UNIVERSITEIT GENT

FACULTY OF PHARMACEUTICAL SCIENCES

ANALYTICAL, PHARMACOKINETIC AND REGULATORY CHARACTERISATION OF SELECTED PLANT *N*-ALKYLAMIDES

Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

Lieselotte VERYSER

Promoter
Prof. Dr. Bart DE SPIEGELEER

Drug Quality & Registration (DruQuaR) lab

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The promoter

Prof. Dr. Bart De Spiegeleer

The author

Lieselotte Veryser

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LIST OF ABBREVIATIONS AND SYMBOLS

Θ	Exposure time
A	Area
A	Skin surface
A.	*Achillea*
ACAT	Acyl-CoA: cholesterol acyltransferase
ACE	Angiotensin-converting enzyme
ACN	Acetonitrile
AEA	Arachidonoylethanolamine
AP	*Anacyclus pyrethrum*
AP-1	Activator protein 1
APCI	Atmospheric pressure chemical ionisation
aPTT	Activated partial thromboplastin time
BBB	Blood-brain barrier
BSA	Bovine serum albumin
$C_{24h,epidermis}$	Concentration in the epidermis after 24h
$C_{24h,vehicle}$	Concentration in the vehicle after 24h
CAM	Cell adhesion molecule
CAM	Complementary and alternative medicine
CBR	Cannabinoid receptor
CBR1	Cannabinoid receptor 1
CBR2	Cannabinoid receptor 2
CD	Capillary depletion
CD14	Cluster of differentiation antigen 14
C_d	Concentration in dose solution
$C_{d,0}$	Initial concentration in donor chamber
CID	Collision induced dissociation
CJEU	Court of justice of the European Union
Cl	Plasma clearance
CLP	Cecal ligation and puncture
CNS	Central nervous system
COX-2	Cyclooxygenase-2
$C_{pl,ss,top}$	Steady state plasma concentration after topical application

C_r	Concentration in the receiver compartment
$C_{r,t-1}$	Concentration in the receiver compartment at the previous time point
C(x)	Concentration at coordinate x in the barrier
CV	Coefficient of variation
d	Skin thickness
D	Diffusion coefficient
D_m	Diffusion coefficient
dC/dx	Concentration differential across a membrane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DS1	Dose solution 1
DS2	Dose solution 2
EC	European Commission
ECETOC	European Centre for Ecotoxicology of Chemicals
EFSA	European Food Safety Authority
EI	Electron impact
EMA	European Medicines Agency
EPM	Elevated plus maze
ESI	Electrospray ionisation
EtOH	Ethanol
EU	European Union
f	Sample replacement dilution factor
FA	Formic acid
FAAH	Fatty acid amide hydrolase
FDA	Food and Drug Administration
FDC	Franz diffusion cell
FOSHU	Food for specified health uses
FuFoSE	Functional Food Science in Europe
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GLP	Good laboratory practices
GMP	Good manufacturing practices
h	Hour
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HMPC	Committee on Herbal Medicinal Products
HPLC	High performance liquid chromatography
HUVECs	Human umbilical vein endothelial cells
IBS	Irritable bowel syndrome
IC_{50}	Half maximal inhibitory concentration
ICR-CD-1	Institute for Cancer Research, Caesarean Derived-1
ICV	Intracerebroventricular
IκB	Inhibitor of κB
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IS	Internal standard
ISM	Isocratic solvent manager
IV	Intravenous
J	Flux
JNK	C-Jun-*N*-terminal kinase
J_{ss}	Steady state flux
K	Net brain clearance
K_1	Unidirectional blood-to-brain clearance
K_{in}	Unidirectional brain influx rate
K_m	Partition coefficient
k_{out}	Efflux rate constant
K_p	Permeability coefficient
$K_{p,aq}$	Aqueous permeability coefficient
$K_{p,v}$	Permeability coefficient in the vehicle
L.	Linnaeus
LC	Liquid chromatography
LD_{50}	Median lethal dose
LLoQ	Lower limit of quantification
LoD	Limit of detection
LoQ	Limit of quantification
LPS	Lipopolysaccharide
LR	Lactated ringer
MD2	Myeloid differentiation protein-2
MeOH	Methanol

MES	Maximal electro shock
Min	Minute
MRM	Multiple reaction monitoring
MP	Medicinal product
MS	Mass spectrometry
MS	Member state
MTR	Multiple time regression
MW	Molecular weight
MyD88	Myeloid differentiation factor 88
m/z	Mass/charge
NAA	*N*-alkylamide
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NME	New molecular entity
NO	Nitric oxide
OECD	Organisation for Economic Co-operation and Development
OTC	Over the counter
P_{app}	Apparent permeability coefficient
$P_{app,ab}$	Apparent permeability coefficient from the apical to basolateral direction
$P_{app,ba}$	Apparent permeability coefficient from the basolateral to apical direction
PARNUTS	Particular nutritional uses
PBS	Phosphate buffered saline
PC	Pheochromocytoma
PD	Pharmacodynamic
PDA	Photo diode array
PDE	Permitted daily exposure
PEA	Palmitoylethanolamide
PG	Propylene glycol
PGE2	Prostaglandin E2
PK	Pharmacokinetic
p.o.	Per os
PPAR-α	Peroxisome proliferator-activated receptor-α
PT	Prothrombin time
PTZ	Pentylenetetrazole
Q_{1d}	Cumulative quantity obtained after 1 day, expressed as % of the effective

	dose applied
QC	Quality control
RP	Reversed phase
R_t	Retention time
SA	*Spilanthes acmella*
SAR	Structure-activity relationship
SC	Stratum corneum
SD	Standard deviation
SEM	Standard error of the mean
SIM	Selected ion monitoring
S/N	Signal-to-noise ratio
SPE	Solid phase extraction
SS	Steady state
SSR	Sum of squared residuals
t	Time
$t_{1/2}$	Half-life
$t_{1/2,brain}$	Efflux brain half-life
$t_{1/2,e}$	Elimination half-life
t_{lag}	Lag time
TEER	Transepithelial electrical resistance
TFA	Trifluoroacetic acid
THMP	Traditional herbal medicinal product
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TRIF	TIR domain-containing adapter-inducing interferon-β
TrkA	Tropomyosin receptor kinase A
TRPV1	Transient receptor potential vanilloid 1
UHPLC	Ultra high performance liquid chromatography
USA	United States of America
UV	Ultraviolet
V_0	Vascular brain distribution volume
V_d	Volume of the donor compartment
V_g	Tissue brain distribution volume

vis	Visible
WE	Well-established
x	Distance from the donor compartment

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CHAPTER I

INTRODUCTION

"Knowledge speaks, but wisdom listens."

Jimi Hendrix
(°1942-†1970, American rock guitarist, singer, and songwriter)

CHAPTER I

INTRODUCTION

1. *N*-ALKYLAMIDES

It is well-known that plants are used all over the world for their medicinal properties. Based upon the ethnopharmacological properties of plants, many drugs have been developed, with aspirin and morphine as renowned examples. Moreover, many of currently used synthetic drugs still originate from plants. Between 2001 and 2010, in the United States of America (USA), the Food and Drug Administration (FDA) approved 15 drugs derived from plants of which even 10 were new molecular entities (NME). The generic names of these NME approvals, with the natural lead compound between brackets, are nitisinone (leptospermone), miglustat (1-deoxynojirimycin), tiotropium bromide (atropine), trospium chloride (atropine), solifenacin (quinine), nabilone (Δ^9-tetrahydrocannabinol), methylnaltrexone bromide (morphine), tetrabenazine (emetine), artemether and lumefantrine (artemisinin) and cabazitaxel (paclitaxel). An important advantage of these and other natural products is the wide structural diversity. As such, natural products are a successful source for drug leads in drug discovery [1-3]. Europe has a very long tradition in herbal medicinal products and has a well-developed market in this area with established criteria for quality control and licensing. It is an important industrial sector in Europe, accounting for half of the worldwide sales of herbal medicinal products. Five billion US dollar was spent in European countries on over-the-counter (OTC) herbal medicinal products. However, the importance of herbal medicinal products varies considerably in the Member States of the European Union (EU). Germany is the leading EU country for herbal drugs with annual sales of 2.5 billion US dollar. About 80% was sold as OTC products and 20% on prescription. *Ginkgo biloba*, Horse-chestnut, Hawthorn and St. John's Wort are the main plants prescribed in Germany. Other countries specialised in herbal drugs are France, Italy and Switzerland [4-7].

The biomedical interest in *N*-alkylamides (NAAs), a large group of secondary metabolites found in various medicinal plants, has increased enormously. NAAs occur in more than 25 plant families, have a wide structural diversity and are potential lead compounds for functional food, food supplements, cosmetics and medicines. They are known to have analgesic, antimicrobial, insecticidal, sensory, anti-inflammatory and immune-modulating properties and are traditionally used to treat toothaches, skin and gastric diseases, sexual dysfunctions and viral infections. Besides these ethnopharmacological

uses in traditional medicine, plants containing NAAs are also used as spices for their pungent and tingling sensations, and are incorporated in topical cosmetics for their wrinkle smoothing, anti-aging properties. These compounds thus have a broad biofunctional spectrum interacting with different targets via several mechanisms [8].

N-alkylamides possess a wide structural diversity with as common feature a central amide bond. Generally, the plant NAAs consist of an aliphatic chain of poly-unsaturated fatty acids linked to a short-chain amine. The NAA structural classification is build up from these two parts, starting with 'F' (indicative for fatty acid part) followed by the fatty acid category (from 1 to 13) and ends with 'M' (indicative for the amino part) followed by the amino category (from 1 to 13). Combining the F part with the M part yields thus different chemical NAA classes (FxMy nomenclature) (Figure 1). A chemical and functional online available database, Alkamid®, has been developed in our research group to give a clear overview of the botanical occurrence of NAAs in the plant families, their chemistry, structural classification, physicochemical properties and their biofunctionalities [8]. At regular time points, this database is updated. Since 2011, 62 new NAAs were added to the database.

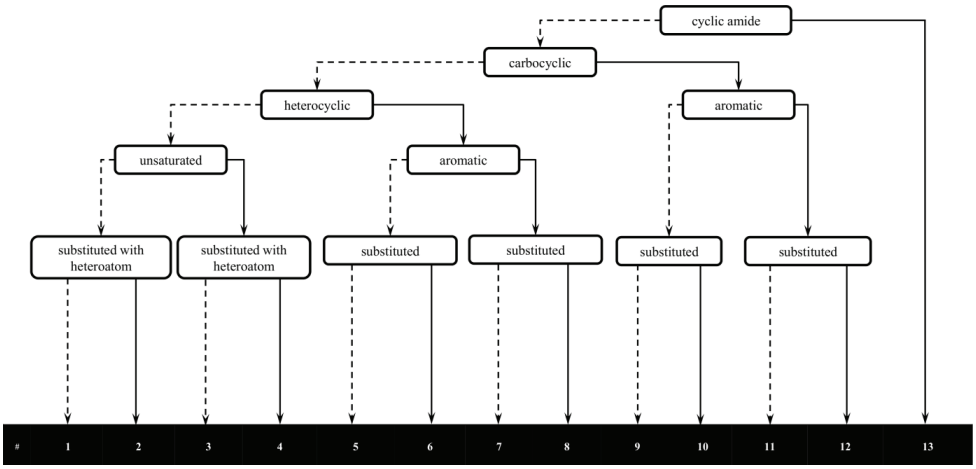

Figure 1: FxMy classification of *N*-alkylamides (solid line: yes, dotted line: no) (source: Boonen *et al.* 2012 [8]).

To date, Alkamid® contains 439 *N*-alkylamides. User information on the database illustrates that researchers originating from 84 countries (3043 sessions) consulted the database between 10 December 2012 and 8 February 2016 (3 years and 2 months). After excluding countries having a bounce percentage of 100, however, 50 countries (1078 sessions) are left. There was a total bounce percentage of 65%, which is between the 40-60% bounce rate benchmark for content websites and 70-98% bounce rate benchmark for blogs [9]. Figure 2 shows the top ten of countries by number of database users.

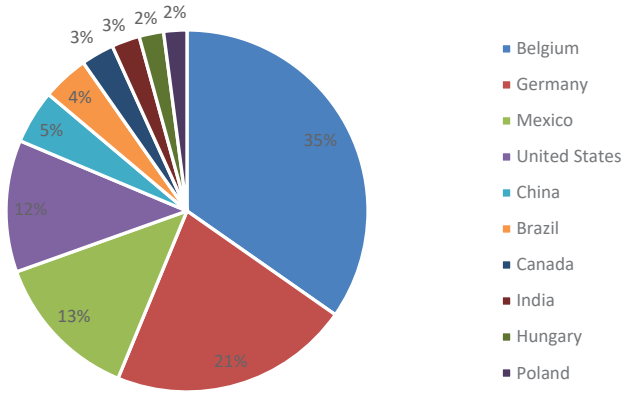

Figure 2: Countries of Alkamid® database users (%).

The percentages in Figure 2 seem to display a correlation with the embeddedness of traditional medicine in local culture, with Mexico, China, Brazil and India as countries well-known for their use of plants consisting of *N*-alkylamides in traditional medicine [8, 10]. A similar correlation can be seen in the citation statistics of the database. Up till now, the paper by Boonen *et al.* (2012), which reported on the Alkamid® database, has been cited 24 times, with authors from Belgium, Mexico, USA, China, Brazil, India, Hungary, Colombia, Jordan, Austria, Finland, Portugal, Taiwan and Turkey (Web of Science 11/06/2016) [8].

In the present research, two important NAAs, *i.e.* spilanthol and pellitorine (Figure 3) were studied for their transdermal and pharmacokinetic properties. Spilanthol (affinin) is an F3M1 *N*-alkylamide present in several plants like *Spilanthes acmella*, in which it is the best known and most abundant NAA. Pellitorine (F3M1 NAA) occurs in different plants and is the most abundant NAA in *Anacyclus pyrethrum*. Spilanthol (deca-2E,6Z,8E-trienoic acid isobutylamide) is a triene NAA, while pellitorine (deca-2E,4E-dienoic acid isobutylamide) is a diene NAA, possessing only two unsaturated carbon bonds. Different pharmacological effects are already described for spilanthol and pellitorine, such as antifungal and insecticidal properties [11-16]. Spilanthol also has analgesic, anti-inflammatory and antimutagenic properties, while pellitorine shows antithrombotic, antiseptic, antiprotozoal and anticancer properties, which will be further discussed in Chapter VII [17-27].

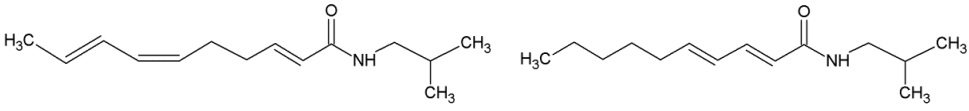

Figure 3: Structures of spilanthol (F3M1 NAA) (left) and pellitorine (F3M1 NAA) (right).

Spilanthes acmella as well as *Anacyclus pyrethrum* belong to the Asteracea plant family, as do *Achillea millefolium* and *Achillea ptarmica*, two plants of which the NAA content will be analytically characterised (Figure 4).

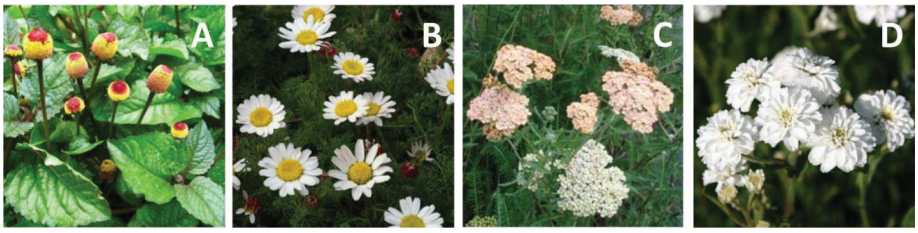

Figure 4: A: *Spilanthes acmella*, B: *Anacyclus pyrethrum*, C: *Achillea millefolium*, D: *Achillea ptarmica*.

2. ANALYTICS OF PLANT *N*-ALKYLAMIDES

The quality control of plants is very important as the consistent efficacy and safety of the plant-derived products on the market are strongly related to the plants' quality. A correct identification of compounds in commercially plant-derived products is vital, as misidentification of compounds can have serious consequences. For the extraction of plants, different extraction methods are applied, such as maceration, hydrodistillation, Soxhlet extraction and supercritical fluid extraction [28, 29]. Various analytical methods are used for the chromatographic profiling of plants, such as thin layer chromatography (TLC), high performance liquid chromatography coupled to ultraviolet / photo diode array detection (HPLC-UV/PDA) and gas chromatography (GC) [30]. Chromatography is an analytical technique which is essential for quality control and standardisation of phytotherapeutics. HPLC is often used in phytochemistry to isolate natural products and to control their purity. HPLC coupled to PDA is widely used as it allows to detect multiple compounds at different wavelengths. It is frequently used for screening of plant extracts in which the UV spectra offers valuable information about the type of compounds. However, liquid chromatography - mass spectrometry (LC-MS) is a more sensitive and selective analytical method to detect and identify compounds [31]. There are different kind of mass analyser systems, such as a single quadrupole and a triple quadrupole MS system. A single quad MS detection system provides additional information compared to UV as the *m/z* values are available of the eluting compounds. A triple quad MS is a tandem MS consisting of three quadrupoles, with two mass analysers in series and in between a cell for collision induced dissociation (CID). Using MS^2, information about the molecular weight of a compound and its structure becomes available.

During the last decades, a growing interest can be observed in bioanalytical methods to quantify NAAs in biological matrices (*e.g.* plasma, serum, tissues). However, to date, there are only few bioanalytical methods reported in literature for the quantification of NAAs. Woelkart *et al.* (2005) for instance investigated the bioavailability and pharmacokinetics of six NAAs from the roots of *Echinacea angustifolia* in humans after oral administration. A sample preparation method was performed on the plasma samples using a solid phase extraction (SPE) technique. 1.0 ml of plasma was applied on a C18 100 mg SPE column. Final samples were analysed on a LiChroCART (55x2 mm, 3 µm) RP-C18 end-capped column with HPLC-electrospray ionisation (ESI) ion trap MS. The injection volume was 10 µl and the run time was 20 min per sample. No ion suppression effects and no interferences of the analytes were observed. The limit of detection (LoD) was 3 ng/ml. After oral intake, NAAs were absorbed with maximum concentration values reached between 20 and 30 minutes [32]. Matthias *et al.* (2005) reported absorption data of NAAs in humans after taking *Echinacea* tablets. 1 ml of supernatant of the plasma samples was needed for the extraction of the NAAs on a SPE C18 cartridge. The samples were analysed with a C18 column (2x100 mm, 3 µm) using a HPLC coupled to a single quad MS with an atmospheric pressure chemical ionisation (APCI) source operating in positive ion selected ion monitoring (SIM) mode. The limit of quantification (LoQ) was 1 ng/ml. Per sample, the run time was 13 min. Resulting from this method, the pharmacokinetic data showed that the maximum NAA concentration in human plasma was reached after 2 hours post ingestion [33]. The same bioanalytical method was used in another study by Matthias *et al.* (2007) in which it was shown that there is no significant difference in bioavailability of NAAs between liquid and tablet preparations in humans [34]. Woelkart *et al.* (*2009*) described the pharmacokinetics of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides in rats after oral administration. For the sample preparation, 200 µl of plasma was needed. Concentrations of the *N*-alkylamides were analysed in tissues (liver and brain) as well. The NAAs were extracted using a SPE C18 100 mg column. Final samples were injected (20 µl) on a C18 column (2x50 mm, 5 µm) and analysed using HPLC-ESI quadrupole MS. There was a short run time of 5 min per sample. The maximum concentration of *N*-alkylamides appeared already within 8 min post administration in the brain [35]. Furthermore, Goey *et al.* (2011) developed a sensitive HPLC-MS/MS method for the quantification of undeca-2-ene-8,10-diynoic acid isobutylamide in human plasma. A liquid-liquid extraction was performed of the plasma samples (1 ml plasma needed). A C18 HPLC column (50x2 mm, 3 µm) and a triple quadrupole MS with ESI source was used. The injection volume was 15 µl. The lower limit of quantification (LLoQ) was 0.05 ng/ml [36]. Goey *et al.* (2012) also developed a LC-MS/MS method for the bioanalysis of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides in human plasma [37]. The same HPLC-MS equipment and column were used as described previously [36]. The injection volume

was 20 µl and a run time of 7.5 min was used. 300 µl plasma volume was needed for the liquid-liquid sample preparation and a LLoQ of 0.01 ng/ml was obtained [37].

The methods described above have a number of important drawbacks. A first disadvantage is the large volume of plasma needed for the analysis of the samples, ranging between 200 µl – 1 ml. Since such quantities of blood are not always available in *in vivo* experiments, there is a need for a sample preparation method requiring lesser volume. When experiments are performed with humans, larger blood volumes are available, but this is however not the case when working with small animals like mice. Secondly, also the long run times in the available studies are problematic. The run times on HPLC-MS vary between 5 and 20 min per sample. Only one method has an acceptable run time of 5 min. This is definitely a drawback that has to be overcome in the future. A third important weakness in the available research is the high injection volumes used. All methods use a HPLC system, requiring higher injection volumes (10-20 µl) compared to ultra high performance liquid chromatography (UHPLC). Moreover, the run time can be shortened using UHPLC. In conclusion, there is a need for a sample preparation method requiring small blood volumes, which can be analysed with a more sensitive UHPLC-MS/MS method.

3. SKIN AS BARRIER

Drugs can be administered in various ways, *e.g.* through oral or dermal route. Several physiological barriers in the human body can however complicate or prevent the absorption of compounds into the systemic blood circulation. Transdermal drug delivery offers several advantages in comparison with the traditional oral administration route, such as: minimisation of the first pass effect and avoiding the acidic environment of the stomach, which could be responsible for drug degradation. This results in a higher bioavailability of the drug, a decrease in side effects and more stable plasma levels. In addition, it provides more controlled drug delivery [38]. However, when topically applied, compounds must be able to penetrate the stratum corneum (SC), the outer layer of the skin, and reach the viable cells of the epidermis and underlying dermis with its blood vessels in order to obtain a biological effect (Figure 5). The 10-20 µm thick SC acts as a penetration barrier, which protects the body against bacterial, enzymatic or chemical impacts and prevents excess of water loss. The SC is mainly composed of several layers (12-16) corneocytes which are surrounded by multilamellar organised lipids. Corneocytes are flattened dead cells, filled with keratin filaments and water, which are encapsulated by the cornified envelope, consisting of protein layers and a covalent bound lipid envelope. The lipid phase is essentially composed of ceramides (50%), cholesterol (25%) and free fatty acids (10%), as well as small amounts of cholesterol esters and cholesterol sulphate. The SC is

often presented as a 'brick and mortar' model, where the corneocytes are the bricks and the lipids correspond to the mortar. The 50-100 µm thick viable epidermis is directly adjacent to the SC. This epithelium is composed of layers (from bottom to top: stratum basale, stratum spinosum, stratum granulosum and stratum lucidum) with various states of differentiation of keratinocytes. Underlying the epidermis, the vascular dermis (1-2 mm thick) offers the mechanical support [39-41]. Often, topically applied compounds are not able to penetrate the SC, because they do not have the ideal physicochemical properties to pass the SC.

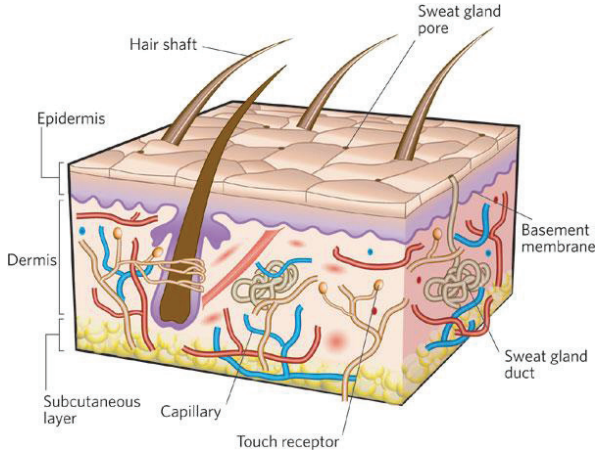

Figure 5: Structure of the skin (source: MacNeil, 2007 [42]).

Besides the permeation of drugs through sweat glands and hair follicles, two other ways to pass the SC constitute the intercellular or the transcellular pathway through the corneocytes. Via the intercellular pathway, diffusion of substances into the body largely occurs through the lipid domains between the corneocytes. Certain drugs can easily pass the skin, such as clonidine, nicotine, estradiol, testosteron, fentanyl, lidocaine, nitroglycerin, ethinyl estradiol, norelgestromin, norethindrone, oxybutynin and scopolamine. These drugs have a molecular weight lower than 500 g/mol, an optimal partition coefficient, low melting point and a certain water-oil solubility [38, 43-45].

The passive diffusion process of compounds for steady state flux (J), to evaluate the drug permeation rate through SC is described by Fick's first law:

$$J = -D \times \frac{dC}{dx}$$

in which D is the diffusion coefficient and dC/dx a concentration differential across a membrane [46].

Previous research by our group already indicated the successful dermal administration of spilanthol in which spilanthol was able to reach the systemic blood circulation [47-49]. When spilanthol is topically applied on pig buccal mucosa, systemic effects can be expected next to local mucosa effects as spilanthol is able to significantly penetrate buccal mucosa. Depending on the formulation of the dose solution (*e.g.* as commercially available products, ethanolic extract or dissolved in propylene glycol/H_2O mixtures), spilanthol mucosal permeability coefficient (K_p) values ranging between 5.3 and $47.5 \cdot 10^{-3}$ cm/h were observed [48]. Spilanthol penetrates through the human stratum corneum and the viable epidermis as well, thereby reaching the dermis and thus the systemic circulation, with K_p values between 0.6 and $53.3 \cdot 10^{-4}$ cm/h [47].

4. BRAIN AS BARRIER

Once present in the blood, compounds distribute to different tissues and some compounds can even cross the blood-brain barrier (BBB) and modulate central nervous system (CNS) effects. The BBB (Figure 6), consisting of capillary endothelia, is almost a continues cell layer differing from endothelial cells elsewhere in the body due to the absence of fenestrae, the presence of tight junctions between the endothelial cells and the fact that the pores in the endothelial cells are much smaller. The capillary endothelia is surrounded by a basal lipid membrane. The layer of astrocytes, glial cells in the brain, biochemically supports endothelial cells of the BBB. Due to the BBB, the passage of drugs from the brain capillaries to the brain tissues is much more complicated than in other places of the body. The brain acts as a physical and metabolic barrier and hence strictly regulates the brain entry of compounds. The BBB is substantially only permeable for good lipid-soluble molecules (passive diffusion) and to molecules that make use of a carrier. The resorption of a drug is influenced by the ionisation degree at pH 7.4, the lipid/water partition coefficient and the plasma protein binding. If there is an infection, the permeability of the barrier increases. Brain penetration of drugs is typically described by the brain to plasma concentration ratio and the influx clearance [50-54].

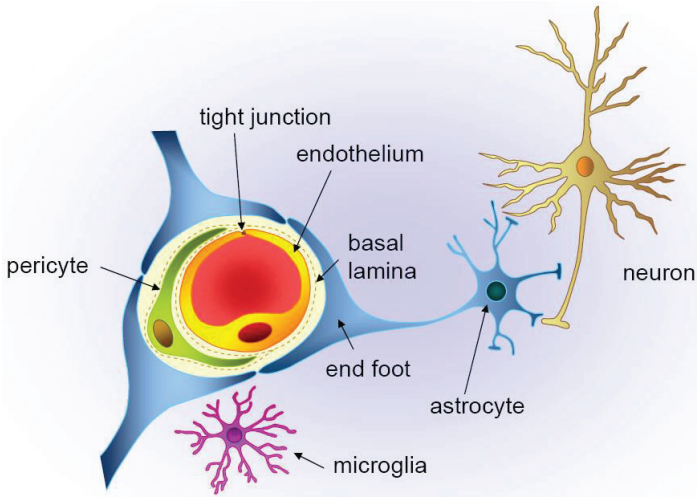

Figure 6: Structure of the blood-brain barrier (source: Abbott and Yusof, 2010 [55]).

Concerning the ability of *N*-alkylamides to penetrate the BBB, only little pharmacokinetic research is at present available. Woelkart *et al.* (2009) present the only study which has already measured the concentration of NAAs in the brain. They demonstrated that dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides (tetraenes originating from *Echinacea*), orally given to rats, are bioavailable with a rapid passage across the blood-brain barrier [35].

Besides this pharmacokinetic study, there are numerous pharmacodynamic studies with NAAs or NAA-containing plant extracts exerting central nervous system effects. Due to the structural similarities of *N*-alkylamides with the endocannabinoid anandamide or arachidonoylethanolamine (AEA) (cannabinoid receptor (CBR) agonist), several binding studies were conducted with NAAs and the cannabinoid receptors. To date, two types of cannabinoid receptors are known: cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R), both belonging to the G-protein coupled receptors. The CB1Rs are predominantly located in the central nervous system and to a lesser extent in peripheral cells, tissues and organs like leukocytes, spleen and heart, while CB2Rs are mainly found on immune cells, in the spleen and to a lesser extent in the brain. In the brain, CB1Rs are present in (1) the cerebellum, basal ganglia and substantia nigra, which are areas involved in the coordination of movement; (2) amygdala and hippocampus, areas involved in emotions, memory and learning and (3) cerebral cortex, which is involved in complex cognitive functions and attention [56]. Due to modulating cytokine release, CB2Rs exert anti-inflammatory effects. In case of cerebral hypoxia-ischemia or neuro-inflammation, CB2Rs expression on microglial cells in brain are upregulated. When activated, CB1Rs reduce cell activity of neurons and modulate the release of neurotransmitters

(gamma-aminobutyric acid (GABA), glutamate, serotonin, dopamine, acetylcholine, noradrenaline and histamine) [56]. The endocannabinoid system is involved in various biological functions like memory, pain, movement, appetite and immune regulation. The endocannabinoid signaling is modified in many neurodegenerative diseases, such as Parkinson's disease, Huntington's disease and Alzheimer's disease. Therefore, it is believed that ligands of CBRs are highly therapeutic interesting [57].

Various binding studies were performed with NAAs to determine the binding affinity to CB1R and CB2R. Woelkart *et al.* (2005) showed that NAAs from *Echinacea angustifolia* roots exhibited affinity to CB1R and CB2R. Modest inhibition of fatty acid amide hydrolase (FAAH) was moreover detected at 25 nM of tetradeca-2E-ene-10,12-diynoic acid isobutylamide, while weak inhibition was observed with dodeca-2E,4E,8Z-trienoic acid isobutylamide and dodeca-2E,4E-dienoic acid isobutylamide. FAAH is an enzyme which hydrolyses anandamide and consequently, inhibition of the enzyme prolongs the activity of CB ligands [58]. Raduner *et al.* (2006) investigated the binding affinity of the *N*-alkylamides dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and dodeca-2E,4E-dienoic acid isobutylamide towards cannabinoid receptors on HEK293 cells. A higher affinity was observed towards CB2R compared to CB1R. Undeca-2E-ene-8,10-diynoic acid isobutylamide did not bind the CB2R [59]. Another study carried out by Matovic *et al.* (2007) demonstrated that dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide from *Echinacea* species is a high affinity binder for the CB2R, while dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide and dodeca-2E,4E,8E,10Z-tetraenoic acid isobutylamide showed lower affinities towards the CB2R. On the contrary, the synthetic dodeca-2E,4E,8E,10E-tetraenoic acid isobutylamide did not exhibit binding affinity for the CB2R [60]. Furthermore, it has been shown that several *N*-alkyl diene and triene amides from *Otanthus maritimus* and NAAs from *Heliopsis helianthoides* and *Lepidium meyenii* exhibited binding affinity for CB1R and CB2R [61, 62]. In the majority of the studies, the binding affinity of the NAA towards the CB2R was higher compared to the CB1R.

Other functional studies into the CNS effects of NAAs are the various investigations related to the analgesic properties of NAAs. Plants containing NAAs have already been used for many years for these medicinal properties. *Spilanthes acmella* for instance, also known as 'toothache plant', is frequently used in traditional medicine for its ability to reduce toothaches [63]. Rios *et al.* (2007) demonstrated analgesic properties of a 10 µg/ml dichloromethane *Heliopsis longipes* extract by means of a GABA release experiment in mice brain slices. Also spilanthol ($1 \cdot 10^{-8}$ M - $1 \cdot 10^{-4}$ M) showed a dose dependent GABA release and thus displayed analgesic properties [17]. It has furthermore been demonstrated that 3-100 mg/kg of an ethanol extract of *Heliopsis longipes* (contains 10% spilanthol) exerted analgesic effects in mice using the acetic acid induced writhing and hot-plate tests

[64]. A study of Nomura *et al.* (2013) showed analgesic effects of an ethanolic *Acmella oleracea* extract in mice by means of the hot plate test (thermal heat hyperalgesia). The underlying mechanism of this analgesic action is still unclear and it is difficult to conclude if its action is centrally or peripherally mediated [65]. Moreover, NAAs present in *Echinacea* inhibit cyclooxygenase-2 (COX-2) enzyme activity in human neuroglioma cells, which can suppress pain and inflammation [66]. Déciga-Campos *et al.* (2012) confirmed the analgesic activity of spilanthol and the *Heliopsis longipes* extract. Other CNS effects were also investigated: a prolonged time of hypnosis induced by sodium pentobarbital, an altered anxiety behaviour and a decrease in time of clonic and tonic pentylenetetrazole (PTZ)-induced seizures (anticonvulsant effect) were observed [19]. Another NAA, capsaicin, which is the major NAA in hot peppers, also showed anti-epileptic properties: it has been shown that capsaicin can prevent epileptogenic seizures induced by kainic acid [67].

