed. The applied gradient ensured good resolution and proper selectivity for parthenolide. The method provided good linearity, precision and accuracy (Table 13,14,15).

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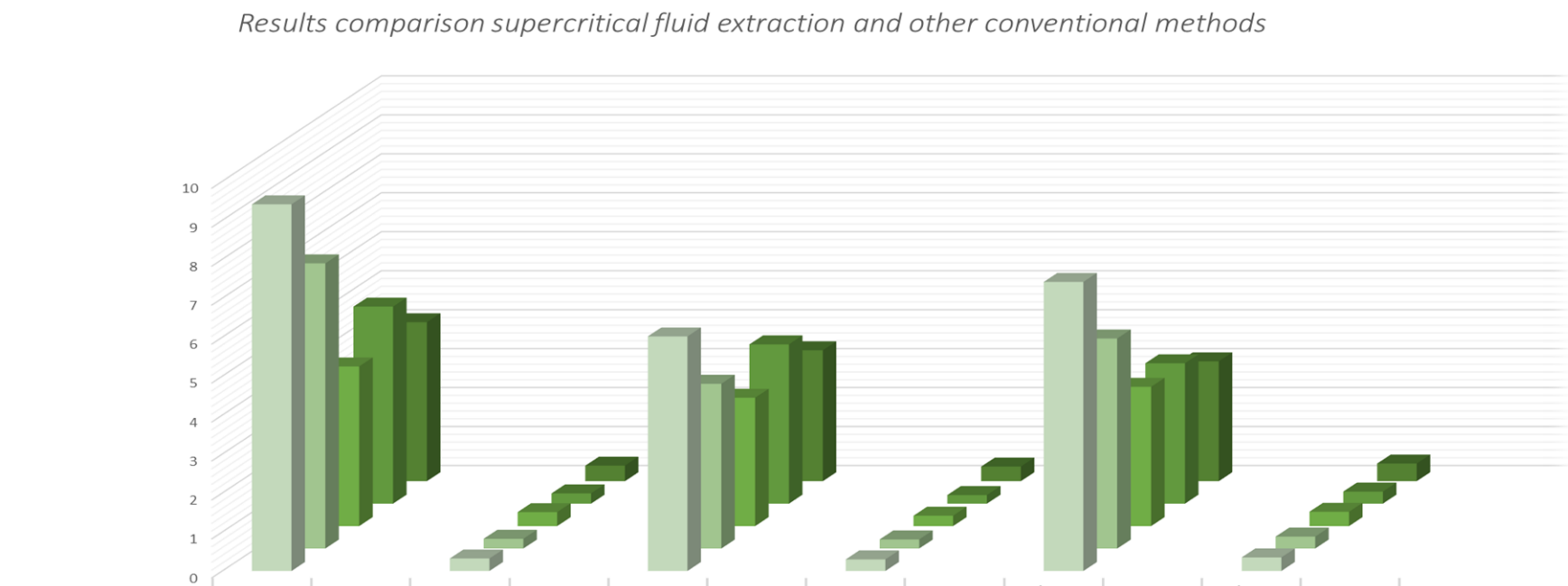

	Leaves before flowering Extraction yield (g/100g)	Leaves before flowering Parthenolide (g/100g)	Leaves during flowering Extraction yield (g/100g)	Leaves during flowering Parthenolide (g/100g)	Flower heads Extraction yield (g/100g)	Flower heads Parthenolide (g/100g)
Soxhlet chloroform extract	9.41	0.321	6.02	0.298	7.42	0.348
Soxhlet methanolic extract	7.32	0.251	4.23	0.234	5.39	0.306
Bottle stirring Fresh samples	4.097	0.358	3.294	0.267	3.574	0.367
Bottle stirring Dried samples	5.057	0.262	4.087	0.221	3.602	0.309
SFE	4.08	0.399	3.36	0.375	3.075	0.453

Figure 23. Comparison of the results of different extraction methods from *Tanacetum parthenium* L. sample

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6.2 Characterization of sesquiterpene lactones and phenolics in the supercritical fluid extracts of *Tanacetum parthenium* L.

6.2.1 HPLC-DAD-MS/MS

The results from the characteristic UV and MS spectra designated seven terpenoid type structure beside parthenolide and thirteen flavonoid type structure in the supercritical fluid extract of feverfew (Figure 16, Table 16.). Although the main sesquiterpene lactone, parthenolide and probably other minor parthenolide derivates are considered to be responsible for the migraine prophylactic effect of feverfew, the pharmacological benefits of the lipophilic flavonoid components cannot be neglected. Luteolin (1), apigenin (2), axillarin (5), parthenolide (14) and casticin (15) were identified by comparison of their chromatographic and spectrometric data to authentic standards, while the structural characterization of the other eight flavonoid derivatives was based merely on the evaluation of their (-) ESI-MS/MS fragmentation data (Table 16.).

Compounds 3 and 4 exhibited molecular ions $[M-H]^-$ at *m/z* 315 and characteristic product ions at *m/z* 300, 271, 255, 227 and 151. The neutral loss of 15 Da (*m/z* 315 → *m/z* 300) indicated the cleavage of a methyl radical ($-CH_3^{\bullet}$), thus the presence of a methoxy group in the molecules. The compounds were tentatively identified as two isomers of methylquercetin based on the comparison of their (-) ESI-MS/MS spectra to that of quercetin. In the case of compound 6 ($[M-H]^-$ at *m/z* 299) the neutral loss of 15 Da between the molecular ion and the product ion with *m/z* of 284 also suggested methoxyflavone structure. The presence of three hydroxyl groups in the molecule was also presumed based on the fact that the *m/z* of the molecular ion ($[M-H]^-$ at *m/z* 315) and the fragment ion mentioned above (*m/z* 284) showed 16 Da difference from those of methyl-quercetin (*m/z* 315 and *m/z* 300, respectively) (Table 16.).