5. REGULATORY STATUS OF PLANT *N*-ALKYLAMIDES

Some unfortunate events have influenced the development of regulations considering the safety of medicines and food. Council Directive 65/65/EEC of 26 January 1965 on medicinal products, for instance, came into force after the thalidomide disaster. In the 1960s, thousands of pregnant woman suffering from nausea, who had taken the sedative drug thalidomide, gave birth to babies with born birth deformities. The implementation of this Directive gave rise to a requirement of prior marketing approval for medicinal products before gaining access to the market. Not only in the pharmaceutical industry, but also in the food industry, serious incidents took place, such as the bovine spongiform encephalopathy and dioxin crises in the 1990s. Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, was adapted in 2002. To guarantee safety in the cosmetic industry, Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products was issued, replacing Directive 76/768/EC [68-72].

A special category of medicinal products are the herbal medicinal products, consisting of herbal substances/preparations. These herbal medicinal products also apply to Directive 2001/83/EC, relating to medicinal products for human use, but for traditional herbal medicinal products, a simplified registration procedure is described in Directive 2004/24/EC, amending Directive 2001/83/EC [73, 74]. In the majority of the EU countries, herbal drugs are generally sold in pharmacies as licensed (non-)prescription drugs. However, in certain Member States (*e.g.* the Netherlands and the United Kingdom), herbal products are sold as food supplements or dietary

supplements. In the latter case, they may not be represented with therapeutic or prophylactic claims [7]. There are different routes to bring herbal products on the market in Europe. They can be classified as (1) medicinal products (well-established used herbal medicinal product or traditional herbal medicinal products), (2) cosmetics, (3) food and (4) medical devices. The classification depends on the route of administration, the formulation, the safety and the intended use and the choice is made by the responsible manufacturer or company. Authorities verify if the right decision is made [75].

Currently, worldwide there are products on the market containing *N*-alkylamides or NAA containing plants and no unequivocal regulation exists for these products. Often, there is no control on the composition, quality and claims of these products. Some of these products are marketed as medicinal products containing the well-known *Echinacea purpurea* plant, famous for its anti-inflammatory properties. A critical discussion of present and advisable future regulations in this respect seems imperative.

6. STUDY OBJECTIVES

N-alkylamides are secondary metabolites occurring in many plants. These bioactive compounds, with many structural variations, are known to exert various pharmacological properties. Many central nervous system pharmacodynamic effects are reported. However, only one study has described the ability of NAAs in *Echinacea* to penetrate the almost impermeable BBB [35]. As spilanthol (deca-2E,6Z,8E-trienoic acid isobutylamide) and pellitorine (deca-2E,4E-dienoic acid isobutylamide) are two promising NAAs, the question rises whether these NAAs are able to cross the BBB and possibly elicit CNS effects. In order to explore physiological barrier properties of these NAAs, the following questions are put forward:

(1) Which *N*-alkylamides are present in the plants *Achillea millefolium* and *Achillea ptarmica*?

Achillea millefolium and *Achillea ptarmica* are plants belonging to the Asteracea family and are traditionally used for their medicinal properties. It has already been shown that some NAAs are responsible for these pharmacological actions. Often, there is no information available on the specific bioactive compounds present in plants. Therefore, the present study sets out to characterise the NAA content of the two plants.

(2) Is the plant *N*-alkylamide pellitorine able to penetrate the human skin?

The transdermal behaviour of pellitorine is questioned, and if so, the quantitative character of its kinetics is searched for to allow pharmacokinetic estimations.

(3) Is the use of a single quad MS detector in high-throughput transdermal research of plant extracts a useful analytical tool?

After performing a Franz Diffusion Cell (FDC) experiment, many samples are obtained and a simple and easy-to-use analytical method is needed to quantify compounds. The use of UHPLC, coupled to a single quad MS detector to quantify NAAs in samples obtained from transdermal research, might be appropriate. By using this method, the local skin pharmacokinetic properties of spilanthol in *Spilanthes acmella* extract and pellitorine in *Anacyclus pyrethrum* extract will be evaluated.

(4) Is the plant *N*-alkylamide spilanthol able to cross the intestinal barrier *in vitro* and *in vivo* and furthermore to cross the BBB?

Are NAAs able to be absorbed from the gastrointestinal lumen after oral administration, thereby passing the oral and/or intestinal mucosal barriers? Few studies about the pharmacokinetic properties of NAAs are available. One study of Matthias *et al.* (2004) investigated the transport of selected NAAs present in *Echinacea* through the Caco-2 cell monolayer. After 90 minutes, more than 50% of the NAAs penetrated the monolayer [76]. In our study, the permeability of spilanthol will be investigated using a Caco-2 cell monolayer. We want to confirm these findings *in vivo*: the intestinal barrier properties of spilanthol will be explored in rats in an oral gavage experiment. Furthermore, the ability of spilanthol, once present in the blood, to cross the blood-brain barrier in mice is also questioned. Moreover, the distribution of spilanthol within the brains (tissue versus capillaries) will be evaluated as well. For this, a sample preparation method must be developed in order to quantify spilanthol in blood/brain samples and to analyse samples with a self-developed bio-analytical UHPLC-MS^2 method.

(5) Is pellitorine able to cross the intestinal barrier *in vitro* and *in vivo* and furthermore to cross the BBB?

Pellitorine is an *N*-alkylamide showing slight structural differences with spilanthol. The same pharmacokinetic properties as mentioned for spilanthol are questioned for pellitorine, *i.e.* is pellitorine able to cross the Caco-2 cell monolayer, the rat intestinal barrier and the BBB of mice?

(6) What is the regulatory status of *N*-alkylamide containing health products?

At present, products containing *N*-alkylamides are available on the market as food supplements, cosmetics or medicinal products. As these NAAs have shown to be able to penetrate physiological barriers and have demonstrated various biological effects, the regulatory status of these various health products has to be questioned.

7. THESIS OUTLINE

The content of this research is presented in six logically ordered chapters. All the chapters can however also be read alone, as a separate introduction covers the content of each chapter.

In **Chapter II**, the *N*-alkylamide profiling of ethanolic *Achillea millefolium* and *Achillea ptarmica* roots, flowers, leaves and stem extracts is performed. NAAs are identified using two analytical techniques, *i.e.* HPLC-ESI-MS and GC-EI-MS.

The skin pharmacokinetic properties of pellitorine are investigated in **Chapter III**. A FDC experiment using human skin is performed using two different pellitorine dose solutions: one containing the *Anacyclus pyrethrum* extract and the other containing purified pellitorine. Pellitorine was isolated and purified from the *Anacyclus pyrethrum* extract using semi-preparative HPLC. Pellitorine is quantified in the receptor fluid at predefined time points and in the different skin layers at the end of the experiment (*i.e.* 24h). Moreover, the permeability coefficient of pellitorine is calculated.

In **Chapter IV**, the transdermal properties of spilanthol and pellitorine in *Spilanthes acmella* extract and *Anacyclus pyrethrum* extract, respectively, are evaluated and quantified using a UHPLC coupled to a new type of single quadrupole MS detector. The advantages of using this type of detector in high-throughput transdermal research are discussed.

Chapter V describes the permeation behaviour of spilanthol through several biological barriers. Transport experiments are performed *in vitro* and *in vivo*. The permeation of spilanthol through a Caco-2 cell monolayer and the rat intestinal barrier after oral administration are investigated. Transport experiments using the Caco-2 cell monolayer are carried out in both the apical-to-basolateral and the basolateral-to-apical direction. The initial blood to brain barrier rate kinetics are investigated using multiple time regression (MTR) after injection of spilanthol in mice. Furthermore, the efflux of spilanthol from the brain to the blood is evaluated in an efflux study with mice. In both experiments (influx and efflux), blood is obtained and brains are collected at specified time points. Quantification of spilanthol in brain and serum samples is conducted using a bio-analytical UHPLC-MS method. Prior to the analyses of the samples, a sample preparation method is developed using solid-phase extraction.

In **Chapter VI**, the physiological barrier penetration properties of pellitorine are investigated. For this purpose, a similar methodology as the one discussed in Chapter V is used. Chapter VI also offers an evaluation of the *in vitro* metabolic stability of pellitorine. Brain and blood samples are analysed using a self-developed bio-analytical UHPLC-MS method.

Lastly, in **chapter VII**, the regulatory status of NAAs or NAA containing plants in health products is discussed, considering their pharmacological properties, the NAA containing products on the market and the current regulatory regulations and guidelines of health products in Europe.

To conclude, the **relevance** of this research and **future** perspectives are discussed.

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CHAPTER II

LIQUID AND GAS CHROMATOGRAPHIC MASS SPECTROMETRIC *N*-ALKYLAMIDE PROFILING OF *ACHILLEA PTARMICA* AND *ACHILLEA MILLEFOLIUM* EXTRACTS

"The beauty of a living thing is not the atoms that go into it, but the way those atoms are put together."

Carl Sagan
(°1934-†1996, American astronomer)

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ABSTRACT

The analytical *N*-alkylamide (NAA) profiling of two plants belonging to the Asteracea family, *i.e. Achillea millefolium* and *Achillea ptarmica*, was performed. The structures of NAAs have been assigned in ethanolic extracts of *Achillea millefolium* and *Achillea ptarmica* using HPLC-ESI-MS and GC-EI-MS. Different extracts were prepared from the roots, the leaves, the stems and the flowers. Using both analytical techniques, the structures of 14 and 15 NAAs have been assigned in *Achillea ptarmica* and *Achillea millefolium*, respectively. Two new NAAs, previously never observed in *Achillea ptarmica*, were assigned: deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) or a related isomeric compound and deca-2E,4E-dienoic acid *N*-methyl isobutylamide. The structure of homospilanthol or a related isomeric compound was also assigned in *Achillea millefolium* for the first time.

CHAPTER II

LIQUID AND GAS CHROMATOGRAPHIC MASS SPECTROMETRIC *N*-ALKYLAMIDE PROFILING OF *ACHILLEA PTARMICA* AND *ACHILLEA MILLEFOLIUM* EXTRACTS

Main focus in this chapter:

- To analytically characterise the *N*-alkylamide content in *Achillea ptarmica* using HPLC-ESI-MS and GC-EI-MS.
- To profile the *N*-alkylamides in *Achillea millefolium* with HPLC-ESI-MS and GC-EI-MS.

1. INTRODUCTION

The genus *Achillea* (*A.*) consists of more than 120 species worldwide, mainly distributed in the Northern Hemisphere. *Achillea* species have been used in traditional folk medicine for many years to treat various diseases and are especially known to cure slow-healing wounds, which explains the name of the genus *Achillea* [1, 2]. It might originate from the Greek mythological character Achilles, as he used the plants to treat the soldiers' wounds during Trojan War [3]. Two species of the *Achillea* genus (*millefolium* and *ptarmica*) will be discussed in detail: *A. millefolium* L. and *A. ptarmica* L., both belonging to the Anthemideae tribe and Asteraceae plant family. *A. millefolium* and *A. ptarmica* plants are both ethnopharmacologically used to treat stomach disorders and stomach ache, respectively [4, 5].

Achillea millefolium, also known as yarrow, consists of several closely related species, named a species complex or aggregate. It is one of the most important plants belonging to the *Achillea* genus, due to its medicinal features. A diversity of pharmacological properties is ascribed to this plant, such

as spasmolytic, anti-inflammatory, analgesic, haemostatic, antidiabetic, cholagogue, antitumor, antioxidant, antiseptic and liver protective effects. Furthermore, tea from *Achillea millefolium* is used to treat diseases of the gastrointestinal tract, like dyspepsia, flatulence, abdominal pain, diarrhea, stomach ache and digestive complaints. *Achillea millefolium* can be consumed as essential oil, infusion or alcohol extract, decoction, hydroalcoholic, methanolic or aqueous extracts [1, 2, 6-10].
An aqueous extract of the aerial parts of the plant protected the gastric mucosa in Wistar rats against ethanol- and indomethacin induced gastric lesions. It also healed acetic acid induced chronic gastric lesions [6]. Potrich *et al.* (2010) reported that the antioxidant properties are at least partly responsible for the gastroprotective effects of the extract [11]. Furthermore, an *Achillea millefolium* extract of the aerial parts (hexane:ether:methanol 1:1:1) showed antimicrobial activity against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa and Salmonella enteritidis* [9]. Safety studies in Wistar rats showed that there were no signs of relevant toxicity after daily treatment with the extract in a concentration of 0.3-1.2 g/kg (p.o.) for 28 or 90 days [6].
Achillea millefolium is used in cosmetics as it has been proven *in vivo* that a 2% *A. millefolium* extract has a rejuvenating effect on the appearance and feeling of the skin surface [12]. The extract is a biological additive ingredient in 65 cosmetic product formulations and creams to accelerate the wound healing rate consist of 2%, 5% or 10% *Achillea millefolium* extract [13, 14]. Furthermore, there are European national pharmacopoeia monographs about *A. millefolium*: Hungarian, German, Austrian, Czech, French, Romanian pharmacopoeias, extra pharmacopoeia Martindale, British herbal pharmacopoeia and the Polish herbal compendium [15].

Another plant belonging to the Asteracea family is *Achillea ptarmica* L. (sneezewort yarrow). Althaus *et al.* (2014) reported that a dichloromethane extract of flowering aerial parts of *A. ptarmica* was found to possess antiprotozoal activity *in vitro*. The extract showed anti-*Trypanosoma brucei rhodesiense* activity (IC_{50} of 0.67 µg/ml) as well as anti-*Plasmodium falciparum* activity (IC_{50} of 6.6 µg/ml) [16].

Of special pharmacological interest, the genus *Achillea* produces several *N*-alkylamides (NAAs), which are secondary metabolites in plants and because of their wide structural diversity, they are classified according to a structural classification system, indicated as FxMy. The F and M stand for the fatty acid chain and the amine part of the NAA, respectively. X and Y represent numbers (1-13), indicative for the structure of the chains. Both chains are linked to each other through an amide bond [17]. *Achillea* NAAs consist of the more widespread isobutylamides and phenethyl amides, and especially saturated and unsaturated 5- and 6-ring alkylamides (piperidides, pyrrolidides, piperideides, pyrrolideides).

C10-, C11- and C14-olefinic and acetylenic alkylamides are characteristic for the genus *Achillea* and are mainly found in the roots of the plant [2, 3, 18-20].

Typical NAAs present in *A. millefolium* are NAAs consisting of a C10 fatty acid chain linked to a piperideide function. 2E,4E,6Z-decatrienoic acid piperideide is the main compound, while the corresponding all trans-isomer only occurs in small amounts. *N*-isobutyl-2,4-decadiene amide (pellitorine); 2,4-decadienoic acid piperidide; 2,4-decadienoic acid piperideide and 2,4,6-decatrienoic acid piperideide are *N*-alkylamides identified using GC-MS in the roots of *A. distans* Willd. subsp. *distans*, however the correct stereoisomer could not be determined. *A. distans* Willd. subsp. *distans* belongs to the *Achillea millefolium* aggregate [2]. Greger and Hofer (1989) identified 17 *N*-alkylamides in *A. millefolium*, while Greger and Werner (1990) were able to identify two additional NAAs, namely undeca-2E,4E-diene-8,10-diynoic acid piperidide and tetradeca-2E,4E,12Z-triene-8,10-diynoic acid isobutylamide [21, 22]. Besides NAAs, other compounds present in *A. millefolium* L. are volatile oils, sesquiterpene lactones, flavonoids, amino acids, polyacetylenes, polysaccharides, phenolic acids, fatty acids, vitamins, alkanes, alkaloids and bases, saponins, sterols, sugars, coumarins, and tannins [15].

The main components of *A. ptarmica* are flavonoids, some essential oils and *N*-alkylamides (carboxamides of olefinic and polyynic carboxylic acids with various amines) [16]. Kuropka *et al.* (1986) and Althaus *et al.* (2014) identified five and six NAAs in *Achillea ptarmica*, respectively [16, 23].

In this study, a thorough *N*-alkylamide profiling of ethanolic extracts from the roots, flowers, leaves and stems of *A. millefolium* and *A. ptarmica* was performed using HPLC-ESI-MS (high performance liquid chromatography electrospray ionisation mass spectrometry) and GC-EI-MS (gas chromatography electron impact mass spectrometry). This study led to the structural assignment of *N*-alkylamides, previously never reported in both plants.

2. MATERIALS AND METHODS

Chemicals and reagents

Ultrapure water (H_2O) of 18.2 MΩ.cm quality was produced by an Arium 611 purification system (Sartorius, Göttingen, Germany). Acetic acid was purchased from Sigma-Aldrich (Diegem, Belgium), while denaturated ethanol (95% ethanol denaturated with 5% diethylether) was obtained from Chem-Lab (Zedelgem, Belgium). Absolute ethanol (EtOH) and HPLC gradient grade acetonitrile (ACN)

came from Fisher Scientific (Erembodegem, Belgium). Pellitorine was purchased from Adipogen Life Sciences (99.8% purity determined by HPLC).

Plant material and extraction

Achillea millefolium (type: Wesersandstein) and *Achillea ptarmica* (type: The Pearl) were bought at tree nursery 'De Bock' in Belgium (Oudenaarde). Plants were harvested in September 2013. The fresh stems, the flowers, the roots and the leaves were collected from the plants and washed with ultrapure water. The plant parts were dried at room temperature for approximately six weeks. The dried plant parts were cut into smaller pieces (parts of approximately 1 cm) with a scissor. Extraction was performed at a ratio of plant part:solvent (w/V), ranging from 1/12 to 1/64 in case of *A. millefolium* and from 1/12 to 1/34 in case of *A. ptarmica*. As extraction solvent, 90:10 denaturated ethanol:H_2O (V/V) was used. After maceration in darkness for approximately 48h at room temperature (20-27°C, 230 rpm), the plant parts were removed by filtration (Whatman). Thereafter, the extraction solvent was removed using a rotavapor (Heidolph and Büchi), protected from light. The extraction yield of *A. millefolium* and *A. ptarmica* was between 3-8% (w/w) and 3-11% (w/w), respectively. The extract was kept in the dark at 4°C until analysis.

HPLC-UV/ESI-MS analysis

The HPLC-MS analyses were done on a HPLC system which consisted of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a Spectra System P1000XR pump, a Spectra System AS3000 autosampler, and a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA) equipped with Xcalibur 2.0 software (Thermo) for data acquisition. The plant extracts (roots, stems, leaves, flowers) were dissolved in 50:50 ACN:H_2O (V/V) (final extract ranging between 5 and 155 mg/ml) and filtered over a 0.45 µm PVDF membrane HPLC filter (Whatman) before analysis. 10 µl of this solution was injected on a Grace Prevail C18 column (250 x 4.6 mm, 5 µm) using a Waters HPLC equipped with a Waters 2487 Dual Absorbance detector set at 260 nm. A gradient with a flow rate of 1.0 ml/min, was applied as follows: t=0 min: 80:20 A:B (V/V), t=0-150 min: 10:90 A:B (V/V), t=150-151 min: 80:20 A:B (V/V), t=151-166 min: 80:20 A:B (V/V) (with A = 1% acetic acid in H_2O and B = ACN). The needle was rinsed with methanol. ESI was conducted with a capillary voltage of 3 kV. Nitrogen was used as sheath and auxiliary gas. The temperature of the heated capillary was kept at 275°C. MS-MS spectra were obtained by collision induced dissociation (CID) of the parent *m/z*, with the relative collision energy set to 35%. Structural assignment was based on the parent *m/z* values and fragmentation ions. Assuming all NAA peaks have a response

factor of 1 relative to pellitorine, the total amount of NAAs is estimated as 0.6 % w/w in *Achillea ptarmica* and 0.2 % w/w in *Achillea millefolium*.

GC-EI-MS analysis

The GC-MS analyses were performed on an Agilent 6890 instrument consisting of an automatic injector 7683 and coupled to a Mass Selective Detector 5973 (Agilent), operating in EI mode at 70 eV. The output signal was recorded and processed using Instrument Analysis MSD Chemstation (Agilent). The root plant extracts were dissolved in absolute ethanol (final extract ranging between 31 and 35 mg/ml) and samples were injected by the instrument's autosampler with an injection volume of 1 µl. Ethanol was used to rinse the syringe between injections (3 x wash post injection). An HP-5MS column (30 m length, 0.25 mm i.d., 0.25 µm film thickness) (Agilent, Belgium) was used for separation. The column oven was programmed with an initial oven temperature of 100°C, and increased to 180°C at a rate of 10°C/min, ramped to 200°C at a rate of 1°C/min, followed by increasing the temperature to 320°C at a rate of 10°C/min and held at 320°C for 1 minute. The total run time was 41 minutes. The split ratio was set at 10:1. The injector and MS transfer line temperature were kept at 210°C and 250°C, respectively. Helium (Air Products and Chemicals, Allentown, PA, USA) was used as a carrier gas with a head pressure of 71.3 kPa resulting in an average velocity of 37 cm/s. The ion source and quadrupole temperature were 150°C and 230°C, respectively. The MS detection scan range was between 40-550 *m/z*.

3. RESULTS AND DISCUSSION

The structures of *N*-alkylamides in the ethanolic *A. millefolium* and *A. ptarmica* extracts were assigned based on their MS spectra. NAAs have characteristic CID fragmentation patterns, related to the amide part of the compound and typical fragment losses are presented in Table 1 [24-26]. The loss of (1) the alkyl group directly attached to the amine; (2) the entire amine functional group, resulting in an acylium ion; (3) the amide portion of the molecule and saturation of one of the double bonds on the alkyl chain and (4) the loss of the amide portion, results in the loss of characteristic *m/z* values. From the corresponding alkyl chain of the two last losses and from the *m/z* value of the acylium ion, the number of carbons present in the alkyl chain can be determined [27]. Furthermore, also with electron impact, characteristic product ions of spilanthol, as a prototypical NAA, are formed [28-30].

Table 1: Characteristic fragment ions of NAAs after CID fragmentation.

Amine alkyl group		Loss alkyl group directly attached to the amine	Loss of entire amine functional group	Loss of the amide portion and saturation of one of the double bonds on the alkyl chain	Loss of the amide portion
Isobutylamide	**Ion**	$[(M+H)-C_4H_8]^+$	$[(M+H)-C_4H_{11}N]^+$	$[(M+H)-C_5H_9NO]^+$	$[(M+H)-C_5H_{11}NO]^+$
	-m/z	56	73	99	101
Phenylethylamide	**Ion**	$[(M+H)-C_8H_8]^+$	$[(M+H)-C_8H_{11}N]^+$	$[(M+H)-C_9H_9NO]^+$	$[(M+H)-C_9H_{11}NO]^+$
	-m/z	104	121	147	149
2-methyl isobutylamide	**Ion**	$[(M+H)-C_5H_{10}]^+$	$[(M+H)-C_5H_{13}N]^+$	$[(M+H)-C_6H_{11}NO]^+$	$[(M+H)-C_6H_{13}NO]^+$
	-m/z	70	87	113	115
***N*-methyl isobutylamide**	**Ion**	$[(M+H)-C_4H_8]^+$	$[(M+H)-C_5H_{13}N]^+$	$[(M+H)-C_6H_{11}NO]^+$	$[(M+H)-C_6H_{13}NO]^+$
	-m/z	56	87	113	115
4-hydroxy phenylethylamide	**Ion**	$[(M+H)-C_8H_8O]^+$	$[(M+H)-C_8H_{11}NO]^+$	$[(M+H)-C_9H_9NO_2]^+$	$[(M+H)-C_9H_{11}NO_2]^+$
	-m/z	120	137	163	165
4-methoxy phenylethylamide	**Ion**	$[(M+H)-C_9H_{10}O]^+$	$[(M+H)-C_9H_{13}NO]^+$	$[(M+H)-C_{10}H_{11}NO_2]^+$	$[(M+H)-C_{10}H_{13}NO_2]^+$
	-m/z	134	151	177	179

Achillea ptarmica

N-alkylamide profiling using HPLC-ESI-MS

The total ion chromatogram (TIC) of the root extract of *A. ptarmica* is given in Figure 1. Peak labels correspond to *N*-alkylamide designations.

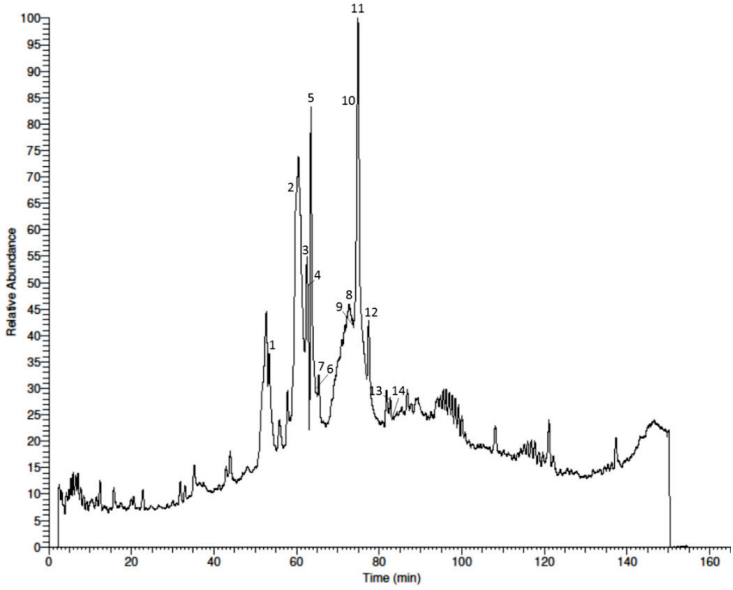


Figure 1: TIC of *Achillea ptarmica* obtained using HPLC-ESI-MS.

Table 2: *N*-alkylamides in the ethanolic *A. ptarmica* extract using HPLC-ESI-MS and/or GC-EI-MS.

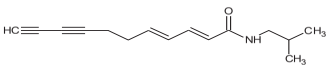
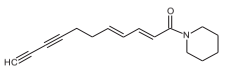
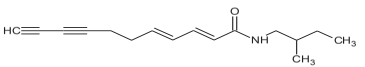
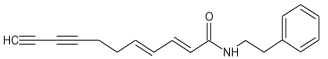
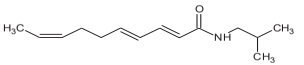
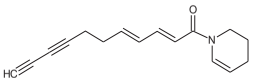
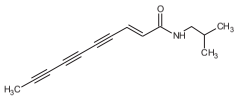
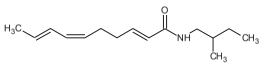
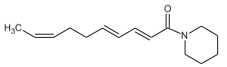
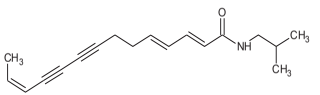
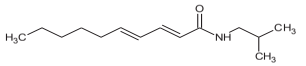
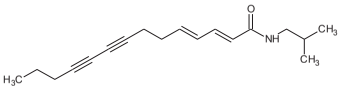
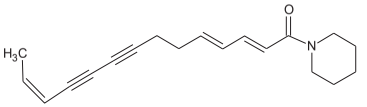
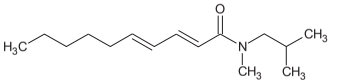
Compound	R_t HPLC (min)[a]	R_t GC (min)	Structure	Chemical name	MW (g/mol)	Classification
P1 (=M1)	53.4 [17.7%]	23.3		Undeca-2E,4E-diene-8,10-diynoic acid isobutylamide	229.32	F3M1
P2	59.7 [14.3%]	-		Undeca-2E,4E-diene-8,10-diynoic acid piperidide	241.33	F3M5
P3	62.4 [3.4%]	-		Undeca-2E,4E-diene-8,10-diynoic acid 2-methylbutylamide	243.35	F3M1
P4	62.6 [0.1%]	-		Undeca-2E,4E-diene-8,10-diynoic acid phenylethylamide	277.37	F3M11
P5 (=M3)	63.4 [73.6%]	15.9		Deca-2E,4E,8Z-trienoic acid isobutylamide (8,9-dehydropellitorine)	221.34	F3M1
P6	65.0 [1.5%]	-		Undeca-2E,4E-diene-8,10-diynoic acid piperideide	239.32	F3M5
P7	65.4 [7.0%]	25.6		Deca-2E-ene-4,6,8-triynoic acid isobutylamide	213.28	F3M1
P8 (=M4)	72.5 [3.4%]	-		Deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol)	235.37	F3M1
P9 (=M5)	73.6 [1.3%]	23.4		Deca-2E,4E,8Z-trienoic acid piperidide	233.35	F3M5

Table 2: *N*-alkylamides in the ethanolic *A. ptarmica* extract using HPLC-ESI-MS and/or GC-EI-MS (continued).

Compound	R_t HPLC (min)[a]	R_t GC (min)	Structure	Chemical name	MW (g/mol)	Classification
P10 (=M17)	74.5 [11.0%]	34.4		Tetradeca-2E,4E,12Z-triene-8,10-diynoic acid isobutylamide	269.39	F3M1
P11 (=M6)	74.9 [100.0%]	15.5		Deca-2E,4E-dienoic acid isobutylamide (pellitorine)	223.36	F3M1
P12 (=M7)	77.5 [13.6%]	34.2		Tetradeca-2E,4E-diene-8,10-diynoic acid isobutylamide (anacycline)	271.40	F3M1
P13	82.4 [1.5%]	-		Tetradeca-2E,4E,12Z-triene-8,10-diynoic acid piperidide	281.39	F3M5
P14	83.3 [4.6%]	-		Deca-2E,4E-dienoic acid *N*-methyl isobutylamide	237.38	F3M1

- : not applicable
a: Between brackets: estimated relative quantity to pellitorine from total ion chromatogram.

The major ions observed in the MS^1 spectra correspond to the protonated forms of NAAs. The MS^2 spectra are shown in Figure 2, while an overview of all the NAAs assigned in *A. ptarmica* is given in Table 2 with their corresponding retention time (R_t), structure, chemical name, molecular weight (MW, average mass) and classification. Structures of fourteen NAAs were assigned with different types of amides in the *Achillea ptarmica* extract: 6 *N*-alkylamides having an isobutylamide function (compounds **P1**, **P5**, **P7**, **P10**, **P11**, **P12**), 3 NAAs with a piperidide function (saturated 6-ring $C_5H_{10}N$, compounds **P2**, **P9**, **P13**), 1 NAA with a piperideide function (unsaturated 6-ring C_5H_8N, compound **P6**), 2 NAAs with a 2-methylbutylamide function (compounds **P3**, **P8**), 1 NAA with a phenylethylamide function (compound **P4**) and 1 NAA having a *N*-methyl isobutylamide function (compound **P14**). Furthermore, the fatty acid chain of the NAAs varies in length (C10, C11, C14) and consists of many sites of unsaturated bonds (double and triple bonds) (Table 2). Complex MS-MS spectra are due to fragmentation of this chain [24]. As can be seen in Table 2, NAAs with terminal alkynes elute early with reversed phase HPLC [27].

Characteristic fragment ions of NAAs with an isobutylamide group are formed by CID (Table 1) and these typical *m/z* values are indicated in bold in Table 3 for compounds **P1**, **P5**, **P7**, **P10**, **P11** and **P12**. In case of compound **P1**, there were cleavages in the fatty acid chain between C1-C2 (*m/z* 129) and between C2-C3 (*m/z* 116). The product ions with *m/z* 174, 157, 131, 129, 116, 91 have been reported previously for undeca-2E,4E-diene-8,10-diynoic acid isobutylamide [24, 26, 31]. For compound **P11** (deca-2E,4E-dienoic acid isobutylamide or pellitorine), product ions with *m/z* 182, 168, 154, 151, 133, 123, 109, 95, 83 and 69 are consistent with values reported in literature [26, 32]. In addition, a cleavage between C4-C5 (*m/z* 140) and C9-C10 (*m/z* 209) of the fatty acid chain was observed. For compound **P12** (tetradeca-2E,4E-diene-8,10-diynoic acid isobutylamide or anacycline), there was a cleavage in the fatty acid chain between C1-C2 (*m/z* 171), C3-C4 (*m/z* 145) and C6-C7 (*m/z* 167). Moreover, the assignment of compounds **P1, P11** and **P12** was also based on comparison of the retention time [26]. For compound **P5** (deca-2E,4E,8Z-trienoic acid isobutylamide), cleavages occurred in the fatty acid between C1-C2 (*m/z* 121) and C8-C9 (*m/z* 194). Furthermore, as the fatty acid chain contains doubly allylic carbon atoms, there is the formation of a distonic radical cation (C6-C7, *m/z* 167) and cationic species with the loss of H, due to cleavage between C5-C6 (*m/z* 152) [32]. There were cleavages between C1-C2 (*m/z* 169), C3-C4 (*m/z* 143), C5-C6 (*m/z* 117) and C12-C13 (*m/z* 242) for compound **P10** (tetradeca-2E,4E,12Z-triene-8,10-diynoic acid isobutylamide).

Figure 2: MS² fragmentation spectra (CID) of *N*-alkylamides in the *A. ptarmica* extract.

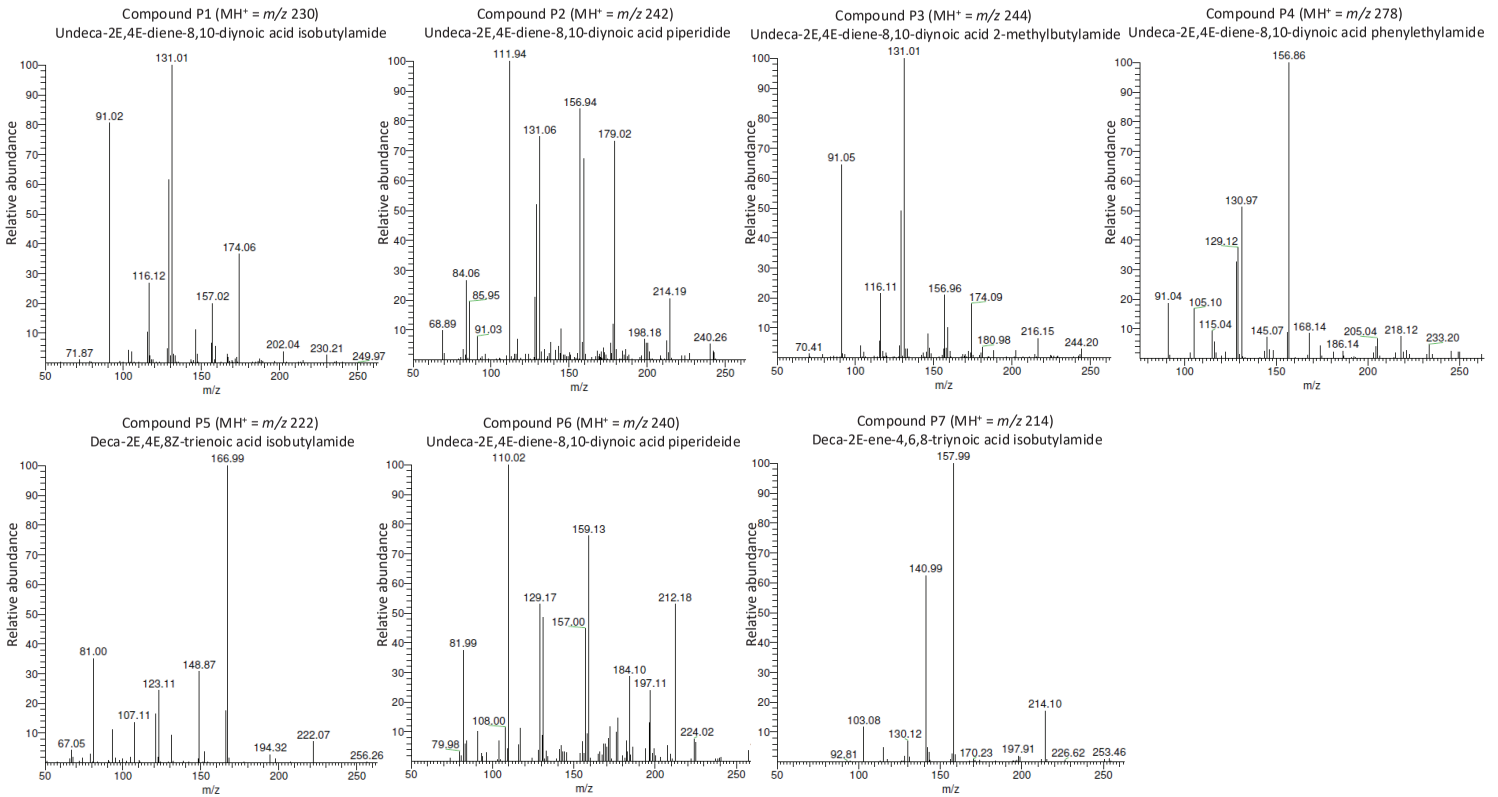


Figure 2: MS² fragmentation spectra (CID) of *N*-alkylamides in the *A. ptarmica* extract (continued).

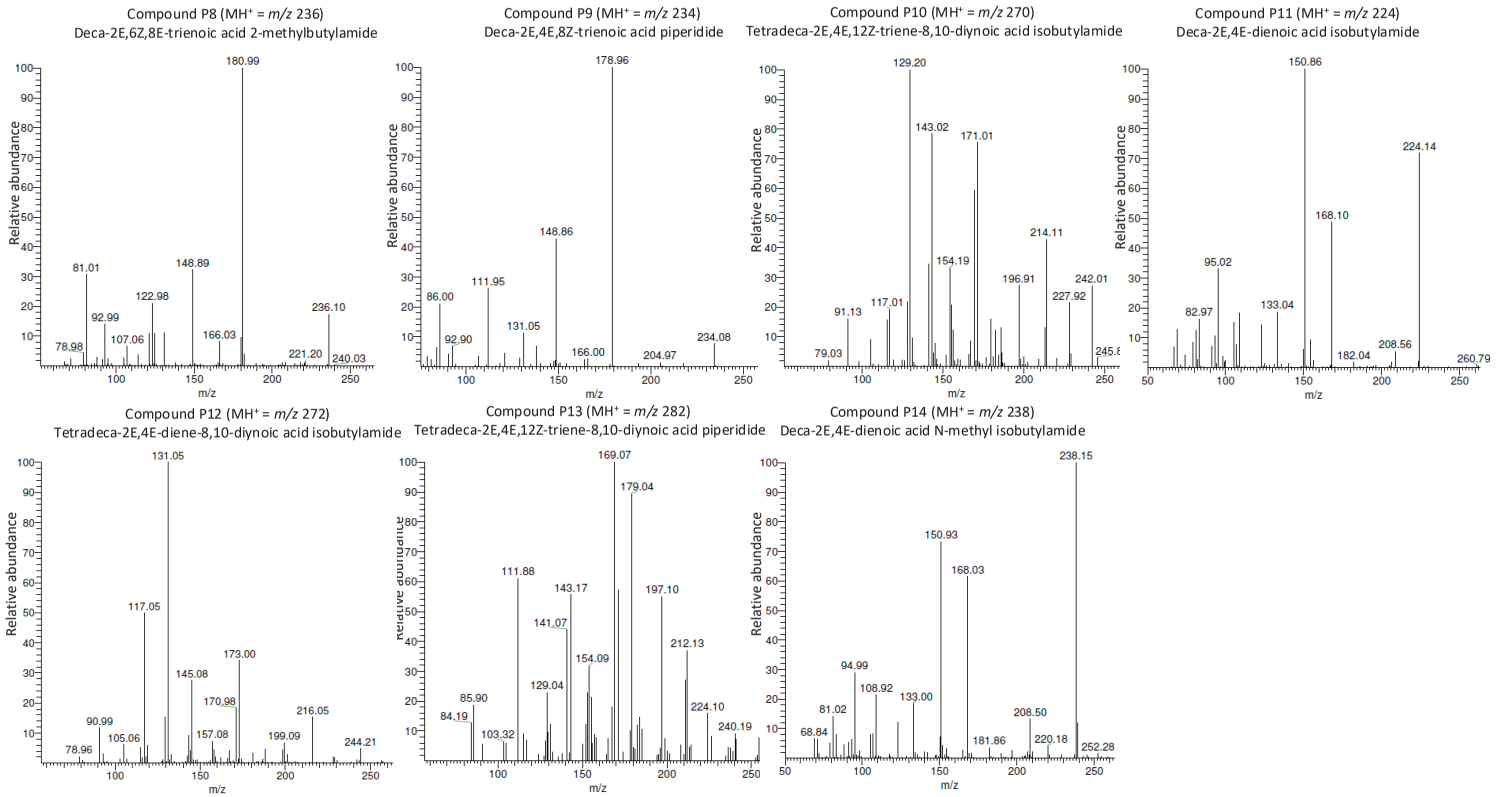


Table 3: MS^1 and MS^2 information of *N*-alkylamides in the ethanolic *A. ptarmica* extract using HPLC-ESI-MS.

Compound	$[M+H]^+$	Product ions (*m/z*)	Losses (*m/z*)
P1	230	215; 202; 174; 159; 157; 146; 133; 131; 129; 128; 123; 121; 117; 116; 115; 110; 105; 98; 93; 91; 79; 72	-15; -28; -**56**; -71; -**73**; -84; -97; -**99**; -**101**; -102; -107; -109; -113; -114; -115; -120; -125; -132; -137; -139; -151; -158
P2	242	222; 214; 198; 179; 176; 159; **157**; 145; 131; 129; 112; 91; 86; 84; 69	-20; -28; 44, -63; -66; -83; **-85**; -97; -111; -113; -130; -151; -156; -158; -173
P3	244	216; 181; 174; 157; 146; 131; 129; 116; 103; 91; 70	-28; -63; -**70**; -**87**; -98; -**113**; -**115**; -128; -141; -153; -174
P4	278	245; 218; 205; 186; 174; 168; 157; 145; 131; 129; 128; 115; 105; 91	-33; -60; -73; -92; -**104**; -110; -**121**; -133; -**147**; -**149**; -150; -163; -173; -187
P5	222	194; 168; 167; 152; 149; 132; 131; 123; 121; 110; 107; 100; 93; 91; 81; 67	-28; -54; -55; -70; -**73**; -90; -91; -**99**; -**101**; -112; -115; -122; -129; -131; -141; -155
P6	240	224; 212; 184; 177; 159; **157**; 155; 129; 110; 108; 82; 80	-16; -28; -56; -63; -81; **-83**; -85; -111; -130; -132; -158; -160
P7	214	198; 172; 159; 158; 141; 130; 115; 103; 89; 72	-16; -42; -55; -**56**; -**73**; -84; -**99**; -111; -125; -142
P8	236	221; 182; 181; 180; 166; 151; 149; 138; 125; 123; 121; 107; 95; 93; 81; 79	-15; -54; -55; -56; **-70**; -85; -**87**; -98; -111; -**113**; -**115**; -129; -141; -143; -155; -157
P9	234	205; 179; 166; **149**; 131; 112; 93; 86	-29; -55; -68; **-85**; -103; -122; -141; -148
P10	270	246; 242; 228; 214; 197; 179; 171; 169; 154; 143; 129; 117; 105; 91; 79	-24; -28; -42; -**56**; -**73**; -91; -**99**; -**101**; -116; -127; -141; -153; -165; -179; -191
P11	224	209; 204; 182; 168; 154; 151; 140; 133; 123; 109; 105; 95; 83; 69	-15; -20; -42; -**56**; -70; -**73**; -84; -91; -**101**; -115; -119; -129; -141; -155
P12	272	244; 216; 199; 173; 171; 167; 145; 131; 117; 91; 81	-28; -**56**; -**73**; -**99**; -**101**; -105; -127; -141; -155;-181; -191
P13	282	254; 240; 224; 212; **197**; 179; 169; 165; 141; 143; 129; 112; 103; 86; 84	-28; -42; -58; -70; **-85**; -103; -113; -128; -141; -139; 153; -170; -179; -196; -198
P14	238	220; 210; 209; 197; 182; 168; 151; 133; 109; 95; 81; 69	-18; -28; -29; -41; **-56**; -70; **-87**; -105; -129; -143; -157; 169

In bold: characteristic product ions or losses.

NAAs having a phenylethylamide alkyl group show similarly formed fragments as the isobutylamide NAAs, *i.e.* the typical losses are presented in Table 1. The typical *m/z* values of these losses for compound **P4** (undeca-2E,4E-diene-8,10-diynoic acid phenylethylamide) are indicated in bold in Table 3. Furthermore, a tropylium ion was formed (benzene and α C) (*m/z* 91). A cleavage was observed in the fatty acid between C1-C2 (*m/z* 129). Product ions of compound **P4** with *m/z* 168, 157, 131, 105 and 91 are consistent with values reported earlier. Moreover, assignment of the NAA was also based upon retention time comparison [26].

Characteristic losses of the NAAs with a 2-methyl isobutylamide function are presented in Table 1 and indicated for compounds **P3** and **P8** in Table 3. For compound **P3**, a cleavage occurred in the fatty acid chain between C1-C2 (*m/z* 129), C2-C3 (*m/z* 116) and C6-C7 (*m/z* 181). The MS spectrum of compound **P8** corroborates well with the structure of deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) or a related isomeric compound and has never previously been reported in this plant (Figure 3). A cleavage was observed in the fatty acid between C6-C7 (*m/z* 182) and C9-C10 (*m/z* 221). Furthermore, structural assignment of compound **P8** was done based on comparison of retention time and product ions. Product ions with *m/z* 166, 149, 123, 121 and 81 were already reported for this NAA [25].

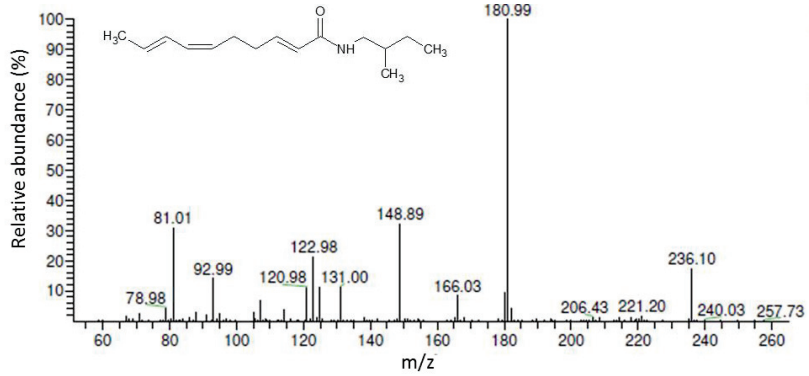


Figure 3: MS^2 spectra of deca-2E,6Z,8E-trienoic acid 2-methylbutylamide or homospilanthol (compound P8) with $[M+H]^+$ = *m/z* 236.

The characteristic losses for NAAs having a *N*-methyl isobutylamide function are shown in Table 1 and indicated in Table 3 for compound **P14**. Interestingly, deca-2E,4E-dienoic acid *N*-methyl isobutylamide is the second NAA, never previously reported in this plant. The MS^2 spectrum is shown in Figure 4 and corroborates well with this structure. A cleavage occurred in the fatty acid part between C8-C9 (*m/z* 209). Moreover, the structural assignment of compound **P14** was also based on

comparison of the retention time and the product ions *m/z* 182, 168, 151 and 109, which were previously reported [26].

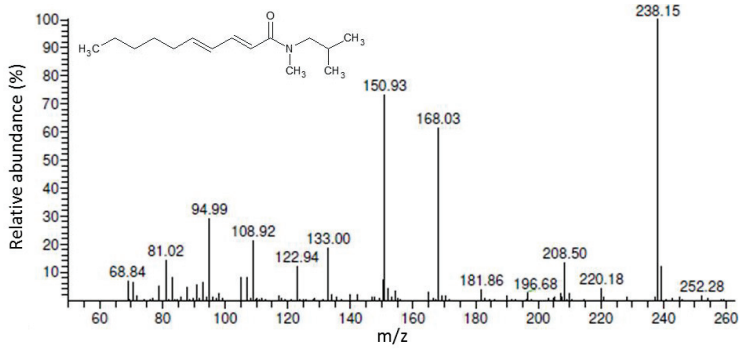


Figure 4: MS^2 spectra of deca-2E,4E-dienoic acid *N*-methyl isobutylamide (compound P14) with $[M+H]^+$ = *m/z* 238.

For compounds **P2** (undeca-2E,4E-diene-8,10-diynoic acid piperidide), **P9** (deca-2E,4E,8Z-trienoic acid piperidide) and **P13** (tetradeca-2E,4E,12Z-triene-8,10-diynoic acid piperidide), having a piperidide function, acylium ions are formed with *m/z* values of 157, 149 and 197, respectively, corresponding with a loss of *m/z* 85. There was a cleavage in the fatty acid chain of these compounds between C6-C7 (*m/z* 179) (Table 3). Furthermore, cleavages were found in the fatty acid chain between C3-C4 (*m/z* 143) and C12-C13 (*m/z* 254) of compound **P13**. Compound **P9** has doubly allylic carbon atoms in the fatty acid part and formed a distonic radical cation due to cleavage between C6-C7 (*m/z* 179) [32]. An acylium ion was also formed in case of compound **P6**, having a piperideide function, with a *m/z* value of 157 (loss of *m/z* 83) and as compound **P6** contains doubly allylic carbon atoms, a distonic radical cation was formed (C6-C7, *m/z* 177) (Table 3) [32].

All the reported NAAs were found in the roots of *Achillea ptarmica*, except for compound **P4**, which was only found in the leaves. Furthermore, compounds **P1**, **P2**, **P3**, **P5**, **P6** and **P7** were also observed in the leaves, while compounds **P1**, **P2**, **P3**, **P5** and **P6** were found in the stem as well (data not shown). No NAAs could be found in the flowers, as the concentration of NAAs was probably too low. In conclusion, the highest amount of NAAs was found in the roots, which is consistent with literature [33].

N-alkylamide profiling using GC-EI-MS

Compounds **P1**, **P5**, **P7**, **P9**, **P10**, **P11** and **P12**, observed using HPLC-ESI-MS, were also observed with GC-EI-MS. The TIC is shown in Figure 5, using the same numbers as indicated in HPLC-MS.

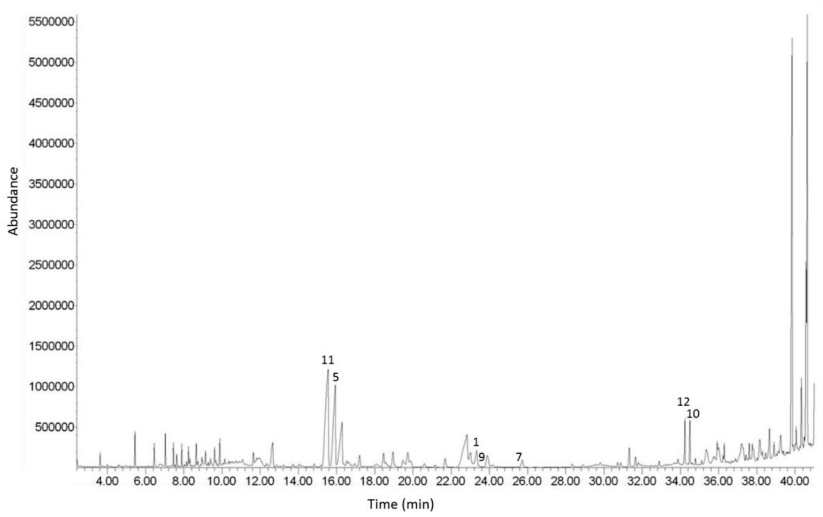


Figure 5: TIC of *Achillea ptarmica* obtained using GC-EI-MS.

The molecular ions were detected for all compounds. Product ions characteristic for isobutylamide NAAs were found for compounds **P1**, **P5**, **P7**, **P10**, **P11** and **P12**. These product ions are indicated in bold in Table 4. This was also done for compound **P9**, an NAA having a piperidide function. Typical product ions for the amide part are also indicated in bold in Table 4. Furthermore, σ-cleavages were observed in the fatty acid chain of all the NAAs. These product ions were for compound **P1**: *m/z* 63/166 (C6-C7), *m/z* 77 (C5-C6), *m/z* 103 (C3-C4); for compound **P5**: *m/z* 206 (C9-C10), *m/z* 41 (C7-C8), *m/z* 55/166 (C6-C7), *m/z* 69 (C5-C6), *m/z* 95 (C3-C4), *m/z* 100/121 (C1-C2); for compound **P7**: *m/z* 198 (C9-C10), *m/z* 63 (C5-C6), *m/z* 87 (C3-C4), *m/z* 113 (C1-C2); for compound **P10**: *m/z* 254 (C13-C14), *m/z* 41 (C11-C12), *m/z* 103 (C6-C7), *m/z* 117 (C5-C6), *m/z* 143 (C3-C4), *m/z* 169 (C1-C2); for compound **P11**: *m/z* 208 (C9-C10), *m/z* 194 (C8-C9), *m/z* 180 (C7-C8), *m/z* 166 (C6-C7), *m/z* 152 (C5-C6), *m/z* 126 (C3-C4); for compound **P12**: *m/z* 256 (C13-C14), *m/z* 242 (C12-C13), *m/z* 43/228 (C11-C12), *m/z* 67 (C9-C10), *m/z* 91 (C7-C8), *m/z* 105/166 (C6-C7), *m/z* 119/152 (C5-C6), *m/z* 100/171 (C1-C2) and for compound **P9**: *m/z* 41 (C7-C8), *m/z* 55 (C6-C7), *m/z* 69 (C5-C6), *m/z* 95/138 (C3-C4), *m/z* 121/112 (C1-C2). In addition, product ions were also detected consistent with a (H-)rearrangement between C4-C5 in the fatty acid chain, for compound **P5**: *m/z* 81, *m/z* 140; for compound **P10**: *m/z* 129; for compound **P11**: *m/z* 83; for compound **P12**: *m/z* 131 and for compound

P9: *m/z* 81, *m/z* 152. The product ions of compound **P11** with *m/*z 223, 208, 180, 166, 151, 113, 110, 96, 81, 66, 57, 55, 53 and 41 were also described by Lazarevic *et al.* (2010) [2]. Compound **P1** was also recognized by the Nist library.

Table 4: MS information of *N*-alkylamides in the ethanolic *A. ptarmica* extract using GC-EI-MS.

Compound	$[M]^+$	Product ions (*m/z*) (% intensity relative to base peak)
P1	229	41 (10%), **43**, 51, 55, **57** (9%), 63 (10%), 66 (23%), 67 (15%), **72**, 77, 81 (8%), 94, 102, 103, 110 (10%), 115, 123, 127 (27%), 128 (64%), 129 (19%), **157** (100%), 166 (14%), **172** (9%), 178, **186**, **214** (9%), 228 (17%), **229** (13%), 233
P5	221	41 (18%), **43**, 53 (10%), 55 (51%), **57** (39%), 65 (10%), 66 (28%), 67 (31%), 68 (12%), 69, **72**, 77 (8%), 79(16%), 81 (21%), 82 (9%), 91 (9%), 96 (11%), 95 (13%), 94 (15%), 93 (19%), **100**, 107 (10%), 110 (26%), **121** (8%), 122 (9%), 131 (8%), 139, 140, **149** (100%), 150 (16%), 152 (18%), 157, **164**, 166 (50%), 167 (17%), **178**, 192, **206** (11%), **221** (41%), 222
P7	213	41 (8%), **43**, **57**, 63 (9%), 69, 73, 77, 81, 85, 86 (11%), 87 (20%), 91, 95, 102, 108, **113** (18%), 114 (6%), 128 (6%), 133, **141** (100%), 142 (15%), 152, **156**, 157 (26%), **170**, 171 (16%), **198**, **213** (43%), 214 (8%)
P9	233	41 (65%), 42 (12%), 43 (41%), 45 (10%), 53 (28%), 54 (28%), 55 (92%), 56 (15%), 57 (33%), 58 (14%), 60 (14%), 63 (9%), 65 (23%), 66 (45%), 67 (81%), 68 (25%), 69 (62%), 70 (14%), 71 (12%), 73 (20%), 77 (26%), 78 (14%), 79 (53%), 80 (25%), 81 (84%), 82 (40%), 83 (49%), **84** (70%), 85 (20%), 91 (29%), 93 (38%), 94 (28%), 95 (54%), 96 (31%), 97 (18%), 98 (16%), 101 (11%), 105 (15%), 106 (9%), 107 (21%), 108 (12%), 109 (24%), 110 (23%), 111 (15%), **112** (38%), 113 (10%), 115 (11%), 117 (9%), 119 (11%), 120 (10%), **121** (17%), 122 (13%), 123 (17%), 124 (12%), 125 (9%), 126 (10%), 127 (24%), 128 (49%), 129 (22%), 131 (14%), 133 (12%), 134 (8%), 135 (12%), 136 (11%), 137 (27%), 138 (46%), 139 (14%), 143 (12%), 144 (10%), 148 (10%), **149** (17%), 150 (29%), 151 (28%), 152 (9%), 157 (75%), 158 (15%), 162 (10%), 164 (29%), 165 (15%), 166 (13%), 167 (8%), 172 (10%), 177 (10%), 178 (100%), 179 (13%), 228 (16%), 229 (16%), 232 (9%), 233 (50%), 234 (11%), 280 (10%)
P10	269	40 (8%), 41 (36%), **43** (24%), 44 (8%), 51 (10%), 53 (11%), 54 (9%), 55 (31%), 56 (12%), **57** (70%), 58 (14%), 63 (11%), 65 (16%), 66 (30%), 67 (40%), 68 (18%), 69 (20%), 71 (11%), **72** (9%), 73 (16%), 74 (8%), 75 (16%), 76 (9%), 77 (80%), 78 (11%), 79 (16%), 81 (19%), 82 (19%), 83 (13%), 84 (8%), 85 (9%), 91 (21%), 93 (15%), 94 (11%), 95 (27%), 96 (12%), 97 (12%), 98 (14%), 102 (15%), 103 (62%), 105 (9%), 107 (11%), 108 (9%), 109 (15%), 110 (16%), 111 (8%), 115 (25%), 117 (13%), 128 (19%), 129 (34%), 131 (10%), 135 (10%), 141 (51%), 142 (17%), 143, 147 (9%), 149 (12%), 152 (25%), 153 (29%), 154 (38%), 155 (25%), 157 (8%), 165 (10%), 166 (20%), 167 (54%), 168 (12%), **169** (24%), 171 (10%), 178, 185 (10%), 187 (10%), **197** (22%), 198 (11%), 207 (15%), **212** (8%), 223, 239 (9%), **254**, 268 (20%), **269** (100%), 270 (23%), 281 (13%), 355 (8%), 410
P11	223	41 (12%), **43**, 53 (8%), 55 (8%), **57**, 60, 66 (11%), 67 (13%), 69 (10%), **72**, 71, 73, 77, 79 (8%), 81 (40%), 83, 89, 94, 95 (12%), 96 (40%), 97, 98 (8%), 103, 109, 110 (12%), 113 (9%), 120, **123**, 126, 138, 145, 152 (35%), **151** (100%), **166** (8%), 167, **180** (8%), 194, **208** (10%), **223** (35%), 224
P12	271	41 (34%), **43** (17%), 51 (10%), 53 (10%), 55 (29%), **57** (80%), 58 (8%), 63 (17%), 65 (26%), 66 (52%), 67 (45%), 68 (15%), 69 (11%), 73 (9%), 77 (58%), 78 (11%), 79 (38%), 81 (17%), 82 (12%), 91 (26%), 93 (9%), 94 (20%), 95 (17%), 96 (9%), 98 (9%), **100** (8%), 103 (18%), 105 (17%), 107 (8%), 110 (29%), 111 (11%), 115 (31%), 117 (18%), 119, 127 (14%), 128 (55%), 129 (100%), 130 (23%), 131 (13%), 134, 141 (35%), 142 (22%), 143 (68%), 144 (23%), 145 (9%), 152 (17%), 153 (10%), 155 (18%), 156 (12%), 157 (27%), 158 (10%), 166 (36%), 167 (55%), 168 (10%), 169 (15%), 170 (11%), **171** (36%), 172 (15%), 173, 186 (10%), **199** (75%), 200 (22%), **214** (13%), 223, **228** (8%), 242 (13%), 243, **256** (33%), 257 (9%), 270 (23%), **271** (57%,), 281 (8%), 297, 355 (8%)

Note: if no % is indicated between brackets = intensity < 7 % of base peak.

Product ions indicated in bold: characteristic product ions for the amide functional group of the NAA.

Achillea millefolium

N-alkylamide profiling using HPLC-ESI-MS

The TIC of the root extract of *A. millefolium* is shown in Figure 6 in which peak labels correspond to *N*-alkylamide designations.

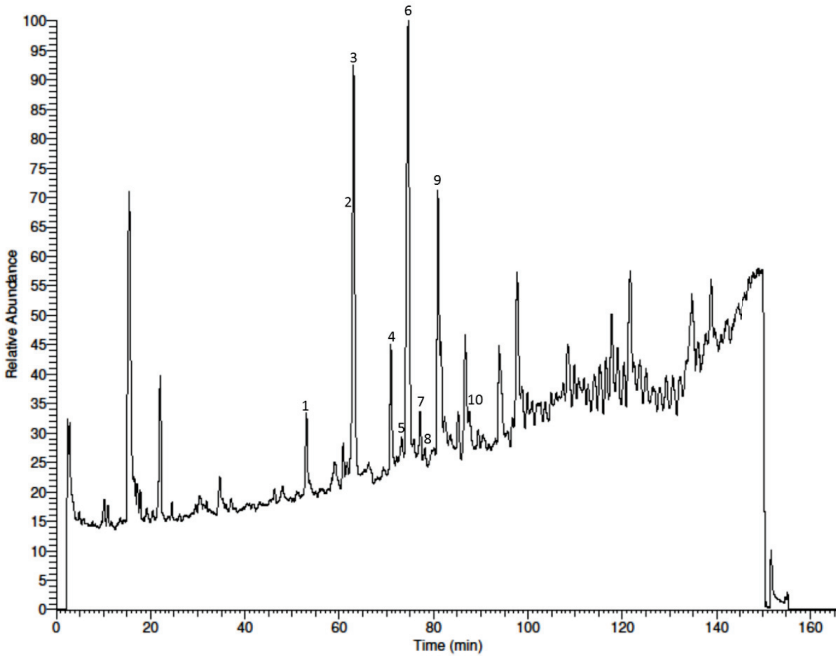


Figure 6: TIC of *Achillea millefolium* obtained using HPLC-ESI-MS.

In the MS^1 spectra, the major ions are the protonated forms of the NAAs. MS^2 spectra are presented in Figure 7. All the NAAs assigned in *A. millefolium* are summarised in Table 5 with their corresponding retention time (R_t), structure, chemical name, molecular weight (MW, average mass) and classification. Structures of ten NAAs were assigned in the *Achillea millefolium* extract with different types of amides: 4 NAAs having an isobutylamide function (compounds **M1**, **M3**, **M6**, **M7**), 2 NAAs with a piperidide function (compounds **M5**, **M10**), 1 NAA with a piperideide function (compound **M8**), 1 NAA with a 2-methylbutylamide function (compound **M4**), 1 NAA with a 4-hydroxyphenylethylamide function (compound **M2**) and 1 NAA having a 4-methoxy phenylethylamide function (compound **M9**).

Table 5: *N*-alkylamides in the ethanolic *A. millefolium* extract using HPLC-ESI-MS and/or GC-EI-MS.

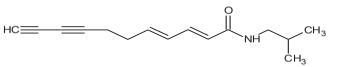
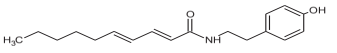
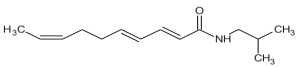
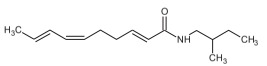
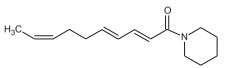
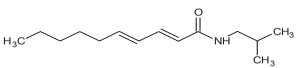
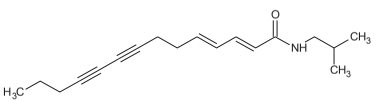
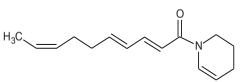
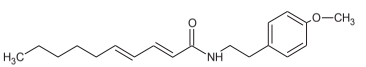
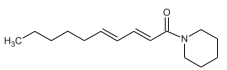
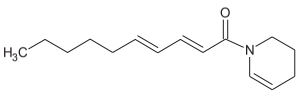
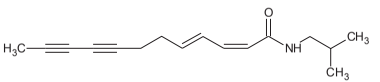
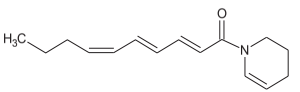
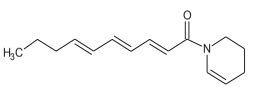
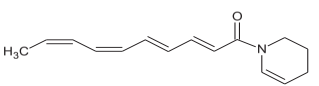
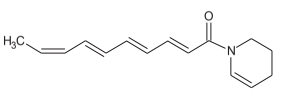
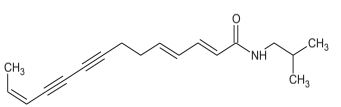
Compound	R_t HPLC (min)[a]	R_t GC (min)	Structure	Chemical name	MW (g/mol)	Classification
M1 (=P1)	53.1 [13.7%]	23.3		Undeca-2E,4E-diene-8,10-diynoic acid isobutylamide	229.32	F3M1
M2	62.9 [21.0%]	-		Deca-2E,4E-dienoic acid tyramide	287.40	F3M12
M3 (=P5)	63.1 [86.6%]	15.8		Deca-2E,4E,8Z-trienoic acid isobutylamide	221.34	F3M1
M4 (=P8)	71.6 [3.7%]	-		Deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol)	235.37	F3M1
M5 (=P9)	73.4 [5.9%]	-		Deca-2E,4E,8Z-trienoic acid piperidide	233.35	F3M5
M6 (=P11)	74.6 [100.0%]	15.4		Deca-2E,4E-dienoic acid isobutylamide (pellitorine)	223.36	F3M1
M7 (=P12)	77.2 [5.8%]	34.2		Tetradeca-2E,4E-diene-8,10-diynoic acid isobutylamide (anacycline)	271.40	F3M1
M8	78.3 [2.3%]	27.1		Deca-2E,4E,8Z-trienoic acid piperideide	231.34	F3M5
M9	80.9 [30.3%]	-		Deca-2E,4E-dienoic acid 4-methoxy phenylethylamide	301.43	F3M12
M10	87.6 [5.4%]	-		Deca-2E,4E-dienoic acid piperidide	235.37	F3M5

Table 5: *N*-alkylamides in the ethanolic *A. millefolium* extract using HPLC-ESI-MS and/or GC-EI-MS (continued).

Compound	R_t HPLC (min) [a]	R_t GC (min)	Structure	Chemical name	MW (g/mol)	Classification
M11	-	23.4		Deca-2E,4E-dienoic acid piperideide	233.35	F3M5
M12	-	25.3		Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	243.35	F3M1
M13	-	26.3		Deca-2E,4E,6Z-trienoic acid piperideide	231.34	F3M5
M14	-	26.3		Deca-2E,4E,6E-trienoic acid piperideide	231.34	F3M5
M15	-	30.3		Deca-2E,4E,6Z,8Z-tetraenoic acid piperideide	229.32	F3M5
M16	-	30.3		Deca-2E,4E,6E,8Z-tetraenoic acid piperideide	229.32	F3M5
M17 (=P10)	-	34.5		Tetradeca-2E,4E,12Z-triene-8,10-diynoic acid isobutylamide	269.39	F3M1

- : not applicable
a: Between brackets: estimated relative quantity to pellitorine from total ion chromatogram.

Figure 7: MS^2 fragmentation spectra (CID) of *N*-alkylamides in the *A. millefolium* extract.