In compound 9 ($[M-H]^-$ at *m/z* 329) the two neutral losses of 15 Da between the molecular ion and the fragment ions at *m/z* 314 and 299, respectively, suggested the presence of two methoxy groups in the molecule. The same conclusion could have been drawn about compound 16 ($[M-H]^-$ at *m/z* 313, product ions at *m/z* 298 and 283). Compound 9 was tentatively identified as dimethylquercetin based on the comparison of

its (-) ESI-MS/MS spectra to that of quercetin, while compound 16 as dihydroxy-dimethoxyflavone considering the 16 Da difference between its molecular ion and fragment ions and those of compound 9 (Table 16.).

The three neutral losses of 15 Da between the molecular ions ($[M-H]^-$) and the characteristic product ions of compounds 10, 11/12 and 17 indicated trimethoxy-flavone structures (*m/z* 359 → *m/z* 334, 329 and 314 in the case of compounds 10 and 11/12 (Figure 24.), *m/z* 343 → *m/z* 328, 313 and 298 in the case of compound 17 (Figure 25.)).

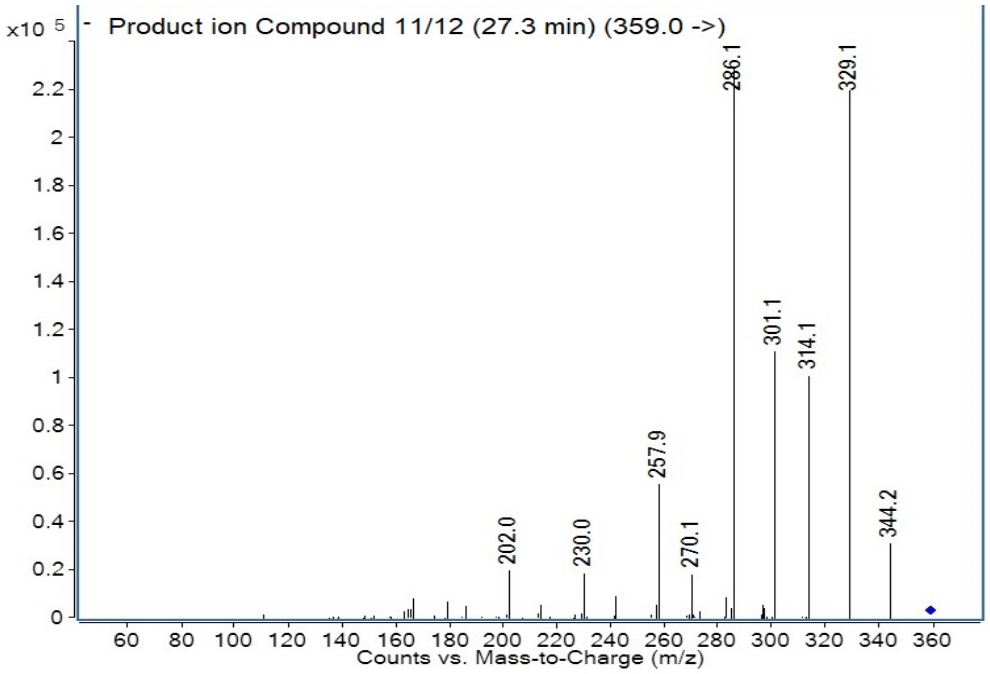

Figure 24. ESI-MS/MS fragmentation data of compound 11/12 from the supercritical fluid extract of feverfew.

Compounds 10 and 11/12 were tentatively identified as isomers of trihydroxy-trimethoxyflavone, while compound 17 as dihydroxy-trimethoxyflavone (Table 16.).

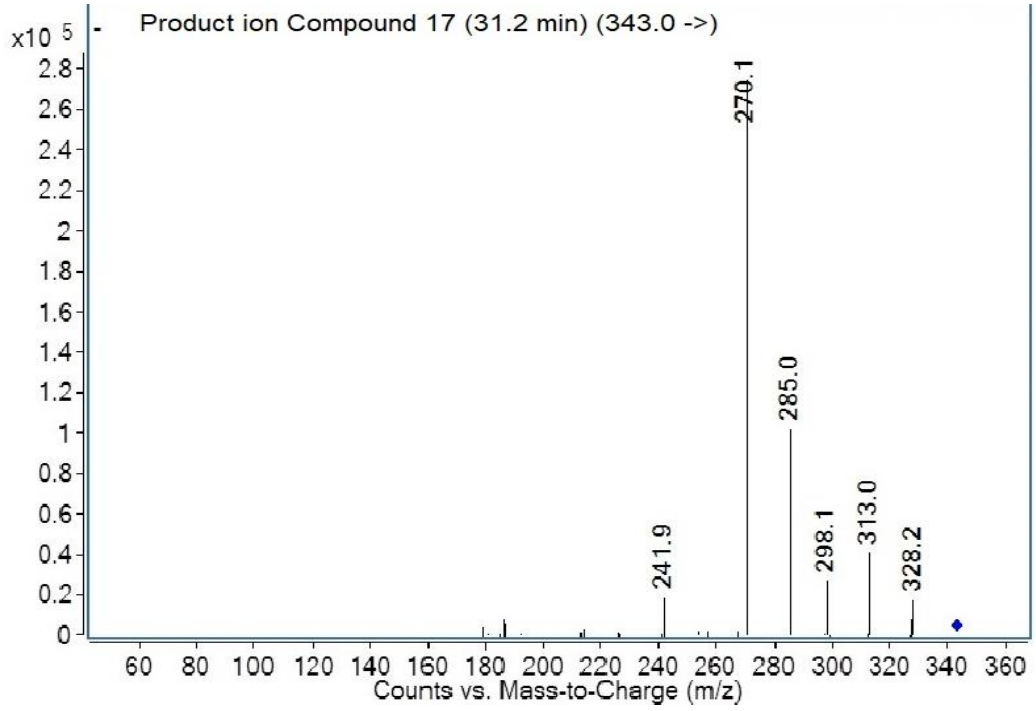

Figure 25. ESI-MS/MS fragmentation data of compound 17 from the supercritical fluid extract of feverfew.