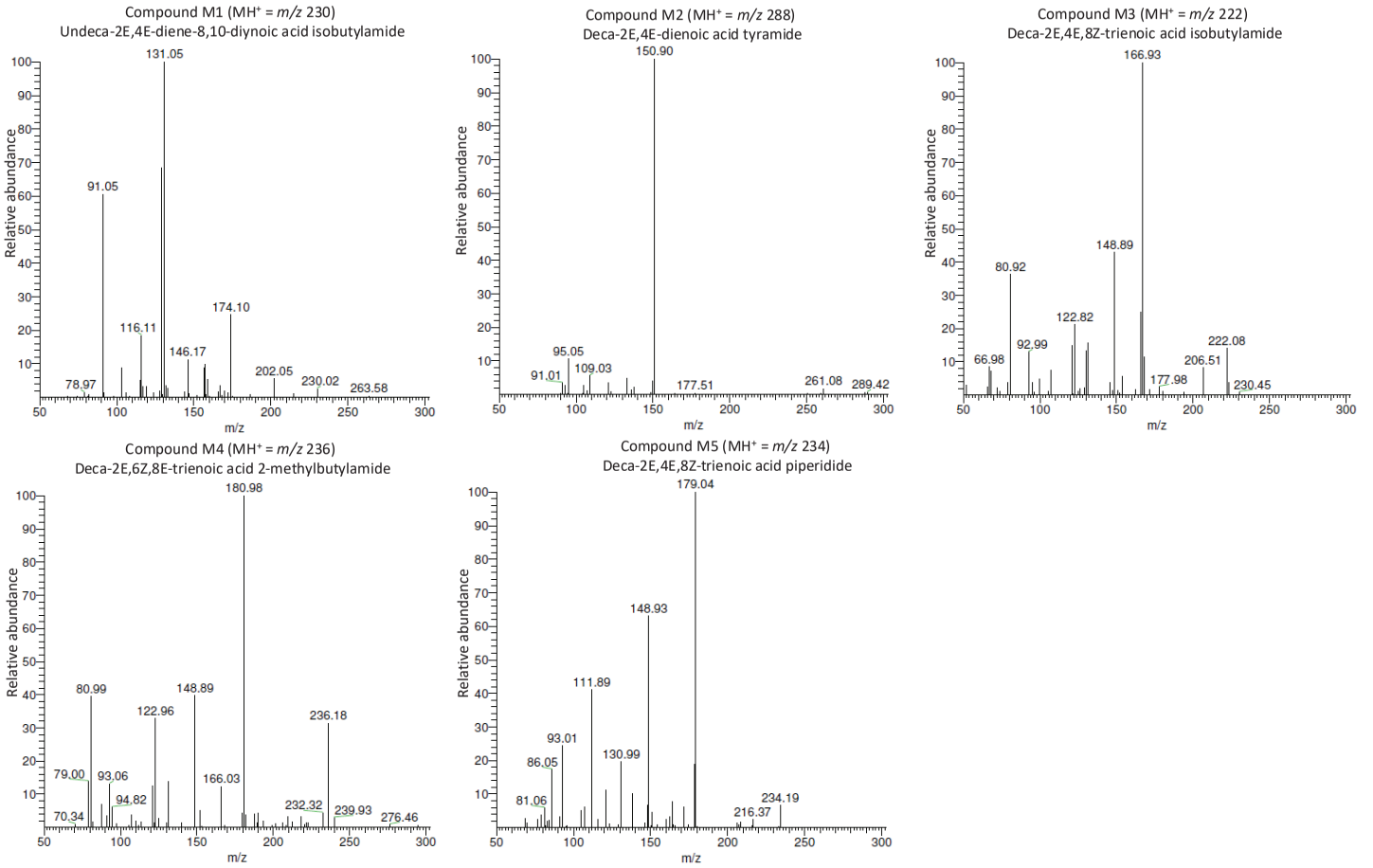


Figure 7: MS² fragmentation spectra (CID) of *N*-alkylamides in the *A. millefolium* extract (continued).

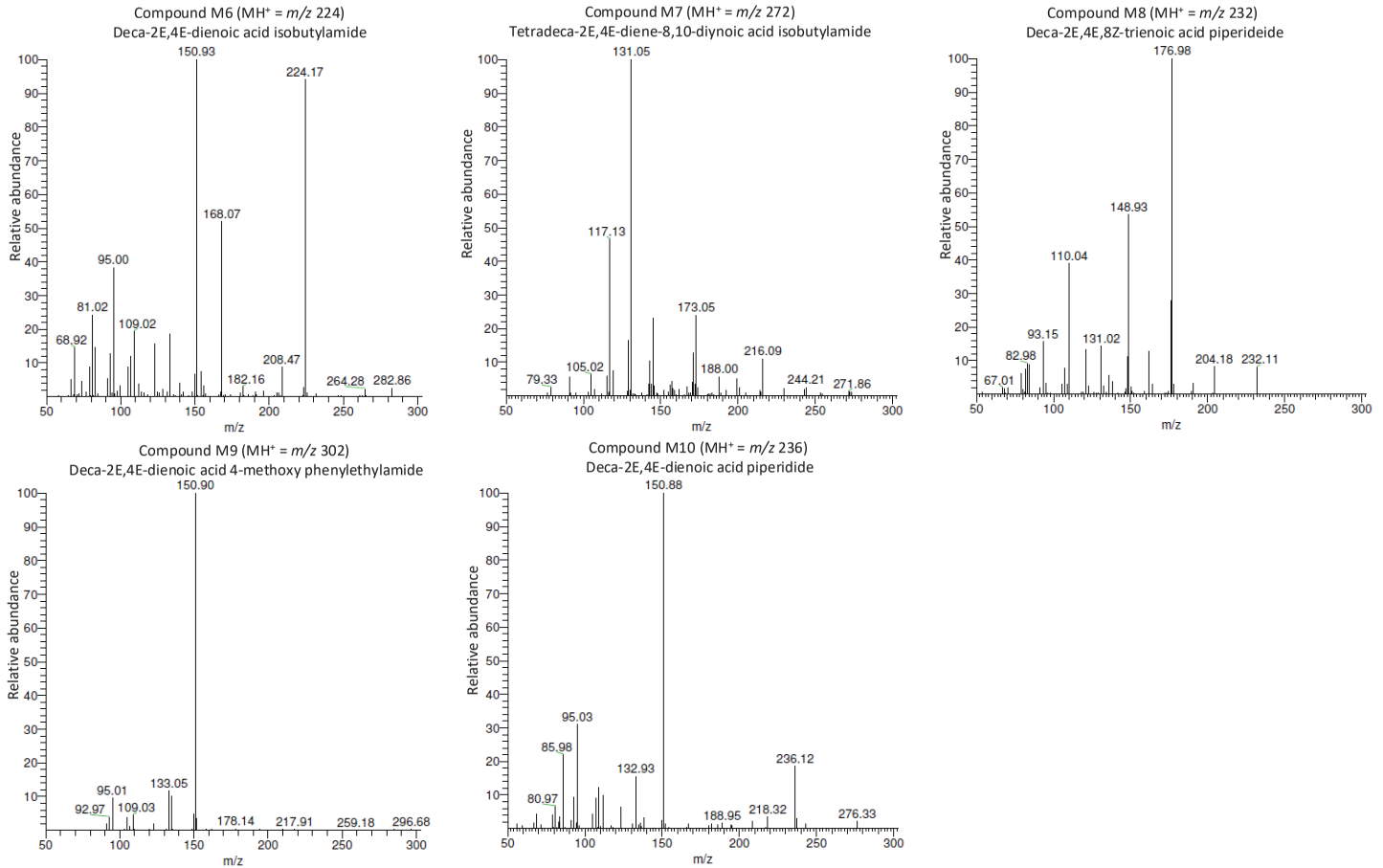


Table 1 contains characteristic fragment ions formed by CID for NAAs with an isobutylamide, a 2-methylbutylamide, a 4-hydroxyphenylethylamide and 4-methoxy phenylethylamide function. Typical *m/z* values of fragment losses of NAAs with an isobutylamide function are indicated in bold in Table 6 for compounds **M1** (undeca-2E,4E-diene-8,10-diynoic acid isobutylamide), **M3** (deca-2E,4E,8Z-trienoic acid isobutylamide), **M6** (deca-2E,4E-dienoic acid isobutylamide or pellitorine) and **M7** (tetradeca-2E,4E-diene-8,10-diynoic acid isobutylamide or anacycline). In case of compound **M1**, there was a cleavage in the fatty acid chain between C1-C2 (*m/z* 129), C2-C3 (*m/z* 116), C3-C4 (*m/z* 103) and C4-C5 (*m/z* 90). Moreover, for compounds **M1** and **M6** of *A. millefolium* and compounds **P1** and **P11** of *A. ptarmica*, identical product ions are previously reported [24, 26, 31, 32]. Furthermore, cleavages between C1-C2 (*m/z* 123) and C4-C5 (*m/z* 140) of the fatty acid chain in compound **M6** were observed. For compound **M7,** there was a cleavage in the fatty acid chain between C1-C2 (*m/z* 171), C3-C4 (*m/z* 145) and C8-C9 (*m/z* 79). Moreover, the assignment of compounds **M1**, **M6** and **M7** was also based on comparison of the retention time [26]. For compound **M3**, cleavages occurred in the fatty acid chain between C1-C2 (*m/z* 121) and C8-C9 (*m/z* 194). As compound **M3** contains doubly allylic carbon atoms, there is the formation of a distonic radical cation due to cleavage between C6-C7 (*m/z* 167). This distonic radical cation undergoes a hydrogen rearrangement to form an acylium ion and the subsequent loss of CO results in a C5 cation (*m/z* 67) [32].

NAAs having a 4-hydroxyphenylethylamide also show typical fragment ions and are described in Table 1 and indicated in bold in Table 6 for compound **M2** (deca-2E,4E-dienoic acid tyramide). A cleavage occurred in the fatty acid chain between C2-C3 (*m/z* 178) and a tropylium ion was formed (benzene and α-C) (*m/z* 91). Moreover, the assignment of compound **M2** was also based on comparison of the retention time and product ions: *m/z* 178, 151, 133, 121 and 95 were previously reported [26].

Characteristic fragment ions for NAAs with a 4-methoxy phenylethylamide function are described in Table 1 and are indicated in Table 6 for compound **M9** (deca-2E,4E-dienoic acid 4-methoxy phenylethylamide). A cleavage occurred in the fatty acid chain between C4-C5 (*m/z* 218) and C7-C8 (*m/z* 259).

Table 6: MS^1 and MS^2 information of *N*-alkylamides in the ethanolic *A. millefolium* extract using HPLC-ESI-MS.

Compound	$[M+H]^+$	Product ions (*m/z*)	Losses (*m/z*)
M1	230	215; 202; 188; 174; 157; 156; 146; 131; 129; 116; 115; 103; 91; 90; 79	-15; -28; -42; -**56**; -**73**; -74; -84; -**99**; -**101**; -114; -115; -127; -139; -140; -151
M2	288	261; 178; 164; 151; 138; 133; 121; 109; 95; **91**; 90	-27; -110; -124; -**137**; -150; -155; -167; -179; -193; -197; -198
M3	222	206; 194; 178; 168; 167; 149; 131; 123; 121; 107; 93; 81; 67	-15; -28; -44; -54; -55; -**73**; -91; -**99**; -**101**; -115; -129; -141; -155
M4	236	232; 190; 181; 166; 149; 131; 123; 95; 93; 81; 79; 70	-4; -46; -55; -**70**; -**87**; -105; -**113**; -141; -143; -155; -157; -166
M5	234	216; 206; 193; 179; 164; **149**; 131; 112; 93; 86; 81	-18; -28; -41; -55; -70; -**85**; -103; -122; -141; -148; -153
M6	224	208; 182; 168; 151; 140; 133; 123; 109; 95; 81; 69	-16; -42; -**56**; -**73**; -84; -91; -**101**; -115; -129; -143; -155
M7	272	244; 216; 199; 188; 173; 171; 157; 145; 131; 117; 105; 91; 81; 79	-28; -**56**; -**73**; -84; -**99**; -**101**; -115; -127; -141; -155; -167; -181; -191; -193
M8	232	204; 177; 162; **149**; 131; 110; 93; 83; 67	-28; -55; -70; -**83**; -101; -122; -139; -149; -165
M9	302	297; 259; 218; 152; 151; 133; 109; 95; 93	-5; -43; -84; -150; -**151**; -169; -193; -207; -209
M10	236	218; 189; 167; **151**; 133; 123; 109; 95; 86; 81	-18; -47; -69; -**85**; -103; -113; -127; -141; -150; -155

In bold: characteristic product ions or losses.

For NAAs having a 2-methyl isobutylamide function, the characteristic fragmentation ions are summarised in Table 1. Typical fragment losses of compound **M4** are marked in Table 6. The obtained MS spectra corroborate well with the structure of deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) or a related isomeric compound. This NAA has never been reported before in *A. millefolium*. A cleavage occurred in the fatty acid part between C3-C4 (*m/z* 95) and C4-C5 (*m/z* 81). Compound **M4** (*A. millefolium*) was also reported for the first time in *A. ptarmica* (compound **P8**) and is shown in Figure 3 together with its MS^2 spectrum.

In case of NAAs having a piperidide function, namely compounds **M5** (deca-2E,4E,8Z-trienoic acid piperidide) and **M10** (deca-2E,4E-dienoic acid piperidide), there was the formation of acylium ions with *m/z* values of 149 and 151, respectively, corresponding with a loss of *m/z* 85. There was a cleavage in the fatty acid chain of compound **M5** between C6-C7 (*m/z* 179), C7-C8 (*m/z* 193) and C8-C9 (*m/z* 206). Furthermore, compound **M5**, containing doubly allylic carbon atoms in the fatty acid part, formed a distonic radical cation due to cleavage between C6-C7 (*m/z* 179) and a cationic species as a result of the cleavage between C5-C6 and loss of a hydrogen atom (*m/z* 164) [32]. For compound **M8**, possessing a piperideide function, there were cleavages in the fatty acid chain between C6-C7 (*m/z* 177) and C8-C9 (*m/z* 204) and the formation of a distonic radical cation (C6-C7, *m/z* 177). This cation undergoes a hydrogen rearrangement to form an acylium ion and the subsequent loss of CO results in a C5 cation (*m/z* 67). There is also the formation of a cationic species (C5-C6 + loss of H, *m/z* 162) [32]. An acylium ion was also formed in case of compound **M8**, with a *m/z* value of 149 (loss of *m/z* 83).

The previously mentioned NAAs in *Achillea millefolium* were all observed in the roots, while in the stems only compounds **M1**, **M3**, **M5**, **M6** and **M10** were found. Due to too low NAA concentrations in the flowers and leaves, no NAAs could be observed.

N-alkylamide profiling using GC-EI-MS

Using GC-EI-MS, compounds **M1**, **M3**, **M6**, and **M7**, observed with HPLC-ESI-MS, were also found. The TIC is shown in Figure 8, with the same numbers as indicated in HPLC-MS. Additional compounds assigned using GC-MS are indicated starting numbering from 11.

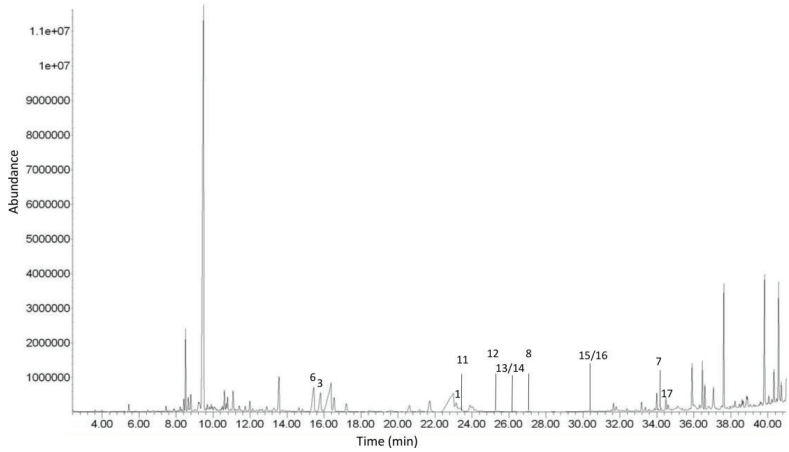


Figure 8: TIC of *Achillea millefolium* obtained using GC-EI-MS.

Other NAAs were structurally assigned using GC-MS and were not observed with HPLC-MS: compounds **M11** (deca-2E,4E-dienoic acid piperideide), **M12** (dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide), **M13** (deca-2E,4E,6Z-trienoic acid piperideide) and **M17** (tetradeca-2E,4E,12Z-triene-8,10-diynoic acid isobutylamide). With the current MS information, no distinction can be made between the isomeric compounds **M8** (deca-2E,4E,8Z-trienoic acid piperideide), **M13** (deca-2E,4E,6Z-trienoic acid piperideide) and **M14** (deca-2E,4E,6E-trienoic acid piperideide) and between compound **M15** (deca-2E,4E,6Z,8Z-tetraenoic acid piperideide) and its isomer **M16** (deca-2E,4E,6E,8Z-tetraenoic acid piperideide). For all compounds, the molecular ions were detected, except for compound **M12**. Characteristic product ions were found for compounds **M1**, **M3**, **M6**, **M7**, **M12** and **M17**, having an isobutylamide function and are indicated in bold in Table 7. This was also done for compounds **M8**, **M11**, **M13/14**, **M15/16**, which are NAAs having a piperideide function. Furthermore, in all NAAs, σ-cleavages were observed in the fatty acid chain. These product ions were for compound **M1**: *m/z* 204 (C9-C10), *m/z* 63/166 (C6-C7), *m/z* 77 (C5-C6), *m/z* 103 (C3-C4), *m/z* 129 (C1-C2); for compound **M3**: *m/z* 206 (C9-C10), *m/z* 41/180 (C7-C8), *m/z* 55/166 (C6-C7), *m/z* 152 (C5-C6)**,** *m/z* 95 (C3-C4), *m/z* 121 (C1-C2); for compound **M6**: *m/z* 208 (C9-C10), *m/z* 194 (C8-C9), *m/z* 43/180 (C7-C8), *m/z* 57 (C6-C7), *m/z* 152 (C5-C6), *m/z* 97 (C3-C4), *m/z* 123 (C1-C2); for compound **M7**: *m/z* 242 (C12-C13), *m/z* 43/228 (C11-C12), *m/z* 67 (C9-C10), *m/z* 91 (C7-C8), *m/z* 105 (C6-C7), *m/z* 171 (C1-C2); for compound **M12**: *m/z* 77 (C6-C7), *m/z* 143 (C1-C2); for compound **M17**: *m/z* 254 (C13-C14), *m/z* 41 (C11-C12), *m/z* 103 (C6-C7); for compound **M8**: *m/z* 41 (C7-C8), *m/z* 55 (C6-C7), *m/z* 69 (C5-C6), *m/z* 95 (C3-C4), *m/z* 121/110 (C1-C2); for compound **M11**: *m/z* 218 (C9-C10), *m/z* 204 (C8-C9), *m/z* 190 (C7-C8), *m/z* 57 (C6-C7), *m/z* 162 (C5-C6), *m/z* 97 (C3-C4), *m/z* 123 (C1-C2); for compound **M13/14**: *m/z* 202 (C8-C9), *m/z* 43 (C7-C8), *m/z* 69 (C5-C6), *m/z* 95 (C3-C4) and for compound **M15/16**: *m/z* 41 (C7-C8), *m/z* 67

(C5-C6), *m/z* 93 (C3-C4), *m/z* 119 (C1-C2). In addition, product ions were also detected consistent with a (H-)rearrangement between C4-C5 in the fatty acid chain, for compound **M3**: *m/z* 81; for compound **M6**: *m/z* 83; for compound **M17**: *m/z* 129; for compound **M8**: *m/z* 81; for compound **M11**: *m/z* 83 and for compound **M13/14**: *m/z* 81. Compound **M6** in the *A. millefolium* extract corresponds to compound **P11** in the *A. ptarmica* extract and product ions were already described in literature for pellitorine. Moreover, the following product ions of compound **M11** were reported as well: *m/z* 233, 162, 151, 95, 81, 81, 69, 67, 66, and 55 [2]. Compound **M1** was also recognized by the Nist library.

Table 7: MS information of *N*-alkylamides in the ethanolic *A. millefolium* extract using GC-EI-MS.

Compound	$[M]^+$	Product ions (*m/z*) (% intensity relative to base peak)
M1	229	41 (27%), **43** (12%), 53 (8%), 54 (8%) 55 (32%), 56, 58 (17%), 63 (11%), 65 (11%), 66 (27%), 67 (39%), 68 (10%), 69 (20%), 77 (11%), 79 (22%), 81 (26%), 82 (26%), 83 (23%), 84 (23%), 91 (14%), 93 (13%), 94 (13%), 95 (23%), 96 (12%), 97 (12%), 103, 107 (8%), 108 (9%), 109 (9%), 110 (14%), 115 (15%), 123, 127 (25%), 128 (69%), **129** (22%,) 133, 138 (12%), 143 (8%), 151, **157** (100%), 158 (15%), 166 (14%), **172** (8%), 178 (18%), **186**, 204, **214** (9%), 222, 228 (16%), **229** (14%), 233, 264, 280
M3	221	41 (18%), 53 (9%), 55 (52%), **57** (37%), 65 (10%), 66 (27%), 67 (30%), 68 (11%), 77 (9%), 79 (16%), 81 (20%), 82 (10%), 91 (8%), 93 (19%), 94 (15%), 95 (12%), 96 (11%), 98, 100, 107 (8%), 110 (26%), **121** (8%), 122 (9%), 131, **149** (100%), 150 (15%), 152 (19%), **164**, 166 (50%), 167 (16%), **178**, 180, 192, **206** (11%), **221** (40%), 222
M6	223	41 (11%), **43**, 53, 55 (8%), **57**, 66 (10%), 67 (12%), 69 (10%), **72**, 77, 79 (8%), 81 (38%), 83, 95 (12%), 96 (38%), 97, 98, 109, 110 (11%), 113 (10%), **123**, 124, **151** (100%), 152 (33%), **166**, **180**, 194, **208** (10%), **223** (33%)
M7	271	41 (4%), **43** (28%), 51 (11%), 53 (13%), 55 (37%), **57** (77%), 58 (8%), 63 (15%), 65 (23%), 66 (49%), 67 (51%), 68 (30%), 69 (22%), 71 (12%), **72** (12%), 73 (20%), 75 (8%), 76 (8%), 77 (48%), 78 (11%), 79 (42%), 80 (11%), 81 (24%), 82 (22%), 83 (16%), 84 (10%), 85 (9%), 91 (31%), 92 (9%), 93 (16%), 94 (20%), 95 (32%), 96 (13%), 97 (13%), 98 (15%), 103 (17%), 105 (17%), 107 (13%), 109 (15%), 110 (25%), 111 (11%), 115 (31%), 116 (10%), 117 (22%), 118 (9%), 119 (10%), 120, 121 (11%), 123 (9%), 127 (16%), 128 (48%), 129 (100%), 130 (17%), 131 (11%), 141 (35%), 142 (19%), 143 (58%), 144 (25%), 145 (11%), 147 (11%), 151 (9%), 152 (15%), 153 (9%), 155 (16%), 156 (11%), 157 (20%), 158 (11%), 159 (11%), 166 (52%), 167 (52%), 169 (18%), **171** (38%), 172 (13%), 175, 186 (15%), 187 (12%), 189 (13%), **199** (69%), 200 (17%), 207 (12%), **214** (12%), **228** (9%), 242 (16%), **256** (40%), 270 (26%), **271** (50%), 272 (12%), 281 (12%), 294, 341, 355, 408
M8	231	41 (54%), 43 (60%), 54 (30%), 55 (88%), 56 (19%), 57 (42%), 60 (24%), 67 (43%), 68 (24%), 69 (56%), 71 (30%), 73 (67%), 77 (23%), 79 (48%), 80 (23%), 81 (79%), **82** (36%), 83 (40%), 85 (23%), 91 (51%), 93 (34%), 95 (59%), 96 (24%), 97 (21%), 107 (100%), 108 (28%), 109 (39%), **110** (23%), 115 (22%), **121** (22%), 122 (20 %), 129 (27%), 133 (21%), 135 (20%), 147 (30%), **149** (64%), 185 (18%), 189 (23%), 231 (73%)
M11	233	41 (49%), 42 (10%), 43 (30%), 53 (21%), 54 (20%), 55 (75%), 56 (18%), 57 (18%), 60 (9%), 65 (13%), 66 (24%), 67 (67%), 68 (26%), 69 (53%), 70 (10%), 71 (8%), 73 (11%), 77 (23%), 78 (9%), 79 (53%), 80 (22%), 81 (100%), **82** (42%), 83 (80%), 84 (43%), 85 (12%), 91 (24%), 93 (31%), 94 (20%), 95 (57%), 96 (25%), 97 (22%), 98 (8%), 105 (11%), 107 (20%), 108 (12%), 109 (23%), 110 (16%), 111 (8%), 112 (16%), 115 (11%), 121 (13%), 122 (8%), **123** (13%), 127 (9%), 131 (9%), 133 (14%), 135 (12%), 136 (9%), 137 (18%), 138 (34%), 139 (10%), 145, 149 (10%), 150 (21%), **151** (73%), 152 (13%), 157 (16%), 162 (28%), 164 (17%), 165 (9%), 167, 176 (9%), 177 (10%), 178 (59%), 179 (14%), 180 (9%), 190, 204 (9%), 209, 218, 233 (82%), 234 (15%), 264 (8%), 280 (10%)
M12	243	41 (17%), 42, **43** (91%), 45 (8%), 53, 55 (21%), 56 (8%), **57** (15%), 60 (11%), 65, 67 (18%), 68 (8%), 69 (19%), 73 (16%), 77 (11%), 79 (14%), 80 (8%), 81 (20%), 82 (12%), 87 (12%), 88 (10%), 89 (11%), 91 (8%), 93 (12%), 95 (16%), 96 (8%), 97 (13%), 98 (8%), 99 (10%), 101 (9%), 105 (9%), 109 (10%), 110, 111 (11%), 114 (10%), 115 (43%), 116 (8%), 121 (8%), 127 (11%), 129 (15%), 135, 139 (9%), **143** (100%), 144 (15%), 151 (10%), 152 (11%), 153 (18%), 157 (15%), 165 (8%), **171** (10%), 182 (25%), 185 (50%), **186** (51%), 187 (8%), 199 (8%), **200**, 207, 213 (8%), 218, 224, 241 (8%), 284 (10%)

Table 7: MS information of *N*-alkylamides in the ethanolic *A. millefolium* extract using GC-EI-MS (continued).

Compound	$[M]^+$	Product ions (*m/z*) (% intensity relative to base peak)
M13 or M14	231	41 (23%), 43 (23%), 53 (8%), 55 (60%), 57, 60, 67 (15%), 68 (12%), 69 (14%), 73 (10%), 77 (28%), 79 (32%), 80 (10%), 81 (14%), **82** (19%), 83 (29%), 85, 91 (30%), 93 (10%), 95 (15%), 96, 105 (10%), 107 (87%), 108 (10%), 109 (11%), 119 (11%), 129 (9%), 135 (10%), **149** (100%), 150 (10%), 163, 174, 189, 202, 207, 231 (77%), 232 (18%), 280
M15 or M16	229	40 (19%), 41 (78%), 43 (90%), 44 (17%), 45 (17%), 53 (21%), 54 (15%), 55 (90%), 56 (25%), 57 (80%), 60 (25%), 65 (18%), 67 (85%), 68 (61%), 69 (58%), 70 (18%), 71 (31%), 73 (30%), 77 (45%), 79 (63%), 80 (32%), 81 (87%), **82** (53%), 83 (43%), 85 (48%), 91 (90%), 92 (20%), 93 (58%), 94 (70%), 95 (93%), 96 (40%), 97 (27%), 103 (21%), 105 (29%), 107 (51%), 108 (26%), 109 (100%), 110 (36%), 111 (20%), 115 (16%), 117 (16%), **119** (61%), 121 (42%), 122 (51%), 123 (30%), 124 (18%), 125 (26%), 129 (28%), 133 (23%), 135 (66%), 136 (18%), 137 (61%), 138 (15%), 145 (16%), **147** (85%), 149 (23%), 151 (34%), 152 (34%), 153 (59%), 159 (17%), 161 (20%), 163 (19%), 164 (19%), 165 (24%), 177 (30%), 189 (28%), 191 (18%), 203 (27%), 205 (19%), 218 (25%), 229 (52%), 259 (17%), 274 (23%), 280 (15%), 284 (16%)
M17	269	41 (17%), 55 (9%), **57** (38%), 63 (10%), 65 (10%), 66 (24%), 67 (20%), 68 (8%), 75 (8%), 77 (58%), 91 (10%), 94 (8%), 95 (9%), 102 (12%), 103 (47%), 110 (12%), 115 (13%), 128 (17%), 129 (20%), 141 (37%), 142 (14%), 152 (18%), 153 (28%), 154 (31%), 155 (24%), 166 (14%), 167 (48%), 168 (11%), 169 (19%), 175, 184, **197** (17%), **212** (9%), **226**, 240, **254**, 268 (16%), **269** (100%), 270 (21%)

Note: if no % is indicated between brackets = intensity < 7 % of base peak.

Product ions indicated in bold: characteristic product ions for the amide functional group of the NAA.

4. CONCLUSION

In this research, the *N*-alkylamide profiling in two ethanolic plant extracts of the *Achillea* genus, namely *Achillea ptarmica* and *Achillea millefolium* was performed using two different analytical techniques, HPLC-ESI-MS and GC-EI-MS, allowing tentative structural assignments. Our obtained MS spectra corroborate well with the structures of these NAAs. Although, full confirmation of the identity can be obtained by NMR and synthetic standards. In the *Achillea ptarmica* extract, in total 14 *N*-alkylamides were assigned: six NAAs having an isobutylamide function, three NAAs with a piperidide function, two NAAs with a 2-methylbutylamide function, one NAA with a phenylethylamide function, one NAA having a piperideide function and one NAA having a *N*-methyl isobutylamide function. Using both analytical methods, compounds **P1**, **P5**, **P7**, **P9**, **P10**, **P11** and **P12** were reported. Compounds **P2**, **P3**, **P4**, **P6**, **P8**, **P13** and **P14** were assigned with HPLC-ESI-MS, but not with GC-EI-MS. Interestingly, it is the first time that compounds **P8** and **P14** are assigned in *Achillea ptarmica*. The MS spectra corroborate well with the structures of deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) or a related isomeric compound and deca-2E,4E-dienoic acid *N*-methyl isobutylamide, respectively. In the *Achillea millefolium* extract, 15 NAAs were assigned: six NAAs having a isobutylamide function, one NAA with a 4-hydroxyphenylethylamide function, one NAA with a 2-methylbutylamide function, two NAAs having a piperidide function, four NAAs with a piperideide function and one NAA with a 4-methoxyphenylethylamide function. Using HPLC-MS and GC-MS, five NAAs were assigned using both analytical techniques: compounds **M1**, **M3**, **M6**, **M7** and **M8**, whereas compounds **M2**, **M4**, **M5**, **M9**, **M10** were only assigned using HPLC-ESI-MS. Furthermore, five additional NAAs were reported using GC-EI-MS: compounds **M11**, **M12**, **M13/14**, **M15/16** and **M17**. Like in the *A. ptarmica* extract, the MS spectra of compound **M4** in *A. millefolium* extract corroborate well with the structure of homospilanthol or a related isomeric compound. This is the first time that homospilanthol has been assigned in *A. ptarmica* and *A. millefolium*.

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CHAPTER III

SKIN PERMEATION KINETICS OF PELLITORINE

"Make everything as simple as possible, but not simpler."

Albert Einstein
(°1879-†1955, physicist, developer the general theory of relativity)

Parts of this chapter were published:

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ABSTRACT

The plant *Anacyclus pyrethrum* (AP) consists of several *N*-alkylamides with pellitorine as main constituent. AP extracts are known to be biologically active and some products for topical administration containing AP plant extracts are already commercially available with functional cosmeceutical claims. However, no transdermal data for pellitorine are currently available. Therefore, our general goal was to investigate the local skin pharmacokinetics of the plant *N*-alkylamide pellitorine using a Franz diffusion cell set-up. Two different forms were applied on human skin: purified pellitorine and the AP extract. Our study demonstrated that pellitorine is able to cross the stratum corneum and the subsequent skin layers. A significantly higher permeability coefficient was observed when the AP extract (K_p = $2.3 \cdot 10^{-4}$ cm/h) was administered, compared to purified pellitorine (K_p = $1.1 \cdot 10^{-4}$ cm/h). With the obtained pellitorine concentrations in the skin layers and the receptor fluid, it is concluded that local and systemic effects can be expected after topical application. Due to these findings and as a regulatory consequence, products containing reasonable concentrations of pellitorine are recommended to be classified as a medicinal product.

CHAPTER III

SKIN PERMEATION KINETICS OF PELLITORINE

Main focus in this chapter:

- To purify and isolate pellitorine from the *Anacyclus pyrethrum* extract.
- To quantitatively investigate the penetration behaviour of pellitorine through the skin.

1. INTRODUCTION

Anacyclus pyrethrum (L.) Lag. (AP) is a plant belonging to the Asteracea family. Several *N*-alkylamides (NAAs) present in the AP extract have already been identified and reported. Thirteen NAAs were identified in an ethanolic AP root extract with HPLC-UV/ESI-MS: *N*-isobutylamides, *N*-methylisobutylamides, 4-hydroxyphenylethylamides and 2-phenylethylamides. The most abundant NAA in this AP extract is pellitorine (deca-2E,4E-dienoic acid isobutylamide) [1]. The structure of pellitorine is given in Figure 1.

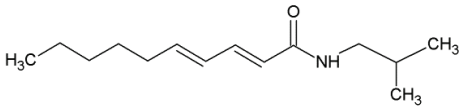

Figure 1. Structure of pellitorine.

Extracts of *Anacyclus pyrethrum* are ethnopharmacologically used to treat various diseases. Root extracts are known to have antimicrobial, local anaesthetical, anti-depressive, insecticidal, and saliva stimulating features and are used to treat epilepsy, paralysis, toothache and rheumatism [2, 3]. Hydro-alcoholic and chloroform extracts of AP have anticonvulsive effects [4, 5]. In addition, aqueous extracts are used as aphrodisiac to improve the libido of men [6].

It was shown that the main biologically active AP constituents, *N*-alkylamides, inhibit cyclooxygenase [7]. This anti-inflammatory effect was confirmed *in vitro* with different AP extracts [8]. In addition, pellitorine was demonstrated *in vivo* to have a strong tingling and saliva stimulating effect [9].

Topical products containing *Anacyclus pyrethrum* extracts are already commercially available with functional, cosmeceutical claims. AP root extracts are used in lotions, creams, toners, gels, moisturizers and bath care products, marketed as a cosmetic not only for moisturizing the skin but also for stimulating cell regeneration to reduce discolouration.

In order to exert a biological effect after topical application, the active *N*-alkylamides must permeate through the stratum corneum into the viable skin layers for a local effect and penetrate through the skin for a systemic effect. Previous research from our group has shown that spilanthol, present in *Spilanthes acmella* extract, can permeate pig mucosa and human skin, with systemic effects very likely to be present [10, 11]. Until now, spilanthol (deca-2E,6Z,8E-trienoic acid isobutylamide), containing three unsaturated double bounds in the fatty acid chain (a triene), is the only plant *N*-alkylamide on which research was done to evaluate the permeation through human skin: no other plant *N*-alkylamides were investigated yet for transdermal purposes. In this study, the skin permeability of another *N*-alkylamide, with a different structure, was investigated: pellitorine, containing two double bounds (a diene). Pellitorine and spilanthol have the same isobutylamide group and both alkylamides possess a 2E double bound in the fatty acid chain, but pellitorine contains 2 double bounds instead of three and the second double bound is on C4, instead of C6 or C8 in case of spilanthol. Both purified pellitorine, as well as the ethanolic extract of *Anacyclus pyrethrum* were used in this study to investigate if there was a difference in penetration of pellitorine through human skin, as co-compounds in the plant extract could influence the skin penetration as well.

2. MATERIALS AND METHODS

Chemicals and reagents

Ultrapure water (H_2O) of 18.2 MΩ.cm quality was produced by an Arium 611 purification system (Sartorius, Göttingen, Germany). Absolute ethanol (EtOH, 99.8% V/V) and acetic acid were purchased from Sigma-Aldrich (Bornem, Belgium). 0.01 M phosphate buffered saline (PBS) was purchased from Sigma-Aldrich as well and prepared according to the instructions of the supplier. HPLC gradient grade methanol (MeOH) and acetonitrile (ACN) came from Fisher Scientific (Erembodegem, Belgium), while formic acid (FA) was bought from Riedel-de Haën (Seelze-Hannover, Germany). Denaturated ethanol (up to 5% ether) came from Chem Lab (Zedelgem, Belgium).

Analytical characterisation of *Anacyclus pyrethrum* extract

The *Anacyclus pyrethrum* root extract was prepared as previously described [1]. For the analytical characterisation of the extract, the extract was dissolved in ethanol, vortexed, sonificated for 2 hours and centrifuged for 15 minutes at 3220 *g* at room temperature. A 30:70 H_2O/EtOH (V/V) solution was prepared, centrifuged again and the supernatant was filtered using a 0.45 µm nylon HPLC filter (Whatman). 25 µl of this solution was injected on a Grace Prevail C18 (250 x 4.6 mm, 5 µm) column using a Waters HPLC equipped with a Waters 2487 Dual Absorbance detector set at 258 nm. A gradient with a flow rate of 1.0 ml/min, was used as follows: t=0 min: 80:20 A:B (V/V), t=0-150 min: 10:90 A:B (V/V), t=150-151 min: 80:10 A:B (V/V), t=151-166 min: 80:20 A:B (V/V), t=166 min: 80:20 A:B (V/V) (with A = 1% acetic acid in H_2O and B = ACN). The identity of the mean peak in the AP extract was determined using HPLC-MS, based upon the precursor ion *m/z* and the fragmentation pattern. The HPLC-MS analysis was done on a HPLC system which consisted of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a Spectra System P1000XR pump, a Spectra System AS3000 autosampler, and a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA) equipped with a Waters 2487 Dual Absorbance detector (Shimadzu, Kyoto, Japan) and Xcalibur 2.0 software (Thermo) for data acquisition. The extract, dissolved in 50:50 ACN:H_2O (V/V), was injected into the LC-MS apparatus and the MS method according to Boonen *et al.* (2012) was used [1].

Purification of pellitorine

Pellitorine was isolated and purified from the *Anacyclus pyrethrum* extract by means of semi-preparative HPLC as follows. The *Anacyclus pyrethrum* root extract was dissolved in acetonitrile, vortexed and sonificated for 2 hours and centrifuged for 15 minutes at 3220 *g* at room temperature. A 1:1 dilution of the supernatant was prepared with H_2O. The solution was centrifuged again and the supernatant was filtered using a HPLC filter. 1.0 ml of the solution was injected on a Vydac C18 monomeric semi-preparative column (Grace, 250 mm x 10 mm, 5 µm) using a Waters HPLC equipped with a Waters 2487 Dual Absorbance detector. The sample compartment and column temperature were maintained at room temperature. An isocratic elution mode was used with as mobile phase 50:50 A:B (V/V) (A: 0.1% FA in H_2O and B: 0.1% FA in MeOH). A flow rate of 6.0 ml/min was used and UV detection was performed at 258 nm. Fractions between 50 and 57 minutes retention time on the semi-preparative HPLC were collected and evaporated to dryness using a rotavapor (Büchi rotavapor R-200 with Büchi heating batch B-490). The obtained dried fractions were dissolved in methanol, combined and evaporated to dryness under nitrogen.

Analytical characterisation of purified pellitorine

The identity of the purified pellitorine was confirmed using HPLC-MS. The equipment is already described in the section 'Analytical characterisation of *Anacyclus pyrethrum* extract'. A prevail RP C18 column (Grace, 250 x 4.6 mm, 5 µm) with a suitable guard column was used. The sample compartment was kept constant at 20°C, while the column temperature was maintained at 30°C. The purified pellitorine was dissolved in 50:50 ACN:H_2O (V/V), 25 µl was injected and the flow rate was set to 1.0 ml/min. A gradient was used as follows: t=0 min: 60:40 A:B (V/V), t=0-5 min: 50:50 A:B (V/V), t=5-35 min: 40:60 A:B (V/V), t=35-40 min: 10:90 A:B (V/V), t=40-41 min: 60:40 A:B (V/V) and t=41-46 min: 60:40 A:B (V/V) (with A = 1% acetic acid in H_2O and B = ACN). ESI was conducted with a capillary voltage of 3 V. Nitrogen was used as sheath and auxiliary gas. The temperature of the heated capillary was set at 275°C. MS-MS spectra were obtained by collision-induced dissociation (CID) of the parent *m/z*, with the relative collision energy set to 35%. Identification was based on the parent *m/z* values and fragmentation ions.

The purity of purified pellitorine was determined using HPLC-UV. 25 µl of purified pellitorine, dissolved in 30:70 H_2O:EtOH (V/V) was injected on a Prevail C18 column (Grace, 250 x 4.6 mm, 5 µm) with guard column. HPLC analysis was performed on a Waters Alliance 2695 HPLC equipped with a Waters 2998 Photo Diode Array detector. The same gradient method was used as previously described above for the characterisation of the extract. The reporting threshold was set on 0.10%.

Preparations of Franz diffusion dose solutions

Dose solutions of the *Anacyclus pyrethrum* extract and the purified pellitorine were prepared in 30:70 H_2O:EtOH (V/V). In these dose solutions, the experimentally determined pellitorine concentration was 816 µg/ml in case of the purified pellitorine (purity factor: 0.93) and 316 µg/ml in case of the *Anacyclus pyrethrum* extract. The purified pellitorine concentration was determined using ultraviolet-visible (UV-vis) spectrophotometry (Ultrospec 4000 Pharmacia Biotech), using $E_{1cm}^{1\%} = 1330$, while the concentration in the AP extract was determined using the purified pellitorine as reference standard [12]. Negative controls were included in the study as well (dose solutions without NAA).

In vitro skin permeation study

Static Franz diffusion cells (Logan Instruments Corp., New Jersey, USA) with a receptor compartment of 5 ml and an available diffusion area of 0.64 cm² were used to determine the skin permeation of pellitorine in the different formulations. Human skin was used and the analyses were done in fourfold for the purified pellitorine and in sextuplicate for the *Anacyclus pyrethrum* extract, using a

randomized blocked design. The skin samples were obtained from aesthetic body contouring surgery of three healthy female patients (40 years old ± 10, mean ± SD), supplied by the Department of Plastic and Reconstructive Surgery of the University Hospital (Ghent, Belgium). Confidentiality procedures with informed consent were applied. Immediately after the surgical procedure, the skin was cleaned with 0.01 M PBS pH 7.4, the subcutaneous fat was removed and the skin was subsequently stored at -20°C for not longer than 6 months. Just before the start of the FDC experiments, the full-thickness skin was thawed, mounted on a template and sliced to obtain split-thickness human skin, using an electrical powered dermatome. An actual skin thickness of 295 ± 15.5 µm (mean ± SEM, n=20), 220 ± 9.35 µm (mean ± SEM, n=30) and 235 ± 9.09 µm (mean ± SEM, n=23) was experimentally determined with a micrometer (Mitutoyo, Tokyo, Japan) from the different patients. The receptor chambers were filled with 0.01 M PBS. The skin samples were visually inspected for skin damage and were mounted on the FDC between the donor and the receptor chambers, with the epidermis side upwards ensuring that no air was present under the skin. A Teflon coated magnetic stirring bar (400 rpm) allowed that the receptor fluid was continuously mixed. Skin integrity was controlled by measuring the skin impedance using an automatic micro-processor controlled Tinsley LCR Impedance Bridge (Croydon, U.K.). Skin pieces displaying an impedance value below 10 kΩ were discarded and replaced by a new skin piece [13]. 500 µl of the dose solutions were applied on the skin surface with a micropipette. The donor chamber was covered with parafilm to prevent evaporation of the dose formulations. During the FDC experiment, the temperature of the receptor compartment was kept constant at 32°C by a water jacket. 200 µl samples of receptor fluid were taken at regular time intervals (0h, 1h, 2h, 4h, 8h, 12h, 18h, 21h, 24h) from the sample port and were immediately replaced by 200 µl fresh receptor fluid. This was taken into account for the calculation of the cumulative permeated concentrations. Immediately after the last sample had been drawn, the remaining dose formulation was removed from the skin surfaces using a cotton swab. The epidermis and dermis were separated with forceps and pellitorine was extracted from the skin layers with ethanol. In addition, the ratio $C_{24h,epidermis}/C_{24h,vehicle}$ was obtained. The concentration of pellitorine in the (epi)dermis was obtained by dividing the amount of extracted pellitorine (experimentally determined) by the volume of the (epi)dermis (thickness of (epi)dermis (cm) x skin surface (0.64 cm^2)). The thickness of the epidermis was assumed to be 50 µm, and the thickness of the dermis was the total measured skin thickness minus the thickness of the epidermis. The concentration of pellitorine in the remaining dose solution after 24 hours was calculated by dividing the amount of pellitorine in the dose solution left after 24h (experimentally determined) by the applied volume of the dose solution, *i.e.* 500 µl. A linear relationship of the individual cumulative amount of pellitorine versus time was observed, confirming steady-state conditions. Sink conditions were confirmed by the data.

Liquid chromatography of the FDC samples

Two methods were used, *i.e.* HPLC-UV and UHPLC-UV. The HPLC-UV method was applied for the FDC experiments with the purified pellitorine. However, this method required too long run times when applied on AP extract samples due to the presence of other compounds which had to be eliminated from the column before a next run. Hence, an adapted UHPLC method was applied on the FDC samples of the extract.

The HPLC-UV method used a Waters Alliance 2695 separation module and a dual absorbance detector 2487, equipped with Empower 2 software (Waters, Millford, USA) were part of the UHPLC apparatus. 25 µl of each sample was injected on a Symmetry C18 column (75 mm x 4.6 mm, 3.5 µm) (Waters, Milford, USA) with an appropriate guard column. The sample compartment was kept constant at 20°C, while the column temperature was maintained at 30°C. A degassed isocratic mobile phase of 0.1% FA in 30:70 H_2O:MeOH (V/V) was used at a flow rate of 1.5 ml/min was used. The run time was 5 min and UV detection was performed at 258 nm with peak areas used for quantification. An analytical validation was performed and the LoD and LoQ, defined as the concentrations equivalent to a signal to noise value of 3 and 10, respectively, were determined to be 6.92 ng/ml and 23.06 ng/ml. Linearity, with determination coefficient R^2 = 1.000, is assured in a working range of 23.06 ng/ml (LoQ) up to 204 µg/ml.

The UHPLC-UV method used a Waters Acquity UPLC with a Waters Photo Diode Array (PDA) detector, equipped with Empower software (Waters, Millford, USA) were part of the UHPLC apparatus. 2 µl of each sample was injected on a Acquity UPLC C18 column (50 mm x 2.1 mm, 1.7 µm) (Waters, Milford, USA) with an appropriate guard column. The sample compartment was kept constant at 5°C, while the column temperature was maintained at 30°C. A gradient was used (t=0-1.8 min: 100% A, t=1.8-2.3 min: 100% B, t=2.3-3.55 min: 100% B, t=3.55-4.05 min: 100% A, t=4.05-6.55 min: 100% A with A: 0.1% FA in 30:70 (V/V) H_2O:MeOH and B: 0.1% FA in MeOH) at a flow rate of 0.5 ml/min was used. UV detection was performed at 258 nm with peak areas used for quantification. An analytical validation was performed and the LoD and LoQ, defined as the concentrations equivalent to a signal to noise value of 3 and 10, respectively, were determined to be 2.97 ng/mL and 9.91 ng/ml. Linearity, with determination coefficient R^2 = 1.000, is assured in a working range of 9.91 ng/ml (LoQ) up to 204 µg/ml.

Calculation of skin permeation parameters

The cumulative amounts of pellitorine (in µg) permeated through human skin were plotted as a function of time (in hours). For the calculations of the transdermal parameters, the individual graphs were used. Steady-state flux (J_{ss}, µg/(cm²·h)) was calculated from the slope of the linear portion of the cumulative amount versus time curve divided by 0.64 to correct for the exposed skin area (cm^2). The lag time (h) was obtained by setting y = 0 in the individual linear regression equation. There exists different interpretations of the term lag time and this will be discussed in more detail in Chapter IV. The Q_{1d} is the cumulative quantity, expressed as % of the effective dose applied, obtained after 1 day. The indicated parameters are the secondary parameters, *i.e.* directly obtained from and dependent on the experimental data. The primary parameters, *i.e.* derived from the experimentally obtained secondary parameters and independent of some operational experimental conditions, are calculated in accordance with ECETOC [14]: the steady-state permeability coefficient (K_p, cm/h) was calculated as follows:

$$K_p = \frac{J_{ss}}{C_d}$$

where C_d (µg/ml) is the concentration of pellitorine in the dose formulation. Furthermore, the apparent diffusion (D_m, cm²/h) and partition (K_m) coefficients were determined using the following equations:

$$D_m = \frac{d^2}{6 \times t_{lag}}$$

$$K_m = \frac{K_p \times d}{D_m}$$

where d and t_{lag} are the measured skin thickness (cm) and the lag time (h), respectively. Furthermore, the steady-state plasma concentration after topical application of pellitorine (ng/ml) was calculated using the following formula:

$$C_{pl,ss}^{top} = \frac{A \times K_p \times C_d}{Cl}$$

in which Cl is the plasma clearance (l/h), A is the skin surface (cm²) and C_d the concentration in the dose solution applied on the skin.

3. RESULTS

Identification and purity of pellitorine

The purity of the *Anacyclus pyrethrum* extract and the purified pellitorine was determined by HPLC-UV. Figure 2 shows the HPLC-UV chromatogram of the used extract. The main peak was pellitorine

(deca-2E,4E-dienoic acid isobutylamide or $C_{14}H_{25}NO$), identified by the protonated molecule *m/z* signal observed in MS^1 (precursor ion *m/z* = 224) and complemented by the CID fragmentation data in MS^2. The product ions (*m/z*) were 74, 83, 123, 133, 151 and 168; with fragment losses of 150, 141, 101, 91, 73 and 56, respectively. Its normalized concentration, based on UV_{258nm} peak area, was 32.0%. The other peaks observed are other *N*-alkylamides, *e.g.* anacycline (tetradeca-2E,4E-diene-8,10-diynoic acid isobutylamide).

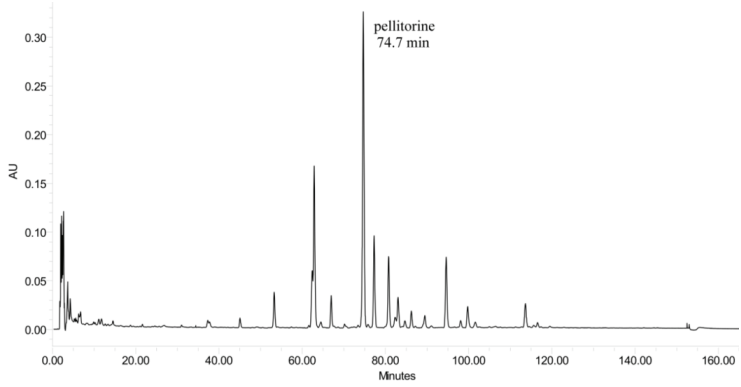

Figure 2. HPLC-UV (258 nm) chromatogram of *Anacyclus pyrethrum* extract.

The isolated pellitorine had a normalised concentration of 93.3%, with only one additional *N*-alkylamide peak observed at 67.0 minutes (Figure 3). MS^1 and MS^2 data confirmed the identity of both peaks. The minor peak observed at 67.0 minutes with a precursor ion of 336 *m/z* ($C_{22}H_{25}NO_2$) resulting in product ions (*m/z*) of 121, 129, 171, 199 and 216 with fragment losses of 215, 207, 165, 137 and 120, respectively, was identified as tetradeca-2E,4E-diene-8,10-diynoic acid 4-OH phenylethylamide.

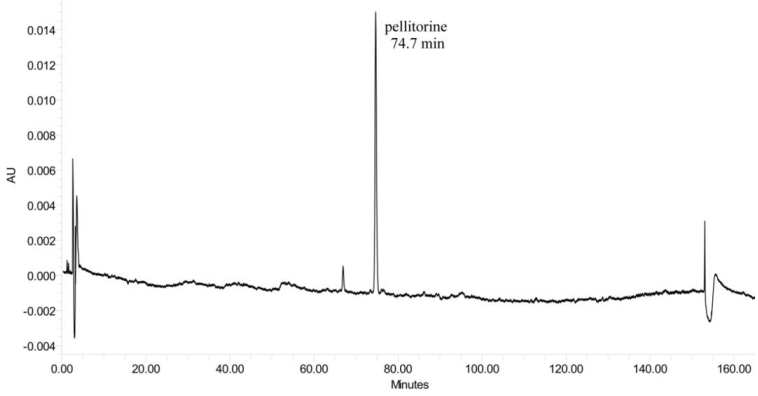

Figure 3. HPLC-UV (258 nm) chromatogram of purified pellitorine.

Franz diffusion cell transdermal results

Our results showed that pellitorine was able to diffuse through human skin when applied in an EtOH:H_2O solution. All individual runs confirmed the sink and steady-state conditions. A linear relationship of the individual cumulative amounts versus time was observed between time points 8 and 24h (R^2 not less than 0.96). The mean percentage of pellitorine of the applied dose solution permeated through the skin versus time, is visually presented in Figure 4 for both the purified pellitorine and pellitorine in the *Anacyclus pyrethrum* extract.

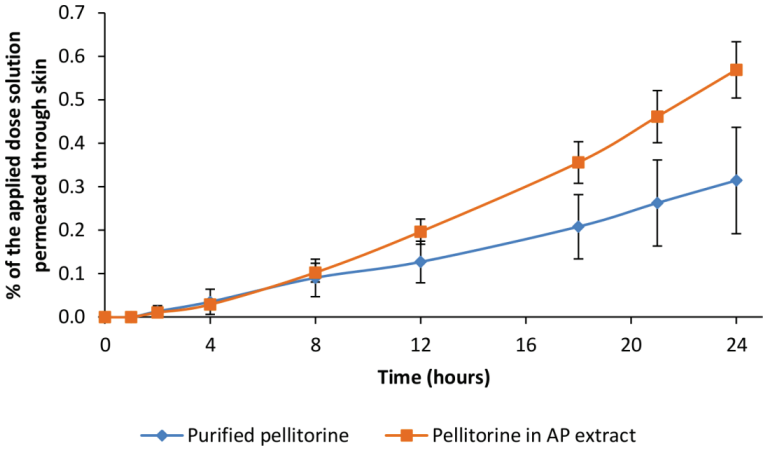

Figure 4. Percentage of the applied dose solution permeated through the skin versus time (mean ± SEM, with n=4 for purified pellitorine and n=6 for pellitorine in AP extract).

During the first hours of the FDC experiment, the percentage of pellitorine permeated through the skin was very similar for both formulations up to 8h. From then on (8-24h), the percentage of pellitorine penetrating the skin was higher for pellitorine in the extract and an increase in difference between the two formulations was seen towards the end of the experiment (24h). The curve of the AP extract exhibited a higher slope than the slope of purified pellitorine.

Linear regression on the linear sections of the individual curves, relating the cumulative amounts of pellitorine versus time, was performed to calculate the skin parameters. The values of the transdermal parameters, calculated on the individual linear trend lines, are given in Table 1.

Table 1. Transdermal parameters for pellitorine in different dose solutions (mean ± SEM, with n=4 for purified pellitorine and n=6 for pellitorine in AP extract).

Dose solution	Observed secondary parameters		
	J_{ss} (µg/cm².h)	Q_{1d} (% of the applied dose solution)	t_{lag} (h)
Pellitorine	0.089 ± 0.032	0.314 ± 0.123	2.445 ± 0.674
Pellitorine in AP extract	0.071 ± 0.008	0.569 ± 0.065	5.050 ± 0.576
Dose solution	**Apparent primary parameters**		
	K_p (10^{-4} · cm/h)	D_m (10^{-5} · cm²/h)	K_m
Pellitorine	1.096 ± 0.397	5.479 ± 1.738	0.067 ± 0.030
Pellitorine in AP extract	2.253 ± 0.246	2.166 ± 0.207	0.265 ± 0.028

The difference in the apparent primary skin parameter K_p between the two formulations was significant at the 0.05 significance level using an independent t-test ($p < 0.05$). The steady-state permeability coefficient of pellitorine in the *Anacyclus pyrethrum* extract was thus significantly higher than the K_p of the purified pellitorine.

After separating the epidermis from the dermis at the end of the FDC experiment (24h), the remaining amounts of pellitorine present in the applied dose solution, in the epidermis and in the dermis were determined. Figure 5 presents an overview of the pellitorine concentrations in the different compartments (dose solution, epidermis, dermis, receptor fluid) obtained under our experimental conditions, applying dose solutions of 816 µg/ml pellitorine (as purified form) and 316 µg/ml pellitorine (as AP root extract).

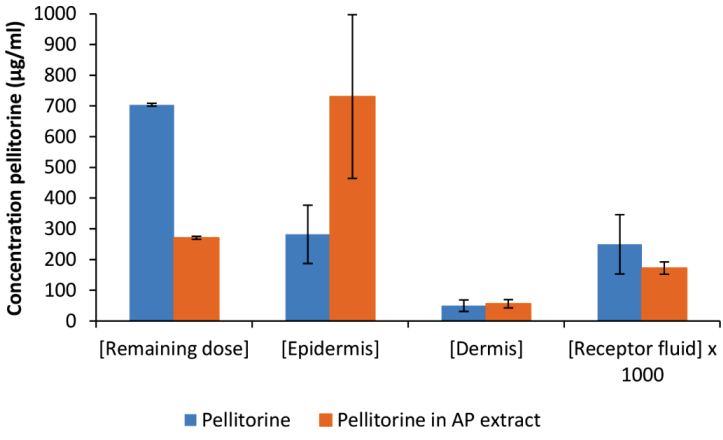

Figure 5. Concentration of pellitorine after 24h (mean ± SEM, n=4-6).

Concentrations of pellitorine in the epidermis and dermis ranged between 282-730 µg/ml and 50-56 µg/ml, respectively. Pellitorine in the purified preparation showed a decreasing concentration gradient from the dose solution over the epidermis and dermis to the receptor fluid, while pellitorine in the plant extract gave a different concentration distribution, with much higher concentrations in the epidermis ('reservoir effect'), compared not only to the dose solution, but also to the purified formulation.

4. DISCUSSION

Our findings demonstrate for the first time that the plant diene *N*-alkylamide pellitorine from an *Anacyclus pyrethrum* extract permeates the skin. This corroborates well with a previous study from our research group, demonstrating the skin permeability of the triene *N*-alkylamide spilanthol [10]. Spilanthol and pellitorine only differ in the place and the number of unsaturated bonds in the fatty acid chain. Spilanthol, having an extra double bond in the fatty acid chain, is slightly more hydrophilic than pellitorine. This is reflected in the logP values: spilanthol has a slightly lower logP value (3.39) than pellitorine (3.65) (calculated with Hyperchem professional 8.0 software). Both these logP values are acceptable values for skin penetrators, with the optimal target logP value currently proposed ranging between 1 and 3 [15]. Due to its slightly more optimal logP value, spilanthol is expected to penetrate the skin more. Indeed, spilanthol has higher K_p values (purified: $1.66 \cdot 10^{-4}$ cm/h and in extract: $4.29 \cdot 10^{-4}$ cm/h) compared to the K_p's of pellitorine (purified: $1.10 \cdot 10^{-4}$ cm/h and in extract: $2.25 \cdot 10^{-4}$ cm/h).

In this study, two different forms were tested: purified pellitorine and pellitorine in *Anacyclus pyrethrum* extract. Upon comparison of the apparent permeability coefficients of pellitorine between the two formulations, a significant difference between the K_p's of the purified pellitorine versus the AP extract was observed: a two times higher K_p was obtained for pellitorine in the extract. As a result of this, the percentage of pellitorine that had penetrated through the skin after 24h was significantly higher when the extract was used as dose solution. This can only be due to the other compounds present in the extract, which improved the penetration of pellitorine. The AP extract does indeed contain other *N*-alkylamides (Figure 2) [1], which can themselves function as skin penetration enhancers. This corroborates well with a previous study, where the NAA spilanthol was demonstrated to act as a compound dependent skin penetration enhancer [16]. The results of the current study with the pellitorine extract suggest that also other NAAs can exert a penetration enhancing effect. Furthermore, based upon these results, a similar study was undertaken comparing the K_p's of purified spilanthol versus the K_p of spilanthol in *Spilanthes acmella* extract. A 2.6 times

higher K_p ($4.29 \cdot 10^{-4}$ cm/h) was obtained for the extract compared to purified spilanthol ($1.66 \cdot 10^{-4}$ cm/h), confirming and generalizing our findings.

In general, two processes have to be considered in skin permeation: (1) the partition of a compound from the dose solution (vehicle) into the SC and (2) the diffusion across the SC and subsequent layers. The first step is thus the partitioning of a compound into the SC, which can be quantified by a thermodynamic equilibrium constant between the concentration of the penetrant in the vehicle and the upper skin layer, *i.e.* the stratum corneum. Partitioning is important for skin absorption because it influences the amount of penetrant in the SC and subsequently this amount affects the thermodynamic gradient across the skin [17].

The partition coefficient K_m for pellitorine in the *Anacyclus pyrethrum* extract (0.265) was higher than the one of purified pellitorine (0.067). This can also be observed in the distribution graph, presented in Figure 5, in which the epidermis contained the highest concentration of pellitorine when the dose solution was the AP extract: the ratio $C_{24h,epidermis}/C_{24h,vehicle}$ was 2.78 for the AP extract and 0.40 for purified pellitorine. The other *N*-alkylamides present in the extract will thus not only influence the pellitorine behaviour in the dose solution, but will also alter the chemical properties of the SC and hence the partition coefficient K_m.

The second stage in skin absorption is the diffusion of the penetrant within the upper skin layers, which depends upon a thermodynamic gradient. Factors that can affect the diffusion are the size, the shape of the penetrant and stickiness of the penetrant to compounds present in the skin (hydrogen bonding) [17]. Furthermore, it is recognized that solvents, such as ethanol, as used in our study, can enhance solubility, influence drug permeation and alter skin barrier properties. Ethanol is a frequently used solvent in topically applied medicines and is known to also have a penetration enhancing effect at lower concentrations with a change in this property observed at concentrations above 70%. At these higher ethanol levels, there is *i.a.* a dehydration of human skin resulting in a decrease of permeability of compounds dissolved in high ethanol formulations [10, 18, 19]. The co-compounds in the extract apparently do not significantly influence the diffusion in the viable skin layers and thus once in the skin, there is almost no difference between the D_m of pellitorine in the two forms.

As there are already some topical products on the market containing pellitorine, it is important to determine the biological relevance of the obtained local skin kinetics data of pellitorine. Until now, there are no integrated pharmacokinetic/pharmacodynamic (PK/PD) models available to characterise the effects of pellitorine. Consequently, for example, the required plasma concentration of pellitorine for having systemic effects is not yet known. Therefore, this information is taken from

another plant *N*-alkylamide, where PK/PD data are available. In the study of Guiotto *et al.* (2008), the apparent clearance of the *N*-alkylamide dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide from *Echinacea purpurea* was 137 l/h and this value was also used for our calculations with pellitorine [20]. With the obtained K_p values in this study ($1.1 \cdot 10^{-4}$ for purified pellitorine and $2.3 \cdot 10^{-4}$ for pellitorine in the extract) and under the assumptions that the exposed skin surface is 20 cm² and the concentration in the dose solution is 1 mg/ml, the steady-state plasma concentration of pellitorine after topical application can thus be calculated: the resulting steady-state plasma concentrations for purified pellitorine and pellitorine in the *Anacyclus pyrethrum* extract were $1.6 \cdot 10^{-2}$ ng/ml and $3.2 \cdot 10^{-2}$ ng/ml, respectively. When comparing these values with the steady-state plasma concentration of the *Echinacea purpurea N*-alkylamide ($4.3 \cdot 10^{-2}$ ng/ml) to have bioactivity, it is concluded that the pellitorine steady-state plasma concentrations are in the same order of magnitude as the bioactive *Echinacea purpurea N*-alkylamide, indicating pellitorine is able to elicit systemic effects after topical application [20]. Furthermore, the epidermis and dermis concentration of pellitorine obtained with a concentration of pellitorine in the dose solution of 816 µg/ml in the purified solution and 316 µg/ml in the extract solution, were between 282 µg/ml – 730 µg/ml and 50 µg/ml – 56 µg/ml respectively. Seen the high skin concentrations of pellitorine, local effects are certainly expected, *e.g.* immune-modulating activity. A topical formulation of pellitorine can thus be interesting for systemic and/or local functionalities.

This transdermal behaviour, coupled to its pharmacodynamic properties, can have consequences for the regulatory classification of products containing plant-based *N*-alkylamides like pellitorine [21-23]. This study clearly demonstrated that pellitorine was able to penetrate the skin and reach not only the viable cells of the skin in this study, but also the systemic circulation, recommending formulations containing reasonable concentrations of this plant *N*-alkylamide as a medicinal product. This will be further discussed in Chapter VII.

5. CONCLUSION

In this study, the *N*-alkylamide pellitorine was isolated from an ethanolic *Anacyclus pyrethrum* extract by means of semi-preparative HPLC. The purity was evaluated and was determined to be 93% using HPLC-UV. The transdermal behaviour of pellitorine was investigated with Franz diffusion cells and pellitorine was applied on human skin in two different forms: as purified pellitorine and as *Anacyclus pyrethrum* extract. The purity of pellitorine in the extract was 32%. In both dose solutions, pellitorine was able to cross the stratum corneum and subsequently reach the viable epidermis and dermis. A higher permeability coefficient was observed for pellitorine when administered as extract

($2.3 \cdot 10^{-4}$ cm/h) than as purified pellitorine ($1.1 \cdot 10^{-4}$ cm/h). After 24h, concentration profiles of pellitorine were obtained in the remaining dose solution, the epidermis, the dermis and the receptor fluid. An increased partitioning of pellitorine from the dose solution into the stratum corneum was observed when using the extract, which is also reflected by the higher partition coefficient (0.265) compared to the K_m value of the purified pellitorine (0.067). Other *N*-alkylamides present in the *Anacyclus pyrethrum* extract thus contribute to the skin penetration of pellitorine. To conclude, due to the high systemic and skin concentrations of pellitorine, local dermal and systemic biological effects are expected, recommending that topically applied *N*-alkylamides like pellitorine are generally better classified as a medicinal product instead of a cosmetic.

6. REFERENCES

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CHAPTER IV

IMPLEMENTATION OF A SINGLE QUAD MS DETECTOR IN HIGH-THROUGHPUT TRANSDERMAL RESEARCH OF PLANT EXTRACTS

"Human science fragments everything in order to understand it, kills everything in order to examine it."