Additionally, costunolide (compound 7), dihydro-β-cyclopyrethrosin (compound 8), a tanacetol A isomer (compound 13), tanaphillin (compound 18), 3-β-hydroxyanhydroverlotorin (compound 19), seco-tanapartholide A (compound 20), seco-tanapartholide B (compound 21), hispidulin (compound 22) has been tentatively identified from UV spectral data obtained by LC-DAD and fragmentation pattern were compared to literature data [Williams et al. 1995, Williams et al. 1999, Williams et al. 1999, Ablajan et al. 2013, Ablajan et al. 2006, Fabre et al. 2001].

6.2.2 Blood-Brain Barrier Specific Parallel Artificial Membrane Permeability Assay (PAMPA-BBB)

The parallel artificial membrane permeability assay was first introduced by Kansy et al. in 1998 to model oral absorption processes [Kansy et al 1998]. Di and coworkers have modified the PAMPA system specifically for blood brain barrier (BBB) application [Di et al 2003]. The assay can serve information about the BBB penetrating ability of various

compounds. These compounds can appear in the central nervous system and have a potential biological effect on it. The lipophilic/hydrophilic nature of the molecules from feverfew makes the penetration through the BBB possible with passive transport.

The system consists of two multiwell microtiter plates, a donor and an acceptor compartment in a "sandwich" like configuration, separated by an artificial lipid impregnated filter membrane (Figure 15.). Initially, the test drug is added in the donor plate and allowed to diffuse across the membrane.

After incubation, typically for 4 hrs at 37 °C, PAMPA sandwich plates are separated and drug concentrations in donor and acceptor solutions are determined by UV spectroscopy or LC-MS.

The whole *T. parthenium* extract was subjected to the PAMPA-BBB analysis without purification or prefractionation. Among the flavonoid constituents, compounds 11/12 and 17 were shown to have BBB permeability potential comparable to that of parthenolide (10) ($logP_e$ -4.56±0.12, -4.40±0.19 and -4.27±0.17, respectively). The positive BBB penetration of parthenolide was consistent with its CNS activity from previous *in vitro* tests. The optimal application conditions of the supercritical fluid extraction enhanced the coextraction of compounds with similar polarity. This explains the presence of BBB positive lipophilic flavonoids in the extract. These flavonoids may contribute to the overall CNS activity of the plant, so the further identification of these were necessary.

6.2.3 NMR analysis of the SemiPrep-HPLC fractions

For the isolation of the compounds capable of crossing theBBB, SemiPreparative HPLC method was applied. The structural identification of these compounds was performed with NMR spectroscopy. The ^{1}H NMR spectrum recorded in $CDCl_3$ reflected H-bonds (in the 12-13 ppm region) between the C5-OH group and the carbonyl C=O of the C ring of the flavonoid skeleton. This finding confirms that both compounds are hydroxylated in position 5 in ring A. According to the mass spectrometric data, ring A should also contain two additional methoxy function. The NMR data confirmed that *O*-methylation occurred at position 6 (3.80 ppm) and at position 8 (3.89 ppm) respectively (Figure 26.).

The lack of NOE cross-peaks between the *O*-methyl resonances in the 2D NOESY spectrum also confirmed this methylation pattern.