Leo Tolstoy, War and Peace
(°1828- †1910, Russian writer)

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ABSTRACT

In this study, a new type of single quadrupole mass spectrometric detector was implemented in transdermal research. The local skin pharmacokinetic properties of the plant *N*-alkylamides (NAAs) pellitorine and anacycline, present in an *Anacyclus pyrethrum* extract, and spilanthol, present in *Spilanthes acmella* extract were investigated. This single quad MS detection method showed great advantages compared to the traditional UV detector. The NAAs could be identified and quantified in the samples with an ultra high performance liquid chromatography (UHPLC)-single quad MS detection system, even if they were not separated, which is a requirement when using an UV-detector. Another advantage of the UHPLC-MS system is that lower limit of detection values could be obtained allowing a more accurate and precise determination of the experimental lag time in the *in vitro* skin permeation experiments. To conclude, this single quad MS detector coupled to UHPLC is a useful analytical tool with improved performance compared to high performance liquid chromatography (HPLC)-UV for biomedical-pharmaceutical purposes in transdermal research.

CHAPTER IV

IMPLEMENTATION OF A SINGLE QUAD MS DETECTOR IN HIGH-THROUGHPUT TRANSDERMAL RESEARCH OF PLANT EXTRACTS

Main focus in this chapter:

- To evaluate the use of a single quad MS detector in transdermal research.
- To compare the advantages of this technique with a traditional UV detector.

1. INTRODUCTION

There are some limitations concerning the use of liquid chromatography – ultraviolet/visible (LC-UV/VIS) for the quantification of chemicals, as there is the possibility for compounds with a very similar structure to co-elute, as well as the relatively high limits of detection (LoD) for most compounds. Therefore, there is a need for a simple technique to overcome these separation problems and to obtain low LoD values. Currently, there is such simple technology available with a single quad MS detector, which is directly compatible with existing LC equipments and software, complying with GLP/GMP requirements. This detector is characterized by easy-to-use smooth lab-integration, increased efficiency and acceptably low cost, providing additional detection possibilities over the traditional UV/VIS detectors. The productivity and information content of the analyses can thus be enhanced. With the single quad MS detector, it is not only possible to make a scan of a selected mass range, but selected ion monitoring (SIM) methods can be used as well, making it possible to look at selected compounds with a known molecular weight. Besides the confirmation of the identity of a compound using the *m/z* of the compound, it can thus also be used for the

quantification of compounds. Recently, this detector has been implemented in the routine QC analysis of some peptide drugs [1].

In this study, biologically active compounds in plant extracts are quantified. Plant extracts are complex mixtures, containing quite often many bioactive compounds. These plant extracts are frequently used in cosmetics for topical use. Investigation of the transdermal behaviour of bioactive compounds is not only important for cosmetics, but also in the development of medicinal compounds to characterize the clinical relevance as well as the toxicological effects of the compounds. A great number of mathematical models are described in the literature to calculate transdermal parameters from *in vitro* and *in vivo* studies. Local skin kinetics of active compounds are investigated *in vitro* using a Franz Diffusion Cell (FDC) set-up, studying permeation, penetration and partition of the absorbed compounds into the different skin layers. The OECD guideline number 428 is widely accepted to perform skin absorption studies [2-4].
After topical application of a compound, the percutaneous absorption begins, but with a delay of entering the systemic circulation ranging from a few minutes to several hours and even days and this time is thus of clinical relevance. The delayed absorption is due to the penetration across the outer layer and main barrier of the skin, *i.e.* the stratum corneum (SC) and the partition into the different viable skin layers. The concept of lag time (t_{lag}) is generally defined as the time it takes for a compound to penetrate the skin. Most therapeutic indications require a drug with a short lag time. The t_{lag} is seldom used alone to describe the transdermal behaviour of a penetrant. Besides the lag time, the most relevant and commonly used transdermal parameters are the steady state (SS) flux (J_{ss}) and the permeability coefficient ($K_{p,v}$) [4-9].

However, there exist different interpretations of the term lag time yielding different calculation approaches (Figure 1). The lag time most often used and calculated in *in vitro* experiments is obtained by the extrapolation to the x-axis of the linear part of the slope of the cumulative amount versus time plot and is called the “extrapolated” lag time. This approach is derived from a steady state mathematical model, based on Fick’s first law of diffusion and used in case of an infinite dose [2, 9, 10]. However, in most cases, a certain amount of the compound has already penetrated the skin before the extrapolated lag time is reached and this time point is called the “experimental” lag time [11].

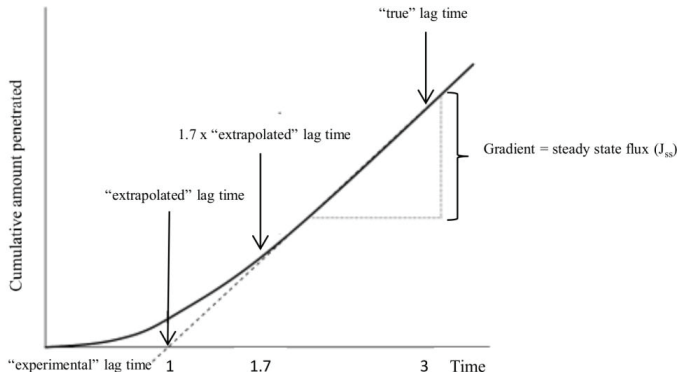

Figure 1. Illustration of a cumulative amount versus time curve with different definitions of t_{lag}.

Another lag time approach is the "true" lag time, *i.e.* the time point when steady state conditions are reached, taking approximately three times the extrapolated lag time. This true lag time reflects the time it takes for the compound's concentration gradient to become stabilized across the skin [12]. Before this true lag time, the permeation rate across the skin continually increases. It follows that SS-flux and permeability coefficient values can only be calculated after three times the extrapolated lag time at SS conditions [5, 13]. After about 1.7 times the extrapolated lag time, there is less than 10% error on the estimation of the actual amount of compound penetrated through the skin at SS [4]. The time it takes to achieve steady state depends on several factors, such as the physicochemical properties of the compound and the permeability of the skin itself, related to the skin thickness on the site of application and the diffusion coefficient [9].

In this study, the *Anacyclus pyrethrum* DC (AP) and the *Spilanthes acmella* (SA) plant extracts are used, containing various *N*-alkylamides (NAAs), showing interesting biological activities [14]. The single quad MS detector coupled to UHPLC was used for the quantification of pellitorine and anacycline present in the AP extract and spilanthol in the SA extract samples obtained during a FDC experiment.

The implementation of the single quad MS detector in transdermal research, overcomes the resolution problems encountered with UV. Moreover, in transdermal research, low concentrations of the compound(s) of interest are often obtained in the FDC experiments. With the MS detector, lower LoD values can be obtained compared to UV. The lower the LoD values, the more accurate transdermal parameters such as the lag time, can be determined.

2. MATERIALS AND METHODS

Chemicals and reagents

The *Anacyclus pyrethrum* root extract came from a local supplier in Sagar (India) and was processed as previously described [14]. The analytical characterisation of the extract was reported earlier by Veryser *et al*. (2014) (see Chapter III) [15]. Spilanthol, supplied as a 30% w/w ethanolic *Spilanthes acmella* flower extract, was a generous gift of Robertet (Grasse, France).

0.01 M phosphate buffered saline (PBS) was bought from Sigma-Aldrich. Absolute ethanol (EtOH, 99.8% V/V) came from Fisher Scientific (Erembodegem, Belgium), while ULC-MS grade formic acid (FA, 99%), ULC-MS grade acetonitrile (ACN, 99%) and ULC-MS grade methanol (MeOH) were purchased from Biosolve (Valkenswaard, the Netherlands). Ultrapure water (18.2 MΩ.cm) was produced using an Arium 611 VF water purification system (Sartorius, Vilvoorde, Belgium).

Preparations of Franz diffusion dose solution

The *Anacyclus pyrethrum* extract was first dissolved in ethanol, vortexed, sonificated for 2h and centrifuged at 3220 × *g* at room temperature for 15 min. Then, a 30:70 H_2O:EtOH (V/V) solution was prepared, centrifuged one more time and the supernatant was filtered using a 0.45 µm Whatman nylon HPLC filter. The experimentally determined concentrations of pellitorine (deca-2E,4E-dienoic acid isobutylamide or $C_{14}H_{25}NO$) and anacycline (tetradeca-2E,4E-diene-8,10-diynoic acid isobutylamide or $C_{18}H_{25}NO$) in the dose solution were 495 µg/ml and 96 µg/ml, respectively. The concentration of pellitorine in the AP extract dose solution was determined using purified pellitorine as reference standard [15].

Spilanthol (deca-2E,6Z,8E-trienoic acid isobutylamide or $C_{14}H_{23}NO$) dose solutions of 1.335 mg/ml and 0.233 mg/ml (experimentally determined) in 30:70 H_2O:EtOH (V/V) were prepared with the *Spilanthes acmella* extract.

In vitro skin permeation study

The skin penetration of pellitorine, anacycline and spilanthol was evaluated using static Franz diffusion cells (Logan Instruments Corp., New Jersey, USA) with an available diffusion area of 0.64 cm^2. The receptor compartment was filled with 5 ml 0.01 M PBS, free of air bubbles. A randomized blocked design was applied and analyses were done in fivefold (pellitorine and anacycline) and in fourfold (spilanthol). Human skin of two healthy female patients of 62 ± 14 years old (mean ± SD) was obtained after aesthetic body contouring surgery, with confidential procedures and informed consent, from the Department of Plastic and Reconstructive Surgery of the University Hospital

(Ghent, Belgium). The skin was cleaned with 0.01 M PBS pH 7.4 immediately after the surgical procedure and the subcutaneous fat was removed. Subsequently, the skin was stored at −20°C for not longer than 6 months. Just before the start of the FDC experiments, the full-thickness skin was thawed, mounted on a template and sliced with an electrical powered dermatome to obtain split-thickness human skin. An actual skin thickness of 492 ± 61.8 µm (mean ± SD, n=15) for donor 1 and 402 ± 39.0 µm (mean ± SD, n=15) for donor 2 was experimentally determined with a micrometer (Mitutoyo, Tokyo, Japan) used for pellitorine and anacycline, while for spilanthol, an actual skin thickness of 530 ± 121 µm (mean ± SD, n = 10) and 377 ± 36.2 µm (mean ± SD, n = 10) were experimentally determined from the different patients. After visual inspection for skin damage, the intact skin pieces with the epidermis side upwards were mounted on the FDC between the donor and the receptor chambers ensuring that no air was present under the skin. During the whole experiment, the receptor fluid was mixed continuously using a Teflon coated magnetic stirring bar (400 rpm). The skin impedance was measured to check for skin integrity using an automatic micro-processor controlled Tinsley LCR Impedance Bridge (Croydon, U.K.). Skin pieces displaying an impedance value below 10 kΩ were discarded and replaced by a new skin piece [16]. With a micropipette, 500 µl of the dose solutions were applied on the skin and afterwards covered with parafilm to prevent evaporation of solvents. Due to a water jacket, the receptor compartment was kept at 32°C. After 1h, 2h, 4h, 8h, 12h, 18h, 21h and 24h (pellitorine and anacycline) and after 15 min, 30 min, 45 min, 1h 30, 2h, 2h 30, 3h, 3h 30, 4h, 4h 30, 5h, 6h (spilanthol), 200 µl samples of the receptor fluid were taken from the sample port and were immediately replaced by 200 µl fresh PBS solution. This was taken into account for the calculation of the cumulative permeated concentrations. At the end of the experiment using the AP extract, *i.e.* after 24h, the remaining dose formulation was removed by swabbing the skin surfaces with cotton wool. Afterwards, the epidermis and dermis were separated with forceps and pellitorine and anacycline were extracted from the skin layers with ethanol to construct a mass balance of pellitorine and anacycline. In addition, the ratio $C_{24h,epidermis}/C_{24h,vehicle}$ was obtained. The concentration of pellitorine/anacycline in the (epi)dermis was obtained by dividing the amount of extracted NAA (experimentally determined) by the volume of the (epi)dermis (thickness of (epi)dermis (cm) x skin surface (0.64 cm^2)). The thickness of the epidermis was assumed to be 50 µm, and the thickness of the dermis was the total measured skin thickness minus the thickness of the epidermis. The concentration of pellitorine/anacycline in the remaining dose solution after 24h was calculated by dividing the amount of pellitorine/anacycline in the dose solution left after 24h (experimentally determined) by the applied volume of the dose solution, *i.e.* 500 µl".

Analysis of the FDC samples using UHPLC-MS

The UHPLC equipment consisted of an Acquity sample manager, an UPLC quaternary solvent manager, an UPLC PDA Acquity isocratic solvent manager (ISM) and a single quadrupole mass detector *i.e.* the Acquity QDa detector with Empower 3 FR 2 software for data acquisition (all Waters, Millford, USA). Positive electrospray ionisation (ESI+) was used for the ionisation. An Acquity UPLC C18 column (50 mm x 2.1 mm, 1.7 µm) (Waters, Milford, USA) with a suitable guard column was used and the injection volume was 2 µl. The column temperature was kept constant at 30°C, while the sample compartment was maintained at 5°C.

For the AP extract, a gradient at a flow rate of 0.5 ml/min was used with mobile phase, consisting of 0.1% FA in 30:70 H_2O:MeOH (V/V) – 0.1% FA in MeOH (t=0-1.8 min= 100:0 (V/V), t=1.8-2.3 min= 0:100 (V/V), t=2.3-3.55 min= 0:100 (V/V), t=3.55-4.05 min= 100:0 (V/V), t=4.05-6.55 min= 100:0 (V/V)). The restrictor module in the ISM divides the post-column mobile phase (0.1% FA in 50:50 MeOH:H_2O (V/V)) at a flow of 0.5 ml/min from the column between the PDA detector and the single quad MS detector with a post-column PDA/MS split ratio of 10/1. The capillary voltage was 0.8 kV and the cone voltage and probe temperature were set at 15 V and 600°C, respectively. A SIM method was used for pellitorine (*m/z* = 224.36) and anacycline (*m/z* = 272.40). UV detection was performed at 258 nm. Analytical validation was performed and the LoD and LoQ of pellitorine, defined as the concentrations equivalent to a signal to noise value of 3 and 10, respectively, were determined to be 0.17 ng/ml and 0.55 ng/ml, in case 100% of the mobile phase was going to the MS detector. Linearity, with determination coefficient R^2 = 0.9999, is assured in a working range of 5.52 ng/ml (LoQ) up to 2.48 µg/ml. The LoD and LoQ of anacycline were 0.02 ng/ml and 0.08 ng/ml, respectively, in case 100% of mobile phase was sent to the MS; while the linearity is assured in a working range of 0.80 ng/ml up to 0.48 µg/ml (R^2 = 0.997). The LoD and LoQ of pellitorine were 3.57 ng/ml, and 11.90 ng/ml, respectively, in case 100% of mobile phase was sent to the UV detector.

For spilanthol, an isocratic method was used with as mobile phase 0.1% FA in ACN - 0.1% FA in H_2O (55:45, V/V) at a flow rate of 0.5 ml/min. The capillary voltage was also 0.8 kV and the cone voltage and probe temperature were set at their optimized values, namely 12 V and 600°C, respectively. A full mass spectrum between *m/z* 50 and 600 was obtained at a sampling rate of 5.0 spectra/sec. In addition, a SIM method was used for spilanthol (*m/z*= 222.15). UV detection was performed at 237 nm. Analytical validation was performed and the LoD and LoQ of spilanthol, defined as the concentrations equivalent to a signal to noise value of 3 and 10, respectively, were determined to be 0.32 ng/ml and 1.07 ng/ml with MS. The LoD and LoQ of spilanthol, determined with UV were 7.10 ng/ml, and 23.67 ng/ml, respectively.

Calculation of skin permeation parameters according to the ECETOC

The cumulative amounts of the NAAs (in µg) permeated through human skin were plotted as a function of time (in hours) and the individual graphs were used for the calculations of the transdermal parameters. The observed secondary parameters are the steady state flux (J_{ss}), the lag time and the Q_{1d} (cumulative quantity, expressed as % of the effective dose applied, obtained after 1 day). Several conditions must be fulfilled in order to apply this method, *i.e.* a constant compound concentration in the vehicle, sink conditions in the receptor compartment and initially a lack of compound in the SC. After a while, SS conditions will be achieved [8]. The formulas used to calculate the transdermal parameters are based on Fick's first law [4]. Steady state flux (µg/(cm²·h)) was calculated from the slope of the linear portion of the cumulative amount versus time curve divided by 0.64 to correct for the exposed skin area (cm^2). The lag time (h) was obtained by setting y = 0 in the individual linear regression equation. These parameters were directly obtained from and dependent on the experimental data. The permeability coefficient of the NAA in the vehicle (ethanol:water) ($K_{p,v}$, cm/h) [17], which is a primary parameter derived from the experimentally obtained secondary parameters and independent of some operational experimental conditions, was calculated in accordance with European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) [9]. The permeability coefficient was calculated as follows: $K_{p,v} = J_{ss}/C_v$, where C_v (µg/ml) is the concentration of pellitorine in the applied vehicle (dose formulation).

3. RESULTS AND DISCUSSION

Chromatograms of the *N*-alkylamides in the *Anacyclus pyrethrum* extract

Figure 2A shows the UHPLC-UV chromatogram of the AP extract at 258 nm; no immediate identification and quantification of the major NAAs pellitorine and anacycline were possible.

Therefore, for the separate quantification of pellitorine and anacycline, the single quad MS detector was used. The main peaks in Figure 2B and 2C are pellitorine and anacycline, respectively, identified by the protonated molecule *m/z* signal observed in MS^1 (precursor ion *m/z* of pellitorine = 224; precursor ion *m/z* of anacycline = 272). Anacycline and pellitorine are eluting around the same time *i.e.* between 1 and 2 minutes. Contrary to the UV detector, it is thus not necessary that compounds are separated from each other at baseline to quantify them.

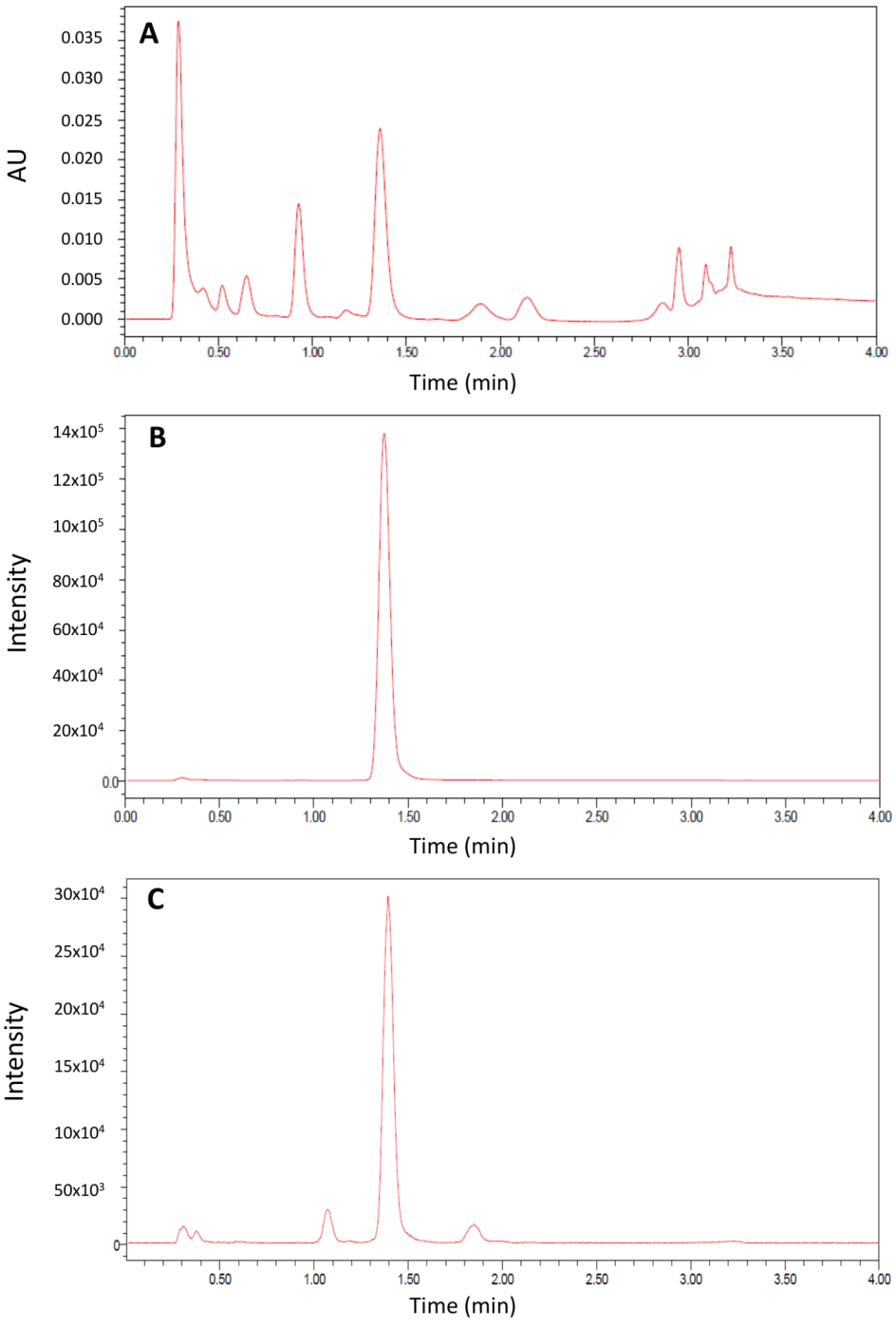

Figure 2. (A) UHPLC-UV (258 nm) chromatogram of *Anacyclus pyrethrum* extract, (B) MS ion chromatogram of AP extract for pellitorine (*m/z* 224), (C) MS ion chromatogram of AP extract for anacycline (*m/z* 272).

Transdermal results

Using the single quadrupole mass detector as quantification technique, the transdermal behaviour of the NAAs was quantified. Steady state conditions were maintained during the experiment for pellitorine. The *in-silico* calculated aqueous solubility of pellitorine was 6.58 mg/l [18]; hence the concentration of pellitorine did not exceed 10% of the solubility in the receptor fluid, confirming sink conditions. Linear regression was performed on the individual cumulative amounts of pellitorine versus time curves from 8 to 24h ($R^2 \geq 0.95$). The cumulative amount versus time curve of pellitorine is shown in Figure 3, as well as the mean cumulative amount of anacycline at 24h.

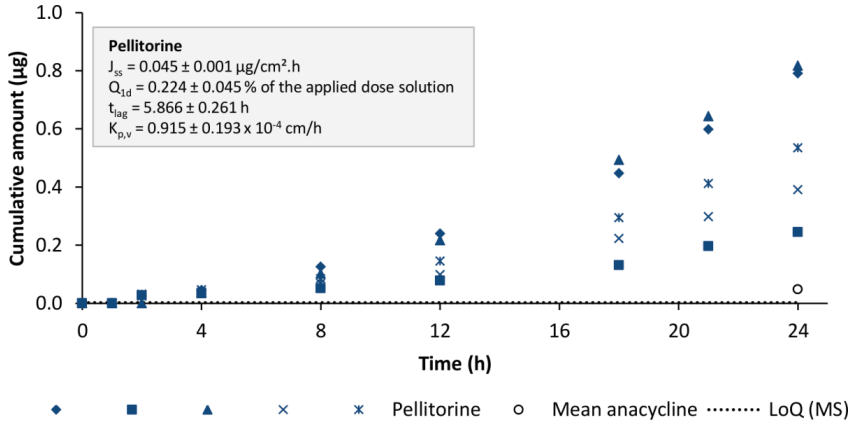

Figure 3. Cumulative amount vs. time curves of pellitorine in the *Anacyclus pyrethrum* extract (n=5), mean cumulative amount of anacycline at 24h (mean ± SEM, n=4) and transdermal parameters of pellitorine (mean ± SEM, with n=5).

A mean amount of 0.56 µg of pellitorine (n=5) permeated through the skin after 24h, which corresponds to 0.22% of the applied dose solution. For anacycline, no cumulative amount versus time curve could be constructed, as the concentration of anacycline in the receptor fluid samples before 24h were below the LoQ. The mean amount of anacycline after 24h is indicated in Figure 3 and amounts 0.05 µg (n=4), corresponding to 0.10% of the applied dose.

Skin parameters of pellitorine were calculated according to the ECETOC and are given in Figure 3. These values are in the same range as the results obtained in a previous study (see Chapter III) [15]. The permeability coefficient $K_{p,v}$ of pellitorine is $0.915 \cdot 10^{-4}$ cm/h. At the end of the experiment (*i.e.* 24h), the concentration of pellitorine and anacycline in the dermis and epidermis were determined. The concentrations of pellitorine in the different compartments, after applying a dose solution of

pellitorine of 495 µg/ml in 30:70 H_2O:EtOH (V/V), are visually shown in Figure 4, while the concentrations of anacycline in the different compartments, after applying a dose solution containing 96 µg/ml anacycline, are graphically shown in Figure 5.

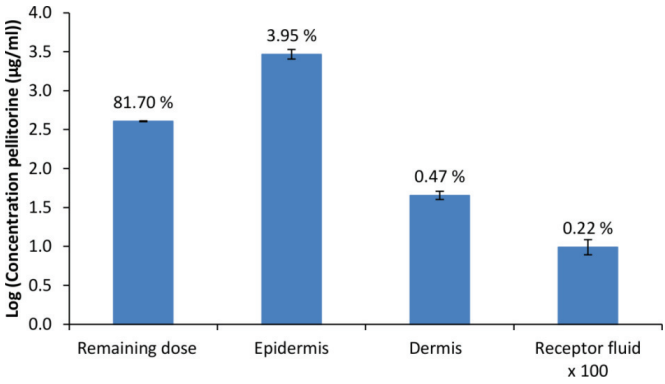

Figure 4. Distribution of pellitorine (mean values ± SEM, n=5) in the different compartments after 24h: remaining dose, epidermis, dermis and receptor fluid. The amount of pellitorine in each compartment as a percentage of the applied dose is indicated above the bars.

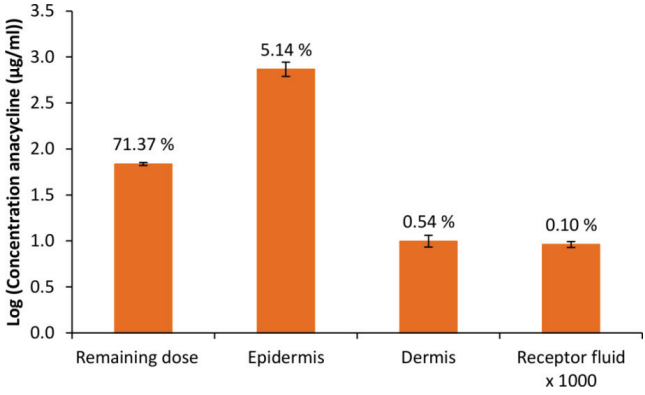

Figure 5. Distribution of anacycline (mean values ± SEM, n=4) in the different compartments after 24h: remaining dose, epidermis, dermis and receptor fluid. The amount of anacycline in each compartment as a percentage of the applied dose is indicated above the bars.

For both pellitorine and anacycline, the concentrations found in the different compartments are in decreasing order: the epidermis < the remaining dose < the dermis < the receptor fluid, respectively. As the epidermis is more lipophilic than the dermis, lipophilic compounds like pellitorine and anacyline will remain there longer. The relative distribution of both NAAs in the different skin compartments is very similar.

Lag time determination

As mentioned in the introduction, different definitions are ascribed to the word 'lag time'. There is the "experimental", the "extrapolated" and the "true" lag time (Figure 1). The real time for a compound needed to cross the skin, is mostly related to the "experimental" lag time and not to the "extrapolated" and "true" lag time, as there is already an amount of compound penetrated through the skin before both these lag times. However, the experimental lag time can be defined in different ways depending on its determination. Theoretically, it can be defined as the time it takes for one molecule of the compound to penetrate (through) the skin, however this is difficult to experimentally observe. Using a particular analytical method, the (highest) observable lag time is related to the LoD or LoQ of the method. Apart from this direct experimentally obtained (highest observable) lag time, mathematical models can be used for calculating the lag time from a discrete set of experimental data. One of the advantages of using these models is the ability to extrapolate from the observable high and method dependent lag time to a lower and method independent lag time.
As this lag time is within the first part of the curve of the cumulative amount versus time curve, this is the non-steady state, transient part of the curve and thus a non-linear model must be used [19-23].

Figure 6 gives the mean cumulative amount curve of pellitorine, quantified with UV (and hence co-elution with anacycline), as well as with the MS detector. The two detection methods are compared here. From an analytical point of view, to determine the observable experimental lag time, the LoD and LoQ must be taken into account. The LoQ (µg) was calculated by multiplying the LoQ (µg/ml, corresponding to signal/noise=10) by the receptor fluid (*i.e.* 5 ml).

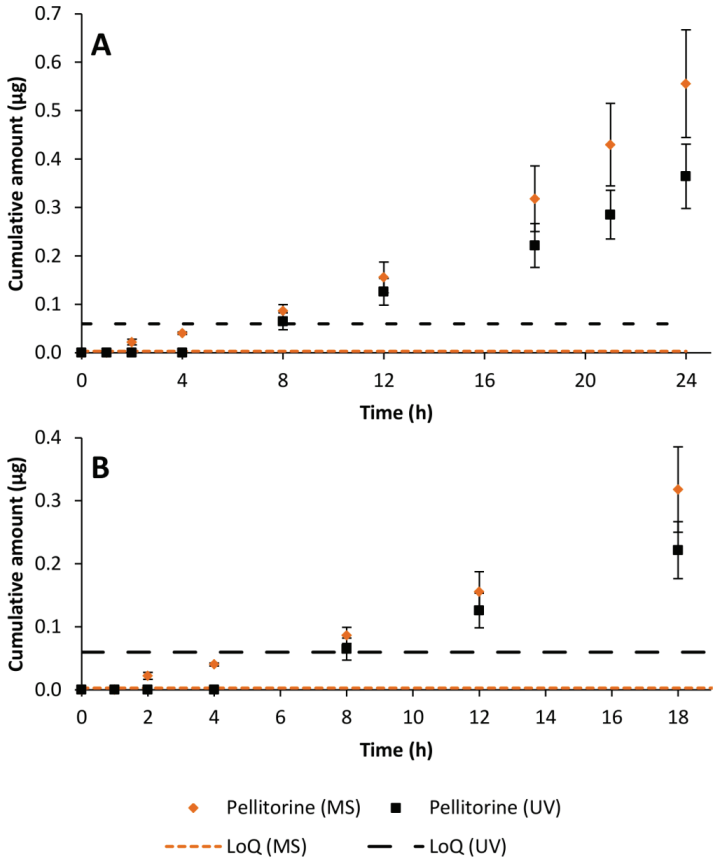

Figure 6. (A) Cumulative amount vs. time curves of pellitorine in the *Anacyclus pyrethrum* extract quantified using UHPLC-UV and UHPLC-MS (mean ± SEM, n=5), (B) Zoomed part of the curve.

From the MS data, it appears that pellitorine already penetrated the skin after 2 hours and it can thus be assumed that pellitorine is present in the systemic blood circulation after 2h. Whereas when using UV, it seems likely that pellitorine penetrated the skin after a longer time, *i.e.* 8h. Significantly lower LoQ values (factor 22) were obtained with the single quad mass spectrometer, compared to UV.

The initial transdermal behaviour of spilanthol, another important NAA [24-26], was also evaluated using UHPLC-MS. The *in-silico* calculated aqueous solubility of spilanthol was 18.63 mg/l [18]; hence sink conditions were maintained during the experiment. The cumulative amount curves of spilanthol versus time are shown in Figure 7.

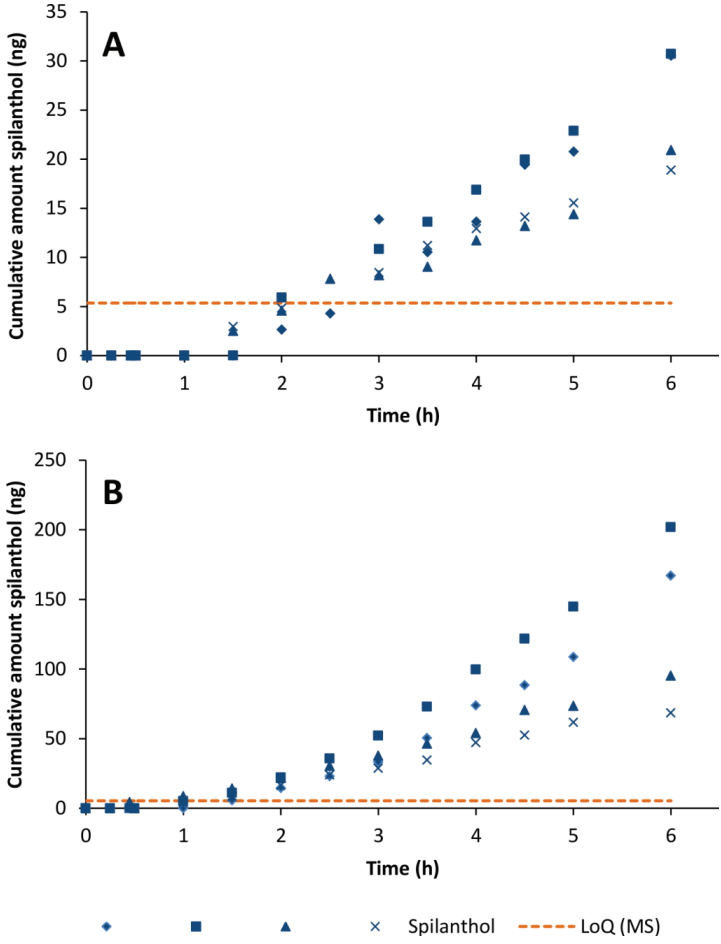

Figure 7. Cumulative amount vs. time curves of spilanthol in the *Spilanthes acmella* extract (n=4) with (A) dose solution (DS) 0.233 mg/ml and (B) DS 1.34 mg/ml.

With UV (237 nm), after 6h, spilanthol was still below the LoD (data not shown). The time after which a compound (in this case spilanthol) is above the LoQ also depends on the concentration used in the dose solution (a higher concentration in the DS results in higher cumulative amounts in the receptor fluid and the LoQ is achieved more quickly at lower time points). With the low concentration DS, spilanthol was above the LoQ after 3h, while already after 2h in case of the higher concentration DS.

By using the single quad MS detector as application for the quantification of NAAs in transdermal research, it has been shown that more accurate lag times are obtained when using the single mass detector due to low LoQ values. Up till now, most researchers estimate the *in vitro* transdermal parameters (*i.e.* J_{ss}, $K_{p,v}$, t_{lag}) based upon visual determination of the linear part of the slope of the

graph of the cumulative absorbed dose and time, although other methods are possible to determine the permeability coefficient and lag time using non-linear regression [27]. However, as this "linear" method is subjective, large variations in time intercept are possible as a result of a minor variation in slope of SS. Moreover, if the concentration of the compound in the vehicle depletes in time or the concentration in the receptor compartment increases enormously (non-sink conditions) the slope method for the determination of the transdermal parameters will underestimate the permeability. In that case, more complex data analysis is needed. In addition, if the slope of the curve was used as a part of the curve in which SS conditions were not yet achieved, there will be an underestimation of permeability coefficient as well.

In the literature, it is reported that permeability coefficient values obtained *in vivo* are larger than permeability coefficient values obtained in *in vitro* experiments. Moreover, the t_{lag}, determined in *in vitro* experiments is not the same as the actual time needed to reach the systemic circulation *in vivo*. However, some caution is advised to simply adopt this, as there are often errors in *in vitro* experiments due to the visual determination of the slope and thus permeability coefficients are often underestimated [3, 8, 28].

The previous described manner for the determination of the lag time *in vitro* is used to compare the transdermal behaviour of chemicals *in vitro*. In this way, it is possible to compare the time needed for compounds to penetrate the skin. As such, the compound which penetrated the skin the fastest can be selected. Compounds with the lowest lag time value are most desirable in therapeutics. However, there is no relevance between this *in vitro* determined lag time and the actual *in vivo* percutaneous absorption. The extrapolated lag time is simply an artificial derivation of the *in vitro* diffusion experimental test. In this study, another approach of the term 'lag time' was used and a more accurate lag time determination was possible, resembling more the *in vivo* percutaneous absorption time and thus of clinical relevance. Before steady state conditions, absorption of the compound has already started and hence the compound can already exert some biological functions in the human body.

4. CONCLUSION

This study has demonstrated the applicability of identifying and quantifying bioactive NAAs *i.e.* pellitorine, anacycline and spilanthol in complex plant extracts by using single quad MS detection. Besides calculation of skin parameters, information about the distribution in the skin was obtained by performing a FDC experiment with human skin. The single quad MS detector shows great advantages compared to the UV detector, as no baseline separation is necessary between the

compounds to be analyzed and lower LoD values are obtained. Additionally, a more accurate determination of the lag time can be achieved. In conclusion, UHPLC coupled to a single quad MS detector is a useful technique with additional advantages compared to traditional HPLC-UV in transdermal research of plant extracts.

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CHAPTER V

QUANTITATIVE *IN VITRO* AND *IN VIVO* EVALUATION OF INTESTINAL AND BLOOD-BRAIN BARRIER TRANSPORT KINETICS OF SPILANTHOL

"Try to learn something about everything and everything about something."

Thomas Henry Huxley
(°1825-†1895, English biologist)

Parts of this chapter were published:

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ABSTRACT

N-alkylamides (NAAs) are a large group of secondary metabolites occurring in more than 25 plant families which are often used in traditional medicine. A prominent active NAA is spilanthol. The general goal was to quantitatively investigate the gut mucosa and blood-brain barrier (BBB) permeability pharmacokinetic properties of spilanthol. *Spilanthes acmella* (L.) L. extracts, as well as purified spilanthol were used to investigate (1) the permeation of spilanthol through a Caco-2 cell monolayer *in vitro*, (2) the absorption from the intestinal lumen after oral administration to rats, and (3) the permeation through the BBB in mice after intravenous injection. Quantification of spilanthol was performed using a validated bio-analytical UHPLC-MS^2 method. Spilanthol was able to cross the Caco-2 cell monolayer *in vitro* from the apical-to-basolateral side and from the basolateral-to-apical side with apparent permeability coefficients P_{app} between $5.2 \cdot 10^{-5}$ and $10.2 \cdot 10^{-5}$ cm/h. This *in vitro* permeability was confirmed by the *in vivo* intestinal absorption in rats after oral administration, where an elimination rate constant k_e of 0.6 h^{-1} was obtained. Furthermore, once present in the systemic circulation, spilanthol rapidly penetrated the blood-brain barrier: a highly significant influx of spilanthol into the brains was observed with a unidirectional influx rate constant K_1 of 796 µl/(g·min). Spilanthol shows a high intestinal absorption from the gut into the systemic circulation, as well as a high BBB permeation rate from the blood into the brain.

CHAPTER V

QUANTITATIVE *IN VITRO* AND *IN VIVO* EVALUATION OF INTESTINAL AND BLOOD-BRAIN BARRIER TRANSPORT KINETICS OF SPILANTHOL

Main focus in this chapter:

- To explore the penetration behaviour of spilanthol through a Caco-2 cell monolayer and rat intestinal gut.
- To evaluate the transport kinetics of spilanthol from blood-to-brain and from brain-to-blood.

1. INTRODUCTION

N-alkylamides (NAAs) are a large group of secondary metabolites occurring in more than 25 plant families, often used in traditional medicine and claimed to possess a diverse range of pharmacological activities such as antimicrobial, analgesic and anti-inflammatory properties [1-8]. The NAAs consist of a short-chain amine linked to an aliphatic chain of poly-unsaturated fatty acids through a central peptide amide bond. Spilanthol (affinin; deca-2E,6Z,8E-trienoic acid isobutylamide; F3M1 according to the FxMy classification of NAAs) is a highly abundant and biologically potent triene NAA found in Asteracea plants such as *Spilanthes acmella*. Traditionally, *Spilanthes acmella* plants are not only used as a food spice, in toothpastes and cosmetics, but have also been used in folk medicine for the treatment of toothaches, stomatitis, rheumatism, fever, fungal skin infections and diverse pain and neuropathic disorders [8]. The pharmacological central nervous system (CNS) activity and physiological mechanisms of spilanthol are only fragmentary documented. Chakraborty *et al.* (2004) and Barman *et al.* (2009) reported a central analgesic activity of a subcutaneously *Spilanthes acmella* extract in an *in vivo* rat tail flick experiment [9, 10]. Rios *et al.* (2007) showed that

a *Heliopsis longipes* extract, in which spilanthol was the main active compound, evoked cortical GABA (gamma-aminobutyric acid) release in an *ex vivo in vitro* mice brain-tissue study [11].

In order to exert these CNS effects, these compounds must be able to cross several physiological barriers in the human body. Using *in vitro* Franz diffusion cell (FDC) experiments, our group has already been shown that the *N*-alkylamide spilanthol can permeate the human skin and pig oral mucosa, reaching the systemic circulation after topical application. The permeability coefficient $K_{p,aq}$ was $3.31 \cdot 10^{-3}$ cm/h for human skin and $11.3 \cdot 10^{-3}$ cm/h for the porcine buccal mucosa [12-14]. After oral administration, compounds must be able to pass through the intestinal barrier to reach the systemic circulation. Matthias *et al.* (2004) investigated the transport of mainly diene *N*-alkylamides from *Echinacea* (*E. angustifolia* and *E. purpurea* root) through a Caco-2 cell monolayer, which is a model of the intestinal barrier, and found that after 90 min, more than 50% of the NAAs permeated through the monolayer [15].

Once present in the blood by passsage through the skin or mucosa, compounds can cross the blood-brain barrier to exert central nervous system effects. This entry of compounds into the brain is strictly regulated and controlled: the BBB forms not only a physical barrier (tight junctions), but also a transport and metabolic barrier (enzymes) to maintain an adequate microenvironment of the neuronal cells [16]. A previous study indicated that tetraene NAAs from *Echinacea* appeared in the brains after a single oral dose of 2.5 mg/kg to rats [17].

Up till now, there is no information about the transport kinetics of the 2,6,8-triene *N*-alkylamide spilanthol through the intestinal barrier after oral application and through the blood-brain barrier, once present in the blood. Therefore, the aim of this study was to quantitatively investigate the intestinal permeability (*in vitro* Caco-2 cell monolayer and *in vivo* oral gavage rat model) and the BBB transport kinetics (*in vivo* mice model) of spilanthol. Moreover, the distribution of spilanthol within the BBB, *i.e.* brain parenchyma versus endothelial cells, was investigated.

2. MATERIALS AND METHODS

Chemicals and reagents

Ultrapure water (H_2O) of 18.2 MΩ.cm quality was produced by an Arium 611 purification system (Sartorius, Göttingen, Germany). Vitamin E-TPGS, Hanks' Balanced Salt Solution (HBSS), trypan blue, potassium chloride (KCl), dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), sodium chloride (NaCl), calcium chloride dehydrate ($CaCl_2.2H_2O$), sodium lactate, dichloromethane, sodium

hydrogen carbonate ($NaHCO_3$), sodium sulphate (Na_2SO_4), sodium dihydrogen phosphate (NaH_2PO_4), hydrochloric acid (HCl) and urethane were purchased from Sigma-Aldrich (Diegem, Belgium), while bovine serum albumin (BSA), disodium hydrogen phosphate dehydrate ($Na_2HPO_4.2H_2O$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), sodium hydrogen carbonate and absolute ethanol (≥ 99.9% V/V) were obtained from Merck KGaA (Darmstadt, Germany). Absolute ethanol (99.8% V/V), HPLC gradient grade methanol (MeOH) and acetonitrile (ACN) came from Fisher Scientific (Erembodegem, Belgium). Dextran was obtained from AppliChem GmbH (Darmstadt, Germany). UPLC-MS grade MeOH and ACN were bought from Biosolve (Valkenswaard, the Netherlands). Phosphoric acid (85%) (H_3PO_4) and dimethylacetamide were purchased from Jansen Chimica (Geel, Belgium). Trypsin-EDTA was obtained from Invitrogen (Ghent, Belgium). Calcium dichloride ($CaCl_2$), LC-MS grade formic acid (FA), polyethylene glycol 400 (PEG 400), tween 80, D-glucose, sodium hydroxide (NaOH) and HEPES were purchased at Fluka (Diegem, Belgium) while propylene glycol (PG) was bought from Riedel-de Haën (Seelze-Hannover, Germany)**.** Triethylamine, decanoyl chloride and isobutylamine were purchased at Acros Organics (Geel, Belgium). The BBB-positive control dermorphin was purchased at Bachem (Bubendorf, Switzerland). NMR solvents were bought from Eurisotop (Saint-Aubin, France).

Products examined

The ethanolic *Spilanthes acmella* (L.) L. flowers extract (batch 0001853516, 30% w/w spilanthol in ethanol) was obtained from Robertet (Grasse, France) and was used for the BBB transport assay. *Spilanthes acmella* (L.) L. is also known as Akarkara (local name) and Para Cress (English name). HPLC analysis confirmed the spilanthol content, as well as a small quantity (9%) of other NAAs [14]. The *Spilanthes acmella* (L.) L. extract (A. Vogel, label claim indicated 2% w/w dry residue of extracts in 69% V/V ethanol; internal assay: 0.11% w/w spilanthol, purity >90%) came from Bioforce AG (Switzerland) and was used for the oral gavage experiment.

Purified spilanthol was used for the Caco-2 cell permeability assay. Semi-preparative HPLC was used for its purification as follows: the *Spilanthes acmella* (L.) L. extract of Robertet was dissolved in 30:70 (V/V) H_2O:ethanol and filtered using a 0.45 µm nylon HPLC filter (Whatman). One ml of this solution was injected on a Vydac C18 monomeric semi-preparative column (Grace, 250 mm x 10 mm, 5 µm particle size, 300 Å pore size) using a Waters HPLC, equipped with a Waters 2487 Dual Absorbance Detector. The sample compartment and column temperature were maintained at room temperature. An isocratic elution mode was used with a 50:50 (V/V) A:B (A: 0.1% FA in H_2O and B: 0.1% FA in MeOH) mobile phase. A flow rate of 6.0 ml/min was used and UV detection was performed at 237 nm. Fractions between 16 and 32 min retention time were collected and lyophilized using a Christ

Gamma 1-16 LSC freeze-dryer (Q-lab, Vilvoorde, Belgium). The purity of the isolated spilanthol was determined by analytical HPLC-UV at 237 nm [12] and was 99.9%.

As analytical internal standard (IS) in the bio-analytics, isobutyldecanamide was used and synthesised as described in Figure 1. The NMR spectra of this internal standard can be retrieved in the Supplementary information S1.

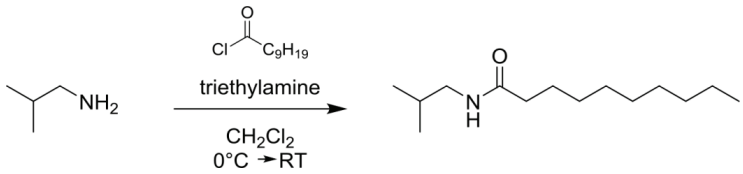

Figure 1. Synthesis of isobutyldecanamide (analytical internal standard).

Triethylamine (6.0 mmol, 608 mg, 837 µl) was added to a solution of isobutylamine (6.0 mmol, 439 mg, 597 µl) in dichloromethane (30 ml). The mixture was cooled to 0°C using an ice bath. Decanoyl chloride (5.0 mmol, 954 mg, 1.0 ml) was slowly added to the solution. The mixture was stirred overnight, allowing the temperature to rise to room temperature. The reaction mixture was extracted with 50 ml dichloromethane which was washed successively with HCl (1.0 M aq.) and $NaHCO_3$ (sat. aq.). The organic layer was dried on Na_2SO_4, filtered and concentrated *in vacuo* to yield the title compound (1.07 g, 4.7 mmol) as an off-white waxy solid. The compound was identified by ^1H and ^{13}C NMR recorded with a Varian Mercury-300BB (300/75 MHz) spectrometer. Chemical shifts are given relative to tetramethylsilane (0 ppm) ^1H NMR (300 MHz, $CDCl_3$): δ 5.47 (s, 1H), 3.07 (t, *J* = 6.45 Hz, 2H), 2.16 (t, *J* = 7.44 Hz, 2H), 1.80-1.69 (m, 1H), 1.68-1.58 (m, 2H) 1.38-1.18 (m, 12H), 0.90 (d, *J* = 6.90 Hz, 6H), 0.87 (t, J = 6.90 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.25, 46.92, 37.16, 32.00, 29.61, 29.51, 29.47, 29.41, 28.67, 26.05, 22.80, 20.24, 14.24.

In vitro permeation study in Caco-2 cell monolayers

Cell culture

Caco-2 cells, originating from a human colorectal carcinoma were maintained in Dulbecco's modified Eagle's medium (DMEM) at 95% humidity and 37°C in an atmosphere of 5% CO_2, supplemented with 10% (V/V) fetal bovine serum, 2 mM L-glutamine and 1% non-essential amino acids (100x), 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen/GIBCO, Ghent, Belgium).

Caco-2 cell permeability assay

The Caco-2 cell intestinal model was used as a model for the gut mucosa to investigate the permeation of spilanthol. Caco-2 cells were seeded at a density of $2.6 \cdot 10^5$ cells/cm² cells for each Transwell (Corning Costar, New York, USA) membrane insert filter (0.4 µm pore size, 12 mm filter diameter) and cultivated in the described supplemented DMEM, also containing 100 units/ml penicillin and 100 µg/ml streptomycin. The medium was changed every second day. The cells were allowed to grow and differentiate for 21-29 days until monolayers were formed. Intact membranes/integrity of the monolayers were checked via the measurement of transepithelial electrical resistance (TEER) of the monolayers using the Millicell-ERS system (Millipore Corp., Bedford, MA, USA) before and after transport experiments. The experiment was performed in duplicate for each dose solution. Transport experiments were carried out in the apical-to-basolateral (ab) direction and in the basolateral-to-apical (ba) direction in Hanks' Balanced Salt solution, according to Hubatsch *et al.* (2007) [18]. Two dose solutions of purified spilanthol at 10 µg/ml were tested, differing in solvent: 0.5% ethanol (further indicated as DS1) and 0.5% of a mixture of vitamin E-TPGS (33.3%), ethanol (11.1%), PEG 400 (33.3%) and PG (22.2%) (further indicated as DS2) dissolved in HBSS.

Final volumes of 0.4 ml apically and 1.2 ml basolaterally were used for 12-mm filter supports during the transport experiment. Samples were taken at 15, 30, 60, 90 and 120 min from the acceptor (= receiver) compartment (basolateral to apical transport: 100 µl; apical to basolateral transport: 300 µl) and were immediately replaced by fresh HBSS. At the last time point, a sample from the donor compartment was taken as well, allowing to obtain the mass balance, which for spilanthol ranged between 104.7 and 120.9% total recovery. Atenolol (50 µM) and propranolol (20 µM) were used as the low- and high-permeability control, respectively, confirming the validity of the test [19]. Quantification of spilanthol was conducted using the ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS²) method as described below.

The apparent permeability coefficient (P_{app} in cm/s) of spilanthol was calculated from a non-sink equation. From Fick's first law: $J = -D \times [dC(x)]/dx$ (D is the diffusion coefficient, J is the flux or transfer rate along the donor-to-receptor side, x is the distance from the donor compartment and C(x) is the concentration in the barrier at the coordinate x in the barrier), the following differential equation is derived: $dM_r(t)/dt = P_{app} \times A \times [C_d(t) - C_r(t)]$, in which M_r is the amount of substance in the receiver chamber, P_{app} is the apparent permeability coefficient (a product of distribution coefficient with diffusion coefficient divided by the barrier thickness), A is the cross-sectional area of the barrier, C_d is the donor concentration and C_r is the receiver concentration. This

differential equation is the basis to calculate P_{app} values of compounds requiring sink, as well as non-sink conditions with different initial conditions to solve this equation. In the case of non-sink conditions, this resulted in the following solution [18-20]:

$$C_r(t) = \left[\frac{M}{V_d + V_r}\right] + \left(C_{r,t-1} \times f - \left[\frac{M}{V_d + V_r}\right]\right) \times e^{-P_{app} \times A \times (\frac{1}{V_d} + \frac{1}{V_r}) \times \Delta t}$$

in which t is the time (s), V_d is the volume of the donor compartment (ml), V_r the volume of the receiver compartment (ml), A the area of the filter (= 1.12 cm²), M the total amount of spilanthol in the system at time t (µg), $C_{r,t-1}$ the concentration of spilanthol in the receiver compartment at the previous time point (µg/ml), f is the sample replacement dilution factor ($1-V_s/V_r$ with V_s is the sample volume), Δt is the time at time t minus the previous time point (s) and $C_r(t)$ the concentration of spilanthol in the receiver compartment at time t (µg/ml). Non-linear curve fitting by minimisation of the sum of squared residuals (SSR) was used to obtain the P_{app}. The efflux ratio is calculated as $P_{app,ba}/P_{app,ab}$, while the uptake ratio is calculated as $P_{app,ab}/P_{app,ba}$.

In vivo **pharmacokinetic experiment with rats**

Animals

The Wistar rats were bred in Bharat Serum and Vaccines pvt. Ltd, Thane, India (registration number 103/99/CPCSEA) and kept for the experiments at C. U. Shah College of Pharmacy, SNDT Women's University, Santacruz, Mumbai, India (registration number 39/99/CPCSEA). The half of the rats used in the experiment were female rats and the other half male rats. Rats of 7-8 weeks old, weighing 220 g, and fasted overnight were used for the experiment.

Pharmacokinetic experiment

The pharmacokinetic oral gavage experiment was carried out at C. U. Shah College of Pharmacy, SNDT Women's University, Santacruz, Mumbai, India. A spilanthol dose solution was prepared with the *Spilanthes acmella* (L.) L. extract (A.Vogel). 1.5 ml of a spilanthol dose solution of 0.73 mg spilanthol/g dose solution in 10:20:30:40 (w/w/w/w) ethanol:PG:vitamin E-TPGS:PEG400 was administered to the rats using a gavage needle made up of stainless steel (length 3 inches and 2.5 mm internal diameter). A blank dose solution was used as well, containing ethanol, PG, vitamin E-TPGS and PEG without spilanthol. For each dose solution, six rats were used of which three were females and three males. Blood was collected (1.5 ml) from the retro orbital vein at specified time points *i.e.* 1, 2, 3, 4, 6, and 8h after oral administration of the dose solution. The collected blood samples were centrifuged using a Remi R-24 centrifuge. At the end of the study, the animals were sacrificed by CO_2 inhalation. All samples were immediately frozen at -80°C until bio-analysis. The elimination rate constant was calculated using a one compartmental model using GraphPad software

(La Jolla, USA), using the following equation: $C(t) = C_0 \times e^{-k_e \times t}$, in which t is time (h), C_0 the concentration of spilanthol at time t=0 (ng/ml), C(t) the concentration of spilanthol at time t and k_e the elimination rate constant (h^{-1}). The elimination half-life (h) is computed as $\ln(2)/k_e$.

In vivo blood-brain barrier experiment with mice

Animals

Female, Institute for Cancer Research, Caesarean Derived-1 (ICR-CD-1) mice (Harlan Laboratories, Venray, the Netherlands) of age 7-10 weeks and weighing 29-32 g, were used during the BBB transport experiments. All animal experiments were performed in accordance with the Ethical Committee principles of laboratory animal welfare as approved by our institute (Ghent University, Faculty of Veterinary Medicine, no. EC2012/157 and no. EC2014/128).

Blood-to-brain transport

An *in vivo* multiple time regression (MTR) analysis was performed to investigate if spilanthol was able to cross the BBB. A dose solution of spilanthol was prepared using the *Spilanthes acmella* (L.) L. extract (Robertet) with a final concentration of 4 mg/ml spilanthol dissolved in 7.0% ethanol, 2.5% dimethylacetamide, 0.6% tween 80 (all w/w) diluted in lactated Ringer's solution containing 1% BSA. The ICR-CD-1 mice were anesthetized by intraperitoneal injection with a 40% (w/V) urethane solution (3 g/kg) and the jugular internalis vein and carotid artery were isolated, 20 µl of the spilanthol dose solution was injected into the jugular vein. Blood was obtained from the carotid artery at regular time points after injection (1, 3, 5, 10, 12.5 and 15 min, with start and end in duplicate), and the mice were immediately thereafter decapitated. After sacrificing the mice, the brains were collected. The collected blood from the carotid artery was centrifuged at 10 000 *g* for 15 min at 21°C, resulting in serum. In order to assure the validity of the MTR method, ^{125}I-BSA and dermorphin diluted in lactated Ringer's solution containing 1% BSA were used as the negative and positive control, respectively [21-23]. Furthermore, to evaluate the functional BBB integrity under our experimental conditions, ^{125}I-BSA was also administered to the mice in a solution containing the same surfactant and co-solvents in equal concentrations as in the spilanthol dose solution. The serum profile of spilanthol was plotted against the time (expressed in min). The curve was fitted using the same one compartmental model as previously described in the pharmacokinetic section of the *in vivo* experiment using rats. In order to determine the BBB permeability of a compound, the ratio of the brain and serum concentration (µl/g) was plotted versus a derived time variable, *i.e.* the exposure time (θ) of the Gjedde-Patlak plot [24, 25].

The exposure time is defined as the integral of the concentration of spilanthol in serum from start (t=0 min) to time T, divided by the concentration of spilanthol in serum at time T: $\theta = \int_0^T \frac{C_s(t)\cdot dt}{C_s(T)}$. The integral of the concentration of spilanthol in serum from zero to time T is the area under the curve until time T.

A biphasic model of blood-brain transfer was used to fit the uptake, as elaborated by Wong *et al.* (1986) [26]:

$$\frac{C_{brain}(T)}{C_s(T)} = K \times \theta + V_g \times \left(1 - e^{\left(-\theta \times \left(\frac{K_1 - K}{V_g}\right)\right)}\right) + V_0 \quad \begin{matrix} K = 0 \\ \cong \end{matrix} \quad V_g \times \left(1 - e^{\left(-\theta \times \left(\frac{K_1}{V_g}\right)\right)}\right) + V_0$$

where K_1 is the unidirectional clearance (µl/(g·min)), K is the net clearance (µl/(g·min)), V_g the tissue brain distribution volume (µl/g), and V_0 the vascular brain distribution volume (µl/g). The negative control BSA has a low vascular distribution volume and this value (14.8 µl/g) is used as V_0 to calculate the brain kinetic parameters of spilanthol. In the equation, $C_{brain}(T)$ is the concentration of spilanthol in the brain at time T (ng/g) and $C_s(T)$ the concentration of spilanthol in serum at time T (ng/µl).

Capillary depletion

To investigate the distribution of spilanthol in the parenchyma and capillaries of the brain, a capillary depletion experiment was performed. By means of this experiment, it is possible to distinguish the transport of spilanthol into the brain (represented by the parenchyma) and part of spilanthol which is trapped by the endothelial cells of the brain (represented by the capillaries). The method of Triguero *et al*. (1990), as modified by Gutierrez *et al.* (1993), was used [27-29]. Briefly, after anesthetizing the mice intraperitoneally with 40% (w/V) urethane solution (3 g/kg), 20 µl of the 4 mg/ml spilanthol dose solution was injected into the jugular vein. Blood was collected from the abdominal aorta ten min after injection and serum was obtained by centrifuging the blood at 10 000 *g* during 15 min at 21°C. Immediately after blood collection, the skin of the mice's chest is removed to clamp the aorta and the jugular veins are severed. Immediately after clamping the aorta, the brain is perfused manually with 20 ml of Lactated Ringer's solution. The mice are decapitated immediately after perfusion and brain is collected. The brains were put into an Eppendorf tube and weighed. 525 µl ice-cold capillary buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 and 10 mM D-glucose adjusted to pH 7.4) was added and homogenized. Then, 1000 µl of 26% ice-cold dextrane solution in capillary buffer was added and vortexed. The Eppendorf tube was centrifuged at 20 000 *g* for 60 min at 4°C. Pellet (capillaries) and supernatant (parenchyma and fat tissues) were separately collected into an Eppendorf tube and weighed. The sample preparation of the pellet is the same sample preparation method as used for the mice brains, while the sample

preparation for the supernatant is the same sample preparation method as used for mice serum, as further described in the section 'bio-analytics'.

The distribution was calculated as follows:

$$\text{Fraction (\%)} = \frac{\text{M}_{\text{tissue}}}{\text{M}_{\text{capillaries}} + \text{M}_{\text{parenchyma}}} \times 100$$

where M_{tissue} represents the amount of spilanthol in the capillaries, respectively parenchyma; $M_{capillaries}$ the amount of spilanthol in the capillaries and $M_{parenchyma}$ the amount of spilanthol in the parenchyma.

Brain-to-blood transport

To evaluate the efflux of spilanthol out of the brain, an *in vivo* method previously described was used [27]. Briefly, after anesthetizing the ICR-CD-1 mice using a 40% (w/V) urethane solution (3 g/kg), the skin of the skull was removed. Thereafter, a hole was made into the lateral ventricle using a 22 G needle marked with tape at 2 mm at the following coordinates: 1 mm lateral and 0.34 mm posterior to the bregma. 1 µl of the 4 mg/ml spilanthol dose solution as used for the blood-to-brain influx experiment was injected intracerebroventriculary (ICV) using a syringe pump (KDS100, KR analytical, Cheshire, UK) at a speed of 360 µl/h for 10 s. At specified time points post-injection (1, 3, 5, 10, 12.5 and 15 min), mice were decapitated. Prior to decapitation, blood was collected from the abdominal aorta. Serum was obtained by centrifuging the blood at 10 000 *g* during 15 min at 21°C and brains were collected. The efflux brain half-life ($t_{1/2,brain}$) (min) was calculated from the linear regression of the natural logarithm of the spilanthol concentration in brain (ng/g) versus time as follows: $t_{1/2,brain} = \ln(2)/k_{out}$, where k_{out} (min^{-1}) is defined as the efflux rate constant calculated as the negative value of the slope of the linear regression, applying first order kinetics.

Bio-analytics

Serum sample preparation

60 µl of the serum samples, 60 µl of 4% (V/V) aqueous H_3PO_4 solution and 30 µl of the IS solution were transferred into a 0.5 ml (LoBind Eppendorf) tube and vortexed. Interfering compounds were removed by solid phase extraction using a positive pressure-96 processor (Waters). 100 µl (rat serum) or 125 µl (mice serum) of the previous sample solution was loaded on the HLB Oasis® µelution 96 well plate (Waters, Zellik, Belgium), which was preconditioned with 200 µl of MeOH and equilibrated using 200 µl of ultrapure water. After the loading step, the HLB Oasis® µelution plate was washed using 200 µl of 5% MeOH in H_2O followed by 200 µl of 20% MeOH in H_2O. Spilanthol was eluted using two times 25 µl ACN. To the eluate, 25 µl of a 80:20 (V/V) H_2O:MeOH solution was

added and analysed with a UHPLC-MS² method, as described further in the section 'UHPLC-MS² method'.

A limited validation of the bio-analytical method for the quantification of spilanthol in serum was performed, based upon the EMA guideline on bio-analytical method validation (EMEA/CHMP/EWP/192217/2009), with limited replicates and concentration levels [30]. The limit of detection (LoD) (S/N=3) and limit of quantification (LoQ) (S/N=10) of spilanthol, determined on the reference standard, were calculated as 0.09 ng/ml and 0.31 ng/ml, respectively, which correspond to 0.17 ng/ml and 0.58 ng/ml in rat serum, respectively. The LoD and LoQ of spilanthol in mouse serum were 0.048 ng/ml and 0.16 ng/ml, respectively. A matrix factor of 1.1 was observed for spilanthol in rat and mouse serum. The quantification of spilanthol in the rat serum samples was performed using a matrix spiked calibration curve (R^2=0.9998). Linearity was assured in a working range from 0.31 ng/ml up to 101 ng/ml, corresponding to 0.58 ng/ml to 189 ng/ml in rat serum. The quantification of spilanthol in the mouse serum samples was performed using a one-point calibration curve with a spilanthol standard in 40:33.33:26.67 (V/V/V) MeOH:ACN:H_2O. The accuracy of the references used for the calibration curves with the pre-extracted spiked matrix samples and the QC reference pre-extracted spiked matrix samples conformed to the specification limits (< 15% of the nominal value and for the lower limit of quantification (LLoQ) < 20% of the nominal value), except for the LLoQ used for the validation of spilanthol in mouse serum (> 20% of the nominal value). These values are slightly higher than the specification limits given by the EMA in their guidelines for formal bio-analytical method validation, but are still acceptable for our purposes in the discovery phase. Precision was expressed as the coefficient of variation (CV). The within-run CV value did not exceed 15% for the pre-extracted spiked matrix samples. Also the LLoQ did not exceed 20% and are thus all conform to the specification limits. The recovery in rat serum was 102.1% (18.8 ng/ml to 189 ng/ml in serum concentration range), calculated from the slopes of the pre-extracted and post-extracted spiked matrix samples. The recovery in mouse serum was 113.3% (3 to 257 ng/ml in serum concentration range). No significant carry-over was observed (< 20% of LLoQ spilanthol and < 5% for IS). The selectivity was conform to the specification limits (placebo rat serum < 20% of LLoQ spilanthol and < 5% for IS).

Brain sample preparation

For the analysis of the mice brain samples, the weighed brains were transferred into a test tube and crushed. 1.0 ml of the IS solution in ACN was added, followed by shaking during 4h at 110 rpm at room temperature using an Eppendorf centrifuge 5810 (Eppendorf, Rotselaar, Belgium). The test tube was centrifuged at 250 *g* for 5 min and 800 µl of the supernatant was transferred into an

Eppendorf tube. Thereafter, a second centrifugation was performed at 20 000 *g* during 5 min at room temperature. 750 µl of the supernatant was evaporated to dryness under nitrogen and 175 µl 90:10 (V/V) H_2O:ACN was added to redissolve spilanthol. Thereafter, 160 µl of this solution was loaded on the HLB Oasis® µelution 96 well plate. The same washing and elution steps were used as for the serum samples.

Again, a limited validation of the bio-analytical method for the quantification of spilanthol in brains was performed, based upon the EMA guideline on bio-analytical method validation (EMEA/CHMP/EWP/192217/2009), with limited replicates and concentration levels [29]. The LoD and LoQ of spilanthol were 6.30 pg/g in brain and 21.0 pg/g in brain, respectively. Ion enhancement (matrix factor = 1.38 > 1) was observed for the quantification of spilanthol in mice brains. Hence, quantification of spilanthol in the brain samples was performed using the matrix spiked calibration curve (R^2=0.9986). Linearity was assured in a working range of 0.107 ng/ml to 175 ng/ml spilanthol, corresponding to 21.0 pg/g to 34.3 ng/g in brain. The accuracy of the references used for the calibration curves with the pre-extracted spiked matrix samples conformed to the specification limits (< 15% of the nominal value), except for the LLoQ used for the validation of spilanthol in brains (> 20% of the nominal value), but was still acceptable for our purposes in the discovery phase. The within-run CV value did not exceed 15% for the pre-extracted spiked samples. Also the LLoQ did not exceed 20% and are thus all conform to the limits. The recovery in mouse brains was 72.7% (0.92 to 98.10 ng/g brain concentration range).