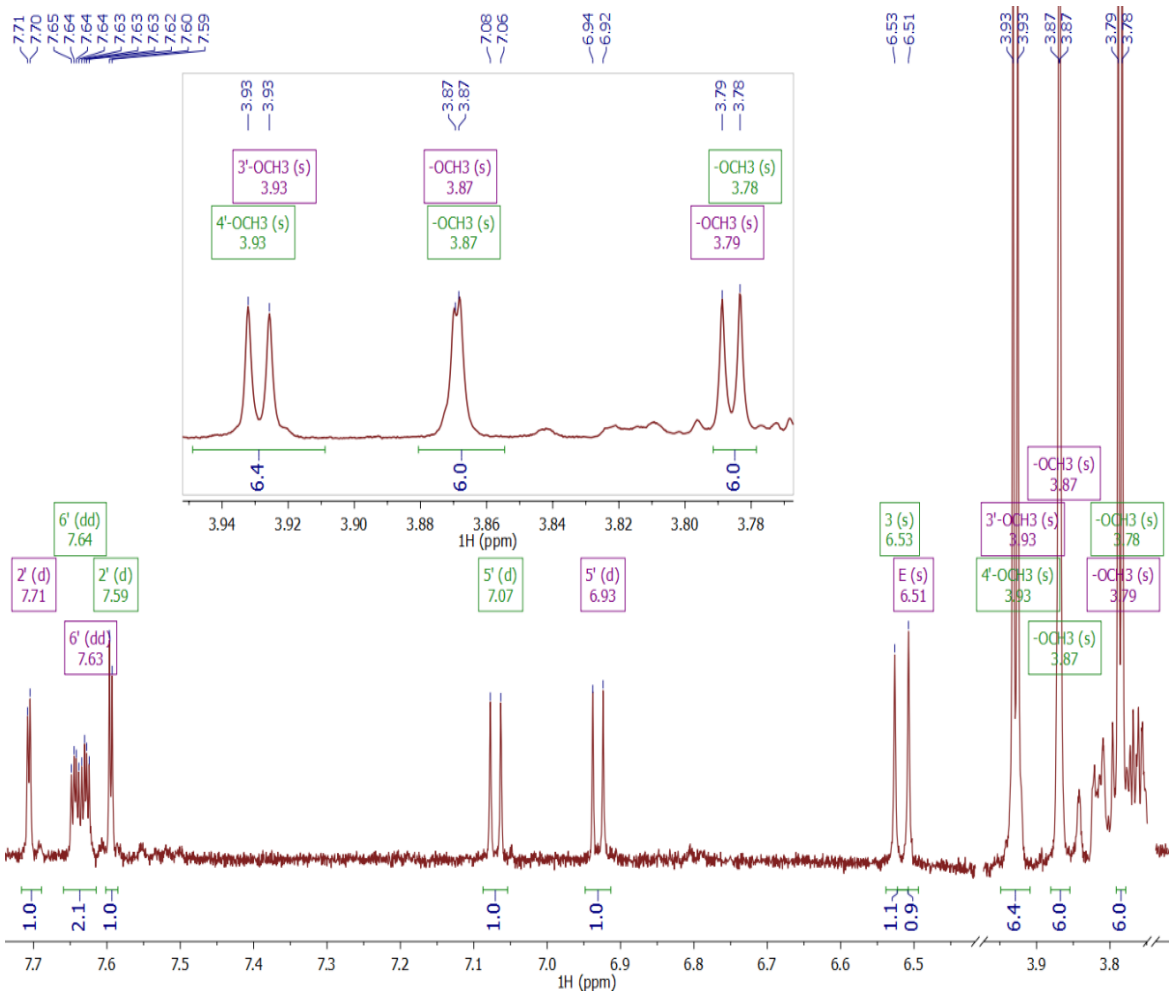

Figure 26. ^{1}H NMR spectrum of sudachitin and acerosin

Consequently, the two isomeric compounds possess the hydroxyl and the *O*-methylated functions at different (meta and para) positions in ring B. The exact location of the *O*-methyl groups was deduced by NOESY experiments. In the case of acerosin (11) the *O*-methyl function (3.95 ppm s, 3H) was found to be in the para position as NOE cross-peak indicated spatial proximity between the methyl protons and H-5’ (at 7.09 ppm d, *J* = 8.6 Hz, 1H) (Figure 26.). The isomeric compound sudachitin (12) was confirmed by the spatial proximity observed between *O*-methyl resonance (3.94 ppm s, 3H) and H-2’ (7.72 ppm d, *J* = 2.1 Hz, 1H) (Figure 27.).

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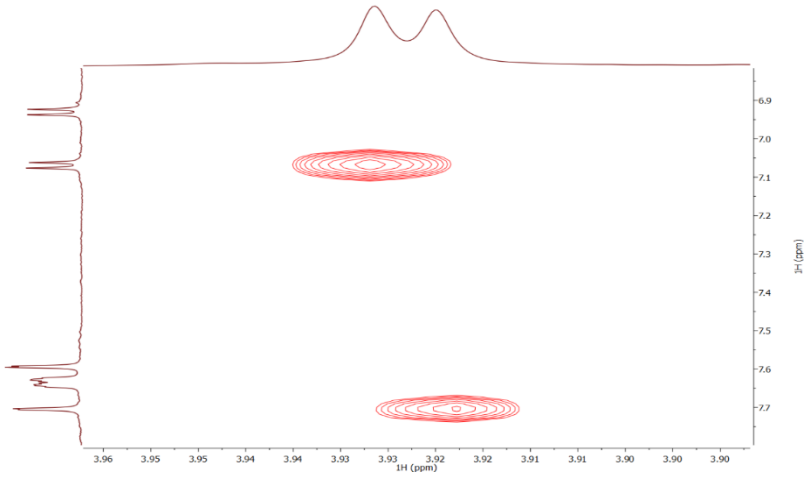

Figure 27. NOE cross-peak between acerosins *O*-methyl group protons (3.95 ppm s, 3H) and H-5' (at 7.09 ppm d, *J* = 8.6 Hz, 1H) and sudachitins *O*-methyl resonance (3.94 ppm s, 3H) and H-2' (7.72 ppm d, *J* = 2.1 Hz, 1H)

In the ^{1}H NMR spectrum of minor fraction 7.5 a similar H-bond was detected (12.93 ppm s, 1H) between the OH function at C5 in ring A and the C=O group in ring C (Figure 28.). The two *O*-methylation in ring A was identified at positions 6 and 8 which is also congruent with biosynthetic considerations. A para methoxy substitution in ring B was deduced from the intensities (2H) and coupling patterns (doublets) of H-2' and H-3'.

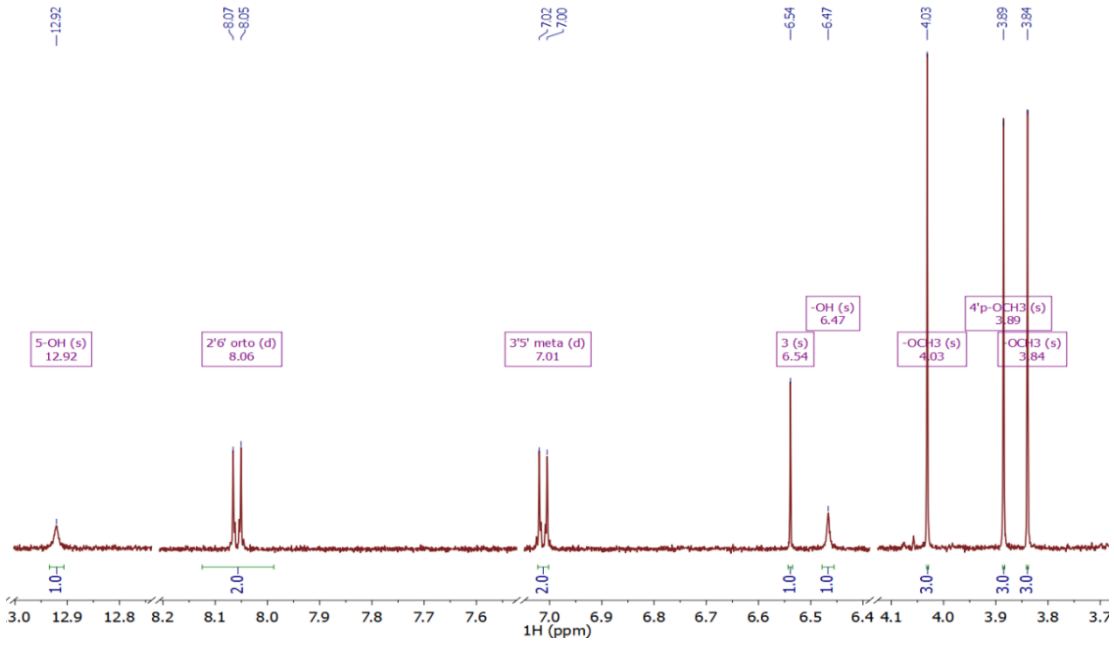

Figure 28. ^{1}H NMR spectrum of nevadensin

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The O-methyl resonance in the para position (at 3.90 ppm) also showed conclusive NOE cross-peak with the H-3’ protons (in meta position) at 7.02 ppm (d, *J* = 9.0 Hz, 2H), therefore this compound was identified as nevadensin (17) (Figure 29.).

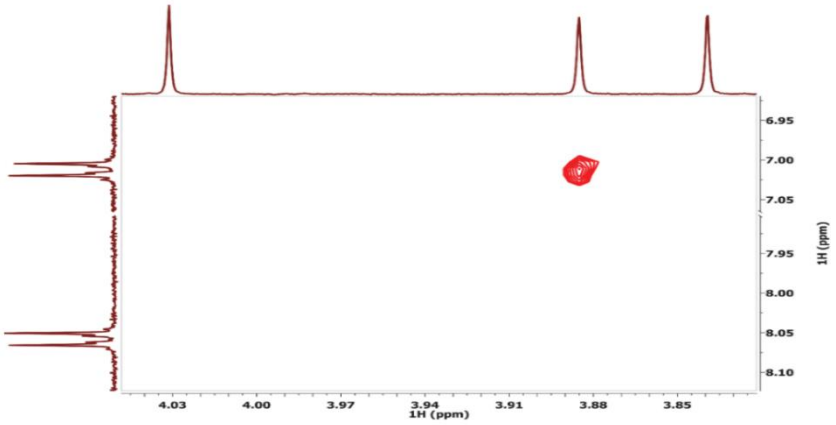

Figure 29. NOE cross-peak between nevadensins *O*-methyl group protons (3.90 ppm s, 3H) and H-3’ (7.02 ppm d, *J*= 9.0 Hz, 2H).

The chemical structures of the three newly identified flavonoids are shown in Figure 30.

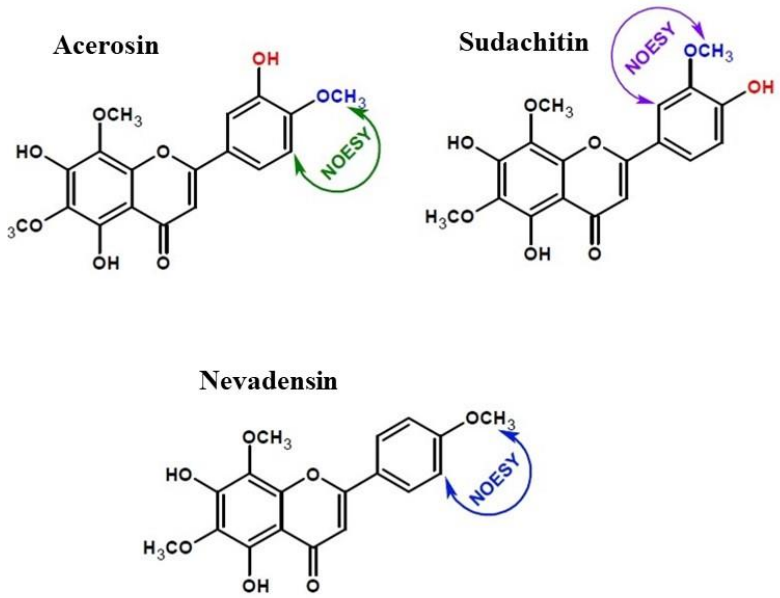

Figure 30. Chemical structures of the three newly identified flavonoids in *Tanacetum parthenium* L. (Acerosin, Sudachitin, Nevadensin)

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6.2.4 PAMPA-BBB of the newly identified compounds

The purified compounds were subjected individually to PAMPA-BBB measurements again together with two flavonoid aglycones luteolin (1) apigenin (2), two methylated flavonoids axillarin (5) and casticin (15) and the sesquiterpene lactone parthenolide (14). As it can be seen in Table 16., the $logP_e$ values of sudachitin/acerosin (11/12) and parthenolide (14) measured in the extract and as individual compounds are not significantly different. Conversely, nevadensin (17) showed higher permeability potential when measured in the extract than as purified compound ($logP_e$ -4.40±0.19 and -4.92±0.04, respectively). This might be attributed to pharmacokinetic interactions occurring among the constituents in the extract. According to the results luteolin (1), apigenin (2) and casticin (15) also fell in the BBB+ region ($logP_e$ > -6.0), although these constituents were not detected on the acceptor side of the PAMPA when the whole extract was investigated. As it can be seen on the chromatogram in Figure 16., all three compounds were present in the extract as minor constituents, which could have led to such low compound concentrations on the acceptor side that fell below the detection limit. Although axillarin (5) was present in the extract in an amount comparable to nevadensin (17), it was not detected on the acceptor side of the PAMPA neither in the whole extract nor when measured as individual compound, which confirms axillarin (5) being BBB-.

6.3 Qualitative and quantitative analysis of the essential oil and supercritical fluid extracts of *Tanacetum parthenium* L.

6.3.1 Qualitative analysis of the essential oil and supercritical fluid extract of feverfew

The essential oil and supercritical fluid extracts were analyzed with HS-SPME-GC/MS method. 11 different compounds were identified according to their retention times and mass spectra. The compounds are listed in Table 19. with retention times and quantitative percentages. The volatile constituents in the essential oil were α-pinene, camphene, p-

cymene, limonene, γ-terpinene, camphor, chrysanthenyl acetate, bornyl acetate, β-caryophyllene, β-farnesene and germacrene D. The compounds were present in all six essential oil samples in different distribution. The supercritical fluid extract of feverfew contained only 7 compounds, these were camphene, p-cymene, limonene, γ-terpinene, camphor, chrysanthenyl acetate and germacrene D. The percentage distribution of the volatile constituents is presented in Figure 31. The main compounds in all samples were camphor and chrysanthenyl acetate. The percentage distribution of the compounds represents only the allocation of these in the extracts.