UHPLC-MS² method

Quantification of spilanthol was performed using a UHPLC-MS² method, which was developed on an Acquity UPLC coupled to a Xevo™ TQ-S mass spectrometer (MS) (Waters, Zellik, Belgium) with electrospray ionisation source and a triple quadrupole mass analyser. An Acquity UPLC RP C18 column (Waters, 50 x 2.1 mm, 1.7 µm) with a suitable guard column was used. The sample compartment was kept constant at 5°C, while the column temperature was maintained at 30°C. 2 µl of the sample was injected and the flow rate was set to 0.5 ml/min. A mobile phase was applied using solvent A (0.1% FA in 30:70 (V/V) H_2O:MeOH) and solvent B (0.1% FA in MeOH) in gradient mode as follows: 0-1.6 min 100:0 (V/V) A:B, 1.6-2 min going from 100:0 (V/V) A:B to 0:100 (V/V) A:B, 2-3 min 0:100 (V/V) A:B, 3-3.4 min going from 0:100 (V/V) A:B to 100:0 (V/V) A:B, 3.4-5 min 100:0 (V/V) A:B. The needle wash solvent was 60:40 (V/V) DMSO:ACN. The MS was operated in the positive electrospray ionisation mode (ESI^+), with an optimised capillary voltage of 3.0 kV, cone voltage of 50 V and source offset of 60 V. Source and desolvation temperatures were set at 150°C and 500°C, respectively, while cone and desolvation gas (N_2) flows were 180 and 1000 l/h, respectively.

Acquisition was performed in the multiple reaction monitoring (MRM) mode with *m/z* 222.15 to *m/z* 80.96 transition. The applied collision energy was 20 eV (collision gas = argon). Data were acquired and analysed through MassLynx® software (V4.1 SCN 843, Waters).

3. RESULTS

Caco-2 cell permeability

Propranolol was used as a positive control and a $P_{app,ab}$ value of $19.1 \cdot 10^{-6}$ cm/s was obtained, which is in good agreement with the values reported in literature [31]. Atenolol served as the negative control and a lower permeability was observed compared to propranolol. Spilanthol was able to diffuse through the Caco-2 cell monolayer from the apical-to-basolateral side, as well as from the basolateral-to-apical side. Figure 2 shows the percentages of spilanthol permeated through the Caco-2 cells versus time plot with DS1 and DS2.

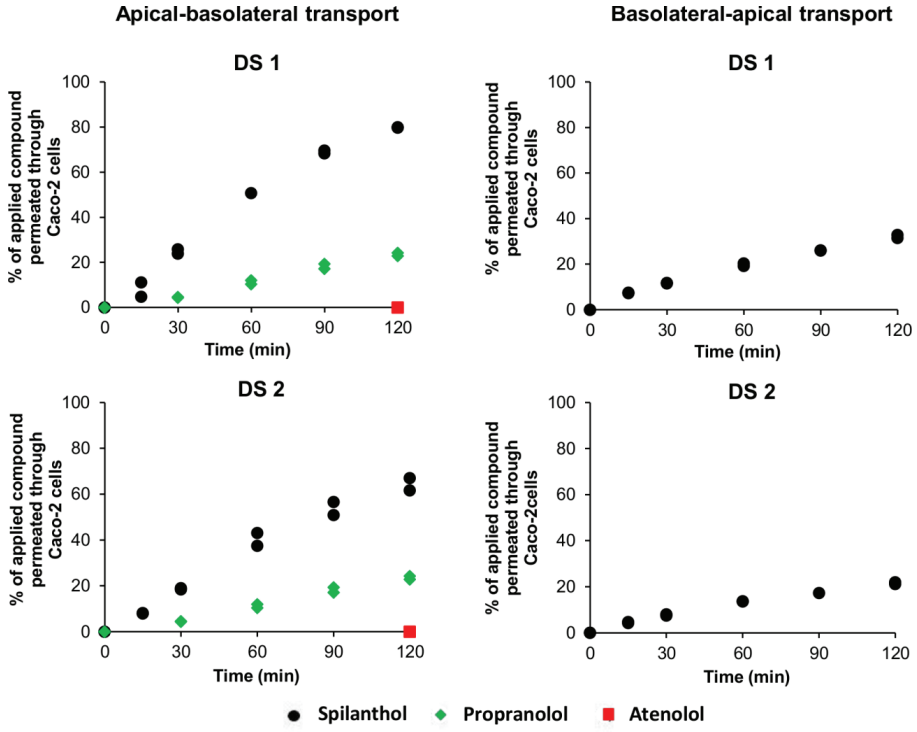

Figure 2. The percentages of spilanthol from the applied dose solutions using dose solution 1 (DS1) and dose solution 2 (DS2) which permeated through the Caco-2 cells monolayer in the course of time. Apical-to-basolateral transport and basolateral-to-apical transport experiments of spilanthol were performed in duplicate (individual results in the same figure).

The percentage of the applied spilanthol which permeated through the Caco-2 cells from the apical-to-basolateral membrane after 120 min is 79.8% (or 3.20 µg spilanthol) with DS1 and 64.3% (or 2.47 µg spilanthol) with DS2. In the opposite direction, the percentage of spilanthol which permeated through the Caco-2 cells from the basolateral-to-apical membrane after 120 min is 32.1% (or 3.86 µg spilanthol) with DS1 and 21.6% (or 2.49 µg spilanthol) with DS2.

The apparent permeability coefficient (P_{app}) of spilanthol obtained with DS1 and DS2 from the apical-to-basolateral side is 8.42 ± 0.59·10^{-5} cm/s (mean ± SD, n=2) and 5.61 ± 0.54·10^{-5} cm/s (mean ± SD, n=2), respectively. The P_{app} of spilanthol obtained with DS1 and DS2 was also determined from the basolateral-to-apical side and is 10.21 ± 0.46·10^{-5} cm/s (mean ± SD, n=2) and 5.20 ± 0.06·10^{-5} cm/s (mean ± SD, n=2), respectively. The efflux ratio for spilanthol with DS1 and DS2 is 1.21 and 0.93, respectively, while the uptake ratio for spilanthol with DS1 and DS2 is 0.82 and 1.08, respectively.

Oral gavage experiment

Figure 3 represents the rat serum data after oral gavage of spilanthol, fitting a one compartmental model with the mean concentration of spilanthol at each time point. These results of the *in vivo* experiment confirm the *in vitro* Caco-2 cell monolayer results of spilanthol.

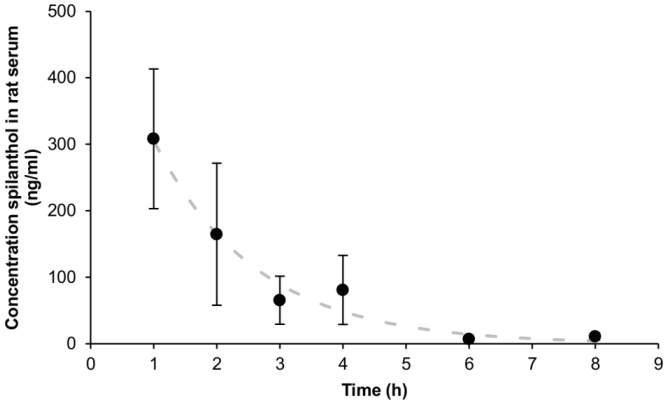

Figure 3. Concentration of spilanthol in rat serum (ng/ml) as a function of time (h) after oral gavage of spilanthol. Data are fitted according to a one compartmental model (n=3, mean, error bars: SEM).

From these data, the elimination rate constant and the elimination half-life were calculated as 0.61 h^{-1} and 1.13 h, respectively.

Blood-brain barrier transport kinetics of spilanthol

The blood-to-brain transport of spilanthol was investigated. The negative and the positive control confirmed the validity of the BBB test. BSA is known to be a vascular marker, while dermorphin is regarded as the positive control as the BBB influx is higher compared to BSA. However, the brain influx of BSA can be explained by the fact that iodinated BSA showed nonspecific binding to cerebral capillaries compared to the tritiated from. Both controls are included in the experiment as a system suitability test [22, 23, 32]. The K_1 value of BSA (negative control) was 0.12 µl/(g·min), while the K_1 value of the positive control dermorphin was 0.26 µl/(g·min), both consistent with previous data [27, 33]. The multiple time regression data indicated that spilanthol crosses the blood-brain barrier. In Figure 4, the ratio of the concentration of spilanthol in brain and serum is plotted versus the exposure time. Furthermore, the kinetic influx data of ^{125}I-BSA with or without the surfactant/so-solvents were not significant different, indicating that the surfactant and co-solvents did not affect the BBB integrity (data not shown).

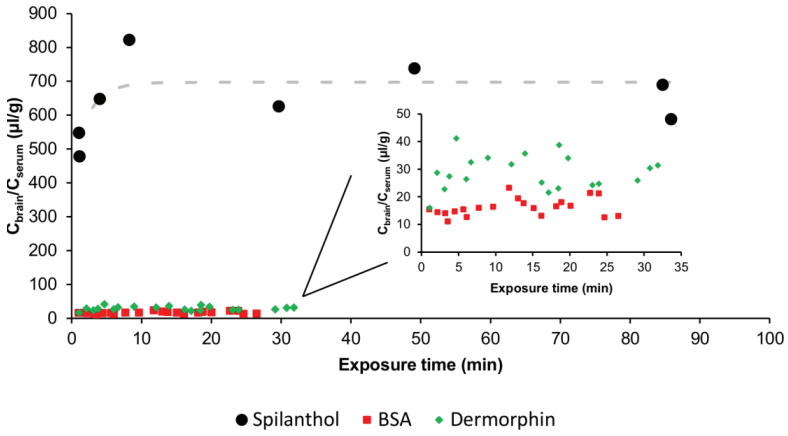

Figure 4. Blood-to-brain transport (multiple time regression experiment) results of spilanthol, BSA and dermorphin in mice. The ratio of spilanthol concentration in brain versus serum (µl serum/g brain) is plotted versus the exposure time (min). Start and end point were performed in duplicate. The data were fitted using a biphasic model.

The MTR data of spilanthol were fitted using a biphasic model, based on the modified Gjedde-Patlak equation according to Wong *et al.* (1986) [26]. A rapid but highly significant influx of spilanthol into the brains was observed with a unidirectional influx rate (K_1) of 796 µl/(g·min). The tissue brain distribution volume (V_g) is 652 µl/g.

The curve reached a plateau-phase after about 10 min exposure time and can be explained by efflux of spilanthol out of the brain.

At the same time, also the elimination kinetics of spilanthol in serum were evaluated in the mice model. The serum profile of spilanthol follows a one compartmental model. The concentration of spilanthol at time zero was 3.05 µg/ml and an elimination rate constant and elimination half-life of 0.22 min^{-1} and 3.16 min were obtained.

Capillary depletion

The capillary depletion method was used to study the distribution of spilanthol *i.e.* the part that was taken up by the brain and the part which was trapped in the endothelial cells of the brain capillaries. A high brain penetration of spilanthol was found: about 98% of spilanthol (corresponding to 137.8 µl/g) was found in the brain parenchyma and only about 2% of spilanthol (corresponding to 2.4 µl/g) that entered the brain remained in the brain capillaries.

Brain-to-blood transport kinetics of spilanthol

The efflux properties of spilanthol out of the brain were investigated by quantifying the concentration of spilanthol in the brain after intracerebroventricular injection of the dose solution. When evaluating the transport of spilanthol out of the brain into the blood, it is confirmed that there is also efflux of spilanthol (Figure 5), which can explain the rapid plateauing observed when evaluating the blood-to-brain kinetics during the influx experiment (Figure 4). The efflux transfer constant k_{out} was derived from the absolute value of the slope of the natural logarithm of the concentration of spilanthol in the brain (ng/g) versus the experimental time curve (min). The k_{out} calculated for spilanthol was 0.11 min^{-1}, equal to a $t_{1/2,brain}$ of 6.4 min.

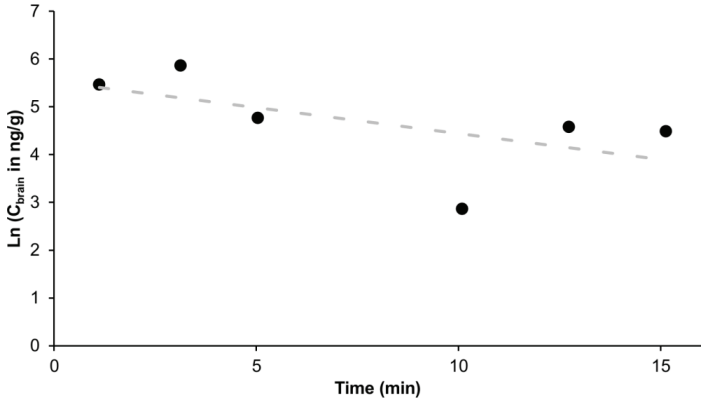

Figure 5. Brain-to-blood transport results of spilanthol in mice. The natural logarithm of the concentration of spilanthol in the brain (ng/g) in function of the time (min) is given.

4. DISCUSSION

In this study, the *in vitro* Caco-2 cell monolayer permeability of spilanthol, a 2,6,8-triene NAA, was investigated using two different dose solutions. The permeation level of spilanthol through Caco-2 cells was the highest from the apical-to-basolateral side: 64-80% of the applied DS permeated through Caco-2 cells after 120 min (absorptive direction). Spilanthol, applied as DS2, did not appear to better permeate the Caco-2 cells compared to spilanthol applied as DS1, suggesting that co-solvents (vitamin E-TPGS, PEG 400, PG) did not enhance the permeation of spilanthol through the cell monolayer. Both dose solutions did not contain more than 0.5% ethanol in buffer, as final ethanol concentrations above 2% can damage the Caco-2 cell monolayer.

As mentioned earlier, Matthias *et al.* (2004) investigated a different type of NAA, namely the transport of 2-ene and 2,4-diene NAAs through the Caco-2 cell monolayer (dissolved in ethanol and diluted in HEPES buffer, final ethanol concentration did not exceed 2%) with P_{app} values ranging from $3 \cdot 10^{-6}$ to $3 \cdot 10^{-4}$ cm/s. The diversity in P_{app} for the different *N*-alkylamides was correlated to structural variations, with saturation and *N*-terminal methylation contributing to lowered P_{app} values [15].

The P_{app} values obtained for spilanthol in this study were all above $1 \cdot 10^{-6}$ cm/s, indicating an almost complete intestinal absorption. Compounds with P_{app} values < $1 \cdot 10^{-6}$ cm/s show a less readily absorption. The P_{app} values obtained for spilanthol in this study ($5 \cdot 10^{-5}$ to $10 \cdot 10^{-5}$ cm/s) are thus consistent with the structural conclusions reported by Matthias *et al.* (2004) based on the mono- and diene-NAAs from *Echinacea* plant species [15].

It has been reported that if there is a more than 2-fold difference in P_{app} values between the apical-to-basolateral and the basolateral-to-apical direction, a high probability of active transport exists [34]. In this experiment, for both dose solutions of spilanthol, this difference was less than 2-fold, indicating that no active transport function can be readily assumed. Studies have proven a correlation between drug permeability coefficients obtained during a Caco-2 cell monolayer experiment and oral absorption in humans [31, 35, 36]. However, as no *in vitro* model can yet totally mimic the *in vivo* intestinal barrier, since many factors such as solubility, formulation, chemical composition, pH of the intestinal secretions, food composition, gastric emptying time, intestinal motility and blood flow play a role [34, 37, 38], an oral gavage experiment in rats was conducted to evaluate the intestinal barrier properties of spilanthol *in vivo*. Rats are one of the most common animals used in preclinical oral absorption studies and are generally considered as good animal models towards humans (*e.g.* F1 modifying factor in permitted daily exposure (PDE) toxicity evaluation is 5 for extrapolation from rats to humans) [37, 39]. Several studies have already shown that there is a good correlation between the absorption of compounds in rat and the absorption in humans [37, 38, 40]. Spilanthol was orally administered to rats in a liquid dose solution containing

the same solvents as used for DS2 of the Caco-2 cell permeability experiment, consisting of ethanol, PG, vitamin E-TPGS and PEG400. For the absorption rate, the formulation plays an important role and compounds solubilized in liquid generally absorb faster compared to solid forms. The elimination phase, on the contrary, is independent of the oral dosage form. Vitamin E-TPGS, an esterified vitamin E derivative, was added to the formulation because of its solubilizing and emulsifying properties to enhance the solubility and facilitate the absorption of spilanthol [41, 42]. In this *in vivo* study, spilanthol was clearly observed in the rat serum and hence, spilanthol was able to diffuse through the gut barrier into the systemic circulation, confirming the *in vitro* Caco-2 cell results. Spilanthol showed an elimination half-life of 1.13h (63 min), corresponding to similar values reported by Woelkart *et al.* (2009), who investigated the plasma concentration of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides from *Echinacea* via oral gavage in rats, resulting in an elimination half-life of 72 min [17].

Besides the lipophilicity of the compound, its passive gut-blood permeability is dependent on the charge and molecular size of the compound. Considering the lipophilic LogP value of 3.39 of spilanthol and the molecular weight of 221.34 g/mol, it seems that transcellular transport is appropriate for spilanthol [43]. These findings were confirmed in the Caco-2 cell permeability experiment, which indicated that spilanthol diffused through the cells via passive diffusion. Once in the systemic circulation, the investigated compound can distribute to extravascular compartments such as the brain to exert CNS activity.

Therefore, the present study investigated the blood-brain barrier transport properties of spilanthol in an *in vivo* mice experiment. In kinetic BBB research, one of the 'golden' methodologies is the MTR using mice for reasons of costs, handling and animal care. In PDE toxicity evaluation, the F1 modifying factor is 12 for extrapolation from mice to humans [39]. Spilanthol was administered to the mice in a solution containing ethanol, dimethylacetamide and tween 80 in lactated Ringer's solution containing BSA. Dimethylacetamide and ethanol are often used in injectable pharmaceutical formulations as co-solvents to solubilize poorly soluble compounds, while tween 80 is a traditional surfactant [44, 45]. It is known that tween 80 in high doses up to 30 mg/kg administered to mice causes blood-brain barrier disruption [46]. In this MTR study, a dose of 3 mg/kg tween 80 was used. Also ethanol, at a concentration of 1-4 g/kg, may cause disruption of the BBB as well [46]. However, in the current study, only 0.04 g ethanol/kg mice was administered. Although transient effects cannot be excluded, our data indicate that the solvents did not affect the BBB integrity. In addition, no toxicity was observed during the experiment. From our results, it is concluded that spilanthol was able to rapidly penetrate the BBB after intravenous administration with a unidirectional influx rate of 796.2 µl/(g·min). This high K_1 value indicates a high initial influx rate of spilanthol into the brain,

which is supported by the lipophilic character of this NAA. Moreover, 98% of spilanthol was found in the parenchyma of the brains, while only 2% was trapped in the capillaries, which makes it possible for NAAs to exert CNS effects. Furthermore, significant efflux out of the brain into the blood was also observed.

It was already mentioned in the introduction that *Spilanthes acmella* extracts showed some central analgesic activities. In general, different central nervous pharmacodynamic activities are attributed to *N*-alkylamide containing plants. Psychotropic effects (anxiolytic effect) in animals were already found by using alkylamide *Echinacea* preparations, which was explained by their affinity for the CB receptors. Furthermore, similar to cannabinoids, alkylamides alter immune cell activity, which open perspectives for the treatment of neuroinflammatory diseases [17, 47]. In addition, capsaicin, the major NAA in hot peppers of the plant genus Capsicum interacts agonistically with the transient receptor potential vanilloid 1 receptor, predominantly expressed in primary afferent fibres and sensory neurones, playing a role in pain sensation [48, 49]. Another study reported an improvement of amyloid beta(1-42)-induced spatial memory impairment after administration of a 50 mg/kg and 100 mg/kg methanolic extract of *Piper nigrum* fruits by gastric gavage to rats. The plant extract, containing piperine, which is a major alkylamide of black pepper, caused an attenuation of the oxidative stress in the rat hippocampus. Other studies demonstrated analgesic, anticonvulsant, antidepressant, anti-oxidant, anti-inflammatory, protection against neurodegeneration and cognitive enhancing effects of cognitive deficit-like condition of Alzheimer's disease of piperine [50, 51].

In addition, in some studies there is an indirect proof of blood-brain barrier influx of *N*-alkylamides. It has been shown that several *N*-alkylamide containing plants showed activity against epilepsy in animal models. Experiments with medicinal plants such as *Anacyclus pyrethrum*, *Nigella sativa*, *Ferula gummosa* and *Pimpinalla anisum* were performed to investigate their effect on seizures of epilepsy. It has been shown that the chloroform fraction of *Anacyclus pyrethrum* roots, 100-800 mg/kg administered intraperitoneally to mice, possess neuropharmacological effects, such as antiseizure activity [52, 53]. Other studies demonstrated the myorelaxation and anticonvulsant activities in mice of the *Anacyclus pyrethrum* extract as well [52, 54]. Treatment with capsaicin resulted in a decrease in behavioral seizure activity and body temperature in a kainic acid induced epileptogenesis mice model. Furthermore, anti-oxidant activity in blood increased and the concentrations of IL-1β and TNF-α in the brain lowered [49].

The majority of the studies reported in literature only look at the effects of *in vivo* or *in vitro* studies and no exposure data are available. Therefore, more detailed studies about exposure levels are required. In the current study, spilanthol concentrations in brains and serum were quantitatively

measured and we demonstrated a high influx of spilanthol into the brain, indicating a possible role in CNS diseases.

5. CONCLUSION

In this study, it is demonstrated that spilanthol is able to cross the Caco-2 cell monolayer *in vitro* and the intestinal membrane *in vivo* in rats after oral administration. After absorption and reaching the systemic circulation, spilanthol is able to rapidly and significantly cross the BBB in mice. Spilanthol also showed a significant efflux out of the brain, which explains partly the biphasic behaviour of the influx. These results suggest possible medicinal applications for spilanthol in central nervous system diseases from a pharmacokinetic point of view.

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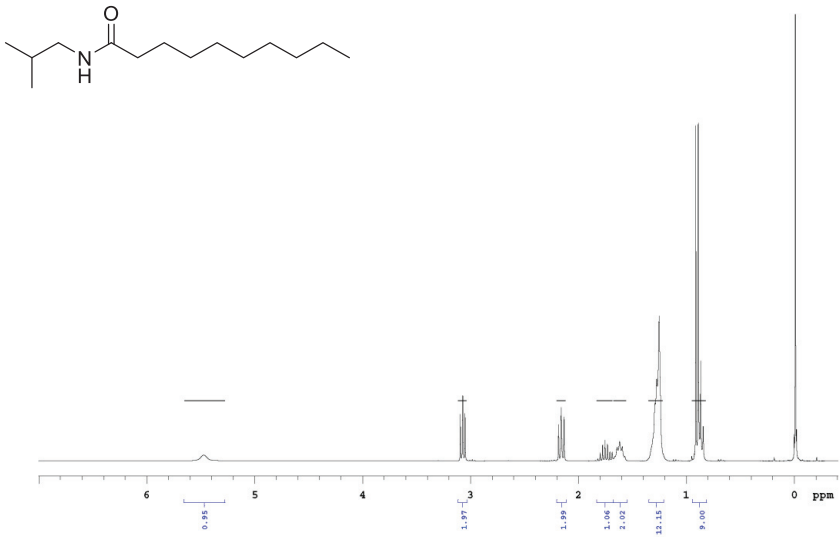
SUPPLEMENTARY INFORMATION

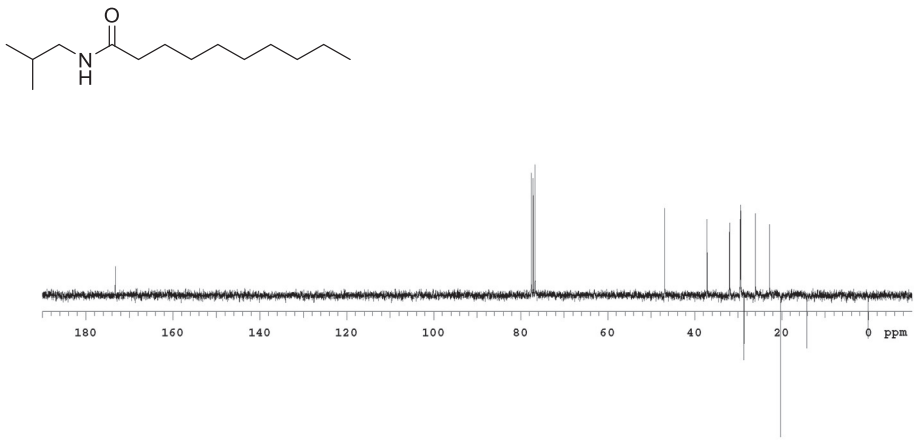
S1. NMR spectra of internal standard isobutyldecanamide.

S1. NMR spectra of internal standard isobutyldecanamide.

***N*-Isobutyldecanamide ^{1}H NMR (300 MHZ $CDCl_3$)**



***N*-Isobutyldecanamide ^{13}C NMR APT (75 MHZ $CDCl_3$)**



CHAPTER VI

QUANTITATIVE *IN VITRO* AND *IN VIVO* EVALUATION OF INTESTINAL AND BLOOD-BRAIN BARRIER TRANSPORT KINETICS OF PELLITORINE

"Discovery consists of looking at the same thing as everyone else and thinking something different."

Albert Szent-Györgyi
(°1893-†1986, Hungarian physician, Nobel Prize winner in Medicine)

Parts of this chapter were published:

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ABSTRACT

The objective was to evaluate the gut mucosa and blood-brain barrier (BBB) pharmacokinetic permeability properties of the plant *N*-alkylamide pellitorine. Pure pellitorine and an *Anacyclus pyrethrum* extract were used to investigate the permeation of pellitorine through (1) a Caco-2 cell monolayer, (2) the rat gut after oral administration, and (3) the BBB in mice after intravenous and intracerebroventricular administration. A validated bio-analytical UHPLC-MS^2 method was used to quantify pellitorine. Pellitorine was able to cross the Caco-2 cell monolayer from the apical-to-basolateral and from the basolateral-to-apical side with apparent permeability coefficients between $0.6 \cdot 10^{-5}$ and $4.8 \cdot 10^{-5}$ cm/h and between $0.3 \cdot 10^{-5}$ and $5.8 \cdot 10^{-5}$ cm/h, respectively. In rats, a serum elimination rate constant of 0.3 h^{-1} was obtained. Intravenous injection of pellitorine in mice resulted in a rapid and high permeation of pellitorine through the BBB with a unidirectional influx rate constant of 153 µl/(g·min). In particular, 97% of pellitorine reached the brain tissue, while only 3% remained in the brain capillaries. An efflux transfer constant of 0.05 min^{-1} was obtained. Pellitorine shows a good gut permeation and rapidly permeates the BBB once in the blood, indicating a possible role in the treatment of central nervous system diseases.

CHAPTER VI

QUANTITATIVE *IN VITRO* AND *IN VIVO* EVALUATION OF INTESTINAL AND BLOOD-BRAIN BARRIER TRANSPORT KINETICS OF PELLITORINE

Main focus in this chapter:

- To quantitatively investigate the Caco-2 cell monolayer and rat intestinal barrier permeability properties of pellitorine.
- To evaluate the ability of pellitorine to cross the blood-brain barrier.

1. INTRODUCTION

Pellitorine (deca-2E,4E-dienoic acid isobutylamide; F3M1 according to the FxMy classification of *N*-alkylamides (NAAs)) is a highly abundant and biologically potent diene NAA found in Asteracea plants such as *Anacyclus pyrethrum* (AP) [1]. Various pharmacological activities of pure pellitorine have already been reported, *i.e*. antiprotozoal, larvicide, antiseptic, antithrombotic, antituberculosis, antibacterial, anticancer, antiplatelet aggregation properties and vascular barrier protective effects [2-10]. It was also demonstrated that pellitorine reduces fatty acid uptake *in vitro* [11]. Central nervous system (CNS) activities of purified pellitorine are not yet described, while a limited number of CNS activities of *Anacyclus pyrethrum* extracts have been reported. Antiseizure activity was demonstrated after intraperitoneally administration of 100-800 mg/kg of the chloroform fraction of *Anacyclus pyrethrum* roots to mice. Other studies with the *Anacyclus pyrethrum* extract in mice showed anticonvulsant and myorelaxation activities [12]. Another study with an ethanolic AP root extract showed anticonvulsant effect against maximal electro shock (MES) induced convulsions in

mice [13]. Sujith *et al*. (2011) found that the AP root extract possesses antidepressant activity in albino Wister rats and improves the learning acquisition of rats [14].

CNS effects of compounds require them to penetrate the blood-brain barrier in order to reach their brain target. Compounds can enter the systemic circulation after topical and oral administration, by crossing several physiological barriers (*i.e.* stratum corneum, intestinal barrier) and further reach the brain. A previous study of our research group demonstrated that pellitorine permeates human skin using an *in vitro* Franz diffusion cell (FDC) experiment (see Chapter III). The permeability coefficient $K_{p,v}$ (with vehicle= 30:70 H_2O:EtOH (V/V)) of pellitorine was $2.3 \cdot 10^{-4}$ cm/h for the crude plant extract and $1.1 \cdot 10^{-4}$ cm/h for purified pellitorine [15]. Moreover, the intestinal barrier and BBB transport kinetics of spilanthol were studied as well (see Chapter V) [16].

Up till now, to our best knowledge, no information is available about the intestinal barrier and blood-brain barrier transport kinetics of the 2,4-diene *N*-alkylamide pellitorine. Hence, the aim of this study was to quantitatively investigate this *in vitro* using a Caco-2 cell monolayer as well as *in vivo* with an oral gavage rat model and a BBB mice model.

2. MATERIALS AND METHODS

Chemicals and reagents

Dextran was bought from AppliChem GmbH (Darmstadt, Germany), while trypsin-EDTA came from Invitrogen (Ghent, Belgium). UPLC-MS grade acetonitrile (ACN), methanol (MeOH) and trifluoroacetic acid (TFA) were purchased at Biosolve (Valkenswaard, the Netherlands). Dimethylacetamide and phosphoric acid (85%) (H_3PO_4) were obtained from Jansen Chimica (Geel, Belgium), while propylene glycol (PG) was bought from Riedel-de Haën (Seelze-Hannover, Germany)**.** Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), sodium hydrogen carbonate ($NaHCO_3$), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$), acetic acid and absolute ethanol (EtOH, ≥ 99.9% V/V) were purchased at Merck KGaA (Darmstadt, Germany), while polyethylene glycol 400 (PEG 400), calcium dichloride ($CaCl_2$), LC-MS grade formic acid (FA), tween 80, sodium hydroxide (NaOH), D-glucose, HEPES, Hanks' Balanced Salt Solution (HBSS), vitamin E-TPGS, trypan blue, dimethyl sulfoxide (DMSO), sodium chloride (NaCl), potassium chloride (KCl), phosphate buffered saline (PBS), sodium hydrogen carbonate ($NaHCO_3$), calcium chloride dehydrate ($CaCl_2.2H_2O$), sodium lactate, sodium dihydrogen phosphate (NaH_2PO_4), sodium sulphate (Na_2SO_4), urethane, Krebs-Henseleit buffer and hydrochloric acid (HCl) were bought from Sigma-Aldrich (Diegem, Belgium). HPLC gradient grade ACN, MeOH and absolute ethanol (99.8% V/V) were obtained from Fisher Scientific (Erembodegem,

Belgium). Ultrapure water (H_2O) of 18.2 MΩ.cm quality was produced by an Arium 611 purification system (Sartorius, Göttingen, Germany).

Products examined

The ethanolic *Anacyclus pyrethrum* root extract was prepared and characterised as previously described [17]. The *Anacyclus pyrethrum* root extract (4.87% w/w NAAs of which pellitorine was the main NAA (1.55% w/w pellitorine)) was used for the oral gavage experiment and the Caco-2 cell permeability assay. Pellitorine was bought from Adipogen Life Sciences (99.8% purity determined by HPLC) and was used for the BBB transport assay. As analytical internal standard (IS), isobutyldecanamide was used, obtained from the Laboratory of Medicinal Chemistry (Ghent University). Dermorphin (> 95% purity determined by HPLC) was obtained from Bachem (Bubendorf, Switzerland) and bovine serum albumin (BSA) from Merck KGaA (Darmstadt, Germany); these compounds were used as positive and negative controls during BBB studies, respectively.

In vitro permeation study in Caco-2 cell monolayers

Cell culture

The Caco-2 cell line originated from a human colorectal carcinoma and was maintained in Dulbecco's modified Eagle's medium (DMEM) (95% humidity, 37°C, 5% CO_2), supplemented with 10% (V/V) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids (all from Invitrogen/GIBCO, Ghent, Belgium).

Caco-2 cell permeability assay

The Caco-2 cell intestinal model was used to investigate the gut mucosa permeation of pellitorine. On each Transwell (Corning Costar, New York, USA) membrane insert filter (0.4 µm pore size, 12 mm filter diameter), the cells were seeded at a density of $2.6 \cdot 10^5$ cells/cm² cells and cultivated in the described supplemented DMEM. Every second day, the medium was changed. During a period of 21-29 days, the cells were grown and differentiated until monolayers were formed. The integrity of the monolayers was checked via the measurement of transepithelial electrical resistance (TEER) of the monolayers with a Millicell-ERS system (Millipore Corp., Bedford, MA, USA) before and after the transport experiments. The experiments were performed in duplicate for each dose solution. Transport experiments were carried out according to Hubatsch *et al.* (2007) in two directions, *i.e.* from the apical-to-basolateral (ab) direction and from the basolateral-to-apical (ba) direction in Hanks' Balanced Salt solution [18]. Two dose solutions of pellitorine (in *Anacyclus pyrethrum* extract) of 1 µg/ml in 0.5% EtOH (further indicated as DS1) and of 56 µg/ml in 0.5% of a mixture of vitamin E-

TPGS (33.3%), EtOH (11.1%), PEG 400 (33.3%) and PG (22.2%) (further indicated as DS2) dissolved