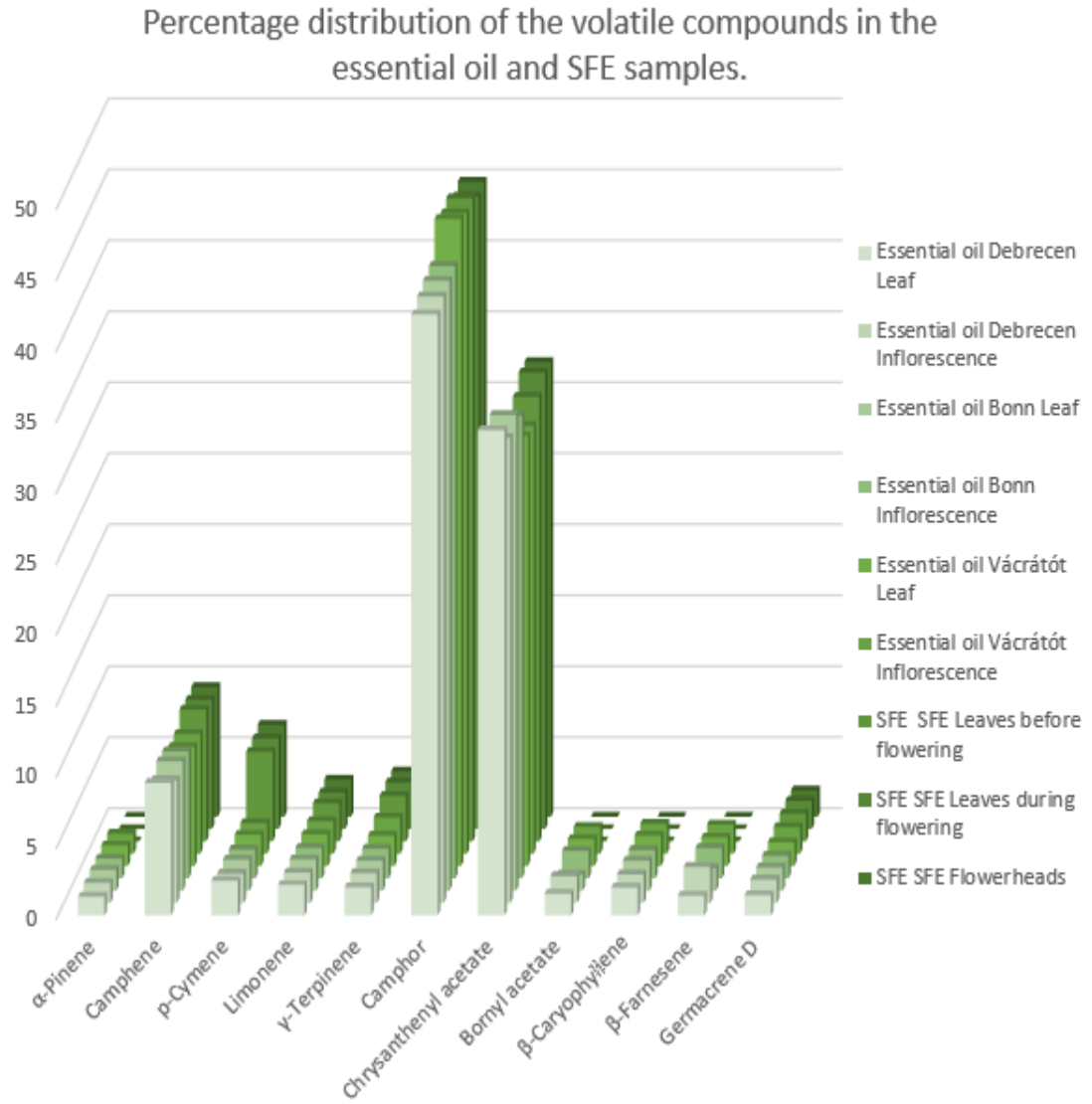

Figure 31. Percentage distribution of the volatile components in the essential oil and supercritical fluid extracts of *Tanacetum parthenium* L. The percentile values were calculated as relative proportion of total peak area from GC-FID chromatogram

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6.3.2 Camphor content of the essential oil and supercritical fluid extract from *Tanacetum parthenium* L.

Although there are several studies about the major and minor components of the essential oil of *Tanacetum parthenium* L., none of them presents a validated quantitative method. Only the relative abundance of the compounds was studied in these reports, including our previously described HS-SPME-GC/MS method too. The previously described high toxicity of camphor indicated a well validated quantitative method for the precise determination of camphor levels in the samples. A method applying supercritical fluids was adequate. The developed UPC^2 method proposes a rapid, sensitive and environment friendly alternative to determine camphor in different samples both in essential oils and supercritical extracts.

The camphor content of the essential oils from the aerial parts of the three feverfew samples from different origin was analyzed by the previously validated convergence chromatographic method.

The total yield and the camphor content of the essential oil were highly dependent on the sample type (Figure 32.).

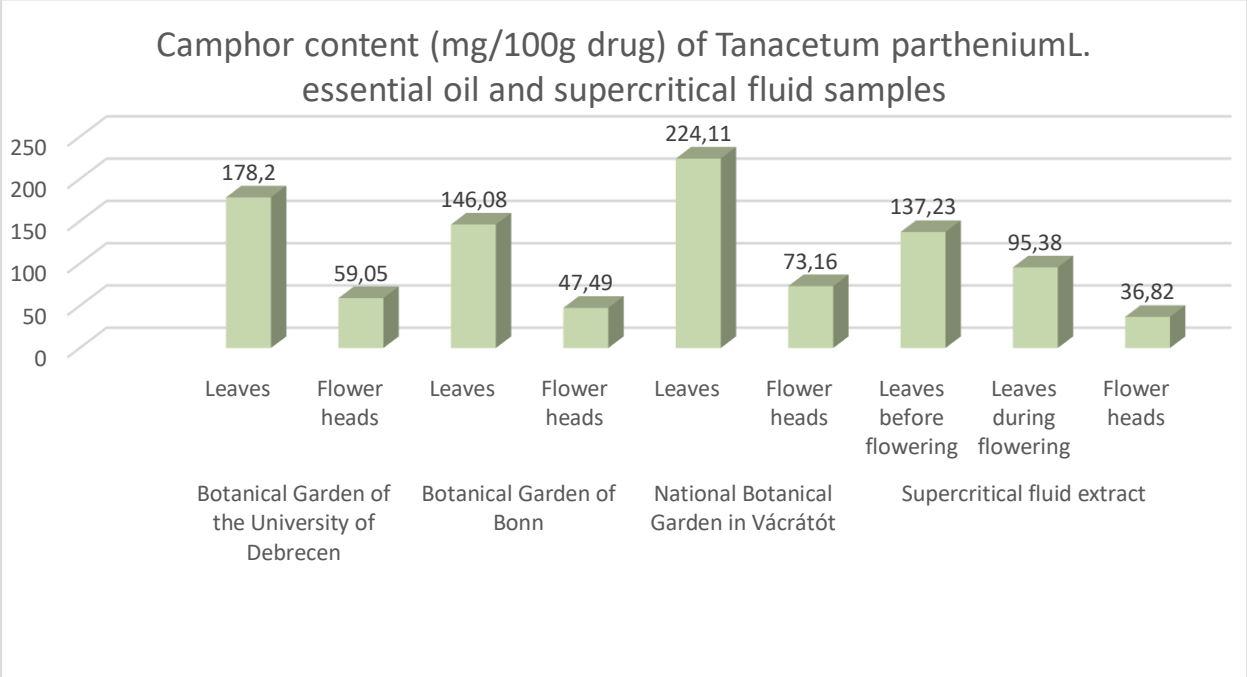

Figure 32. Camphor content of *Tanacetum parthenium* L. samples

The essential oil content of the leaves (from Debrecen, Budaörs and Vácrátot) amounted from 0.59 to 0.69 g/100g drug. The flower heads yielded from 0.32 to 0.39 g/100g drug. The highest amount of camphor was found in the leaves (178.2, 146.08, 224.11 mg/100g drug). These results are in accordance with previous studies [Izadi et al 2010]. Due to the heavy CNS side effects of camphor, it is vital to keep its level low in the extracts and to monitor it with with an accurate and precise method. Beside the biologically beneficial components, the SFE extracts can provide a product with the avoidance of toxic underaction. With the developed and validated SFC method, the camphor content can be determined quantitatively with advantages of sensitivity and very good reproducibility.

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7 CONCLUSION

1. For the preselection and enrichment of the plausibly bioavailable compounds of *Tanacetum parthenium* L. supercritical fluid extraction was applied. The optimum conditions of the SFE of feverfew were determined, in order to maximize the parthenolide recovery of the extracts. The experiments were carried out at different temperature (40°C, 60°C and 80°C), pressure (10MPa, 20MPa and 30MPa) and modifier content (0%, 5% and 10% ethanol). The design of experiment (DOE) was based on a full factorial 3^3 design. Three feverfew samples were investigated (leaves collected before flowering, leaves collected during flowering and flower heads). For the extraction yield no optimum was found. The critical values of parthenolide content were 7 % EtOH, 22 MPa and 64 °C. Our results indicated, that the flower heads contained the highest amount of parthenolide (0.604%). It was confirmed, that the optimum conditions of the extraction, where the maximum parthenolide content and extract yield can be reached, do not coincide.

2. A simple and rapid isocratic Ultra Performance Convergence Chromatographic (UPC^2) method was developed for the qualitative and quantitative analysis of *Tanacetum parthenium* L. supercritical fluid extracts and parthenolide which was proved to be reliable and accurate. The method was successfully validated with advantages of high resolution, sensitivity, very good reproducibility and short analysis time. Therefore, it could be concluded that the developed UPC^2 methods can be successfully utilized for the analysis of the feverfew extracts.

3. Complexity of the extracts was proved by tentative identification of the compounds using HPLC-DAD-MS/MS methods. 7 terpenoids beside parthenolide were identified, namely costunolide, dihydro-β-cyclopyrethrosin, a tanacetol A isomer, tanaphillin, 3-β-hydroxyanhydroverlotorin, seco-tanapartholide A, seco-tanapartholide B Twelve flavonoid components were detected in the extract. Besides apigenin, luteolin, casticin and axillarin, eight further methylated flavonoids were identified.

4. An *in vitro* parallel artificial membrane permeability assay for the blood-brain barrier (PAMPA-BBB) was successfully utilized for the selection of brain-penetrable compounds of the extracts.
    - Beside the main BBB positive component parthenolide, minor sesquiterpene lactones and some methylated flavonoids were enriched in the acceptor side.
    - Based on the NMR analysis of the isolated methylated flavonoids sudachitin/acerosin isomers and nevadensin were identified as BBB positive flavonoids.
    - The presence of sudachitin and nevadensin were previously proven in the Asteraceae family, but neither of the three flavonoids were found in *Tanacetum parthenium* L. before. The components were also tested individually in the PAMPA-BBB model to investigate the occurring pharmacokinetic interactions. The $\log P_e$ values of sudachitin/acerosin and parthenolide measured in the extract and as individual compounds were not significantly different, but nevadensin showed higher permeability potential when measured in the extract than as purified compound. Luteolin, apigenin, axillarin and casticin were found to be BBB negative.
5. The essential oil yield of three *Tanacetum parthenium* L. samples was studied.
    - Leaves collected during flowering and flower heads were investigated. Our studies supported the presumption that the leaves collected during flowering contained the highest amount of volatile oil.
    - The essential oil and supercritical fluid extracts were analyzed with HS-SPME-GC/MS method. 11 volatile compounds were identified in the essential oil and 7 in the supercritical fluid extract. The main compounds in all samples were camphor and chrysanthenyl acetate.
    - A simple and rapid method was developed and validated for the qualitative and quantitative analysis of the camphor content in steam distilled samples and supercritical fluid extracts of *Tanacetum parthenium* L. The UPC^2 method was validated for selectivity, accuracy,

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repeatability and intermediate precision, linearity, limit of detection, limit of quantification and robustness. The validation was successful with advantages of high resolution, sensitivity and very good reproducibility and the method was proven to be reliable and accurate. Very short sample preparation and analysis time have been achieved, the latter with a remarkable 0.785 min retention of camphor. The highest camphor content was measured in the essential oil of the leaves during flowering and the lowest in the supercritical fluid extracts. The presence of nonvolatile apolaric and semipolaric compounds in the extracts beside the volatile terpenoids gives preference to the chromatographic method using supercritical fluid.

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8 SUMMARY

Herbal medicine has always maintained its popularity worldwide. Patients are looking for complementary or alternative medicine more and more often in the over the counter category to substitute synthetic drugs.

The primary aim of our study was to complement and reevaluate the phytochemical, pharmaceutical and biological knowledge about *Tanacetum parthenium* L. Feverfew is a well-known medicinal plant due to its pharmacological activities, particularly the migraine prophylactic effect. Despite the several researches about the plant, new phytochemical, methodological and pharmacological results could have been presented.

A supercritical fluid extraction method was optimized to maximize the parthenolide recovery. A detailed experimental program of the SFE conditions (temperature, pressure, co-solvent content) was planned in order to evaluate the individual and combined effects.

A validated quantitative supercritical fluid chromatographic method was developed for the determination of the parthenolide content of the feverfew supercritical fluid extracts.

For the identification of simultaneously present other co-extracts, a high-performance liquid chromatographic separation was optimized. Different detection methods, diode array detection and electrospray ionisation-tandem mass spectrometry were acquired to gain structural information about the compounds, with particular attention on the lipophilic flavonoids. Twelve flavonoid components were detected in the extract. Besides apigenin, luteolin, casticin and axillarin, eight further methylated flavonoids were identified. We also found 7 terpenoids beside parthenolide, namely costunolide, dihydro-β-cyclopyrethrosin, a tanacetol A isomer, tanaphillin, 3-β-hydroxyanhydroverlotorin, seco-tanapartholide A, seco-tanapartholide B.

The active flavonoid compounds possessing positive permeability rates through blood-brain barrier (BBB+) were isolated and identified, then retested individually in the parallel artificial membrane permeability assay (PAMPA) model. Nevadensin, sudachitin and acerosin were found as new compounds in the feverfew extract.

In order to gain information about the volatile oil of the herb steam distillation was applied for the extraction and a simple and rapid supercritical fluid chromatography method was developed and validated.

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9 ÖSSZEFOGLALÁS

A gyógynövények gyógyászati alkalmazása régóta nagy népszerűségnek örvend világszerte. A betegek egyre gyakrabban keresnek szintetikus gyógyszerelésük kiegészítésére vagy akár helyettesítésére is komplementer/integratív gyógymódokat.

Vizsgálatunk elsődleges célja a *Tanacetum parthenium* L. fitokémiai, gyógyszerészeti és biológiai ismereteinek kiegészítése és újraértékelése volt. Az őszi margitvirág számos farmakológiai tulajdonsága, különösen a migrén-profilaktikus hatása miatt jól ismert gyógynövény. A számos kutatás ellenére új fitokémiai, módszertani és farmakológiai eredményeket mutatunk be munkánkkal.

Optimalizáltuk a szuperkritikus fluid extrakciós módszert a drog kivonatok partenolid tartalmának maximalizálása céljából. Részletes kísérleti programot terveztünk, amellyel vizsgálni tudtuk az SFE-feltételek (hőmérséklet, nyomás, társoldószer-tartalom) egyedi és kombinált hatását a kivonásra. Ezen extraktumok partenolid tartalmának mennyiségi vizsgálatára validált szuperkritikus fluid kromatográfiás módszert dolgoztunk ki.

A kivonat további alkotóinak elválasztására nagy hatékonyságú folyadékkromatográfiás módszert fejlesztettünk. A kivonatok összetételét különböző detektálási módszerekkel vizsgáltuk (DAD, tömegspektrometria), hogy szerkezeti információkat kapjunk az alkotó vegyületekről, különös tekintettel a lipofil flavonoidokra. Tizenkét flavonoidot detektáltunk, melyek közül apigenin, luteolin, casticin és axillarin került azonosításra. A parthenolid tartalom mellett 7 terpenoid származékot találtunk, melyeket costunolid, dihidro-β-ciklopiretrozin, tanacetol A izomer, tanaphillin, 3-β-hidroxi-anhidroverlotorin, seco-tanapartolid A és seco-tanapartholide B-ként valószínűsítettünk.

A vér-agy gáton keresztül pozitív permeabilitási képességgel rendelkező BBB+ hatóanyagokat izoláltuk és azonosítottuk, majd azok átjutását monokomponensként is vizsgáltuk párhuzamos mesterséges membránpermeabilitási modellben (PAMPA). Nevadenzint, sudachitint és acerosint azonosítottuk új vegyületként az extraktumban. Ezen komponensek elsőként kerültek leírásra az őszi margitvirágban.

Annak érdekében, hogy információt nyerjünk az őszi margitvirág víz-gőz-desztillációval előállított illóolajának kámfor tartalmáról, egyszerű és gyors szuperkritikus folyadékkromatográfiás módszert dolgoztunk ki és validáltunk.

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11 LIST OF PUBLICATIONS

Publications related to the thesis

- Végh K, Riethmüller E, Hosszú L, Darcsi A, Müller J, Alberti Á, Tóth A, Béni Sz, Könczöl Á, Balogh Gy T, Kéry Á. (2018). Three newly identified lipophilic flavonoids in *Tanacetum parthenium* supercritical fluid extract penetrating the Blood-Brain Barrier. J Pharm Biomed Anal, 149:488-493.

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Further publications

- Tóth A, Riethmüller E, Végh K, Alberti Á, Béni Sz, Kéry Á. (2018). Contribution of individual flavonoids in Lysimachia species to the antioxidant capacity based on HPLC-DPPH assay. Nat Prod Res, 32(17):2058-2061.

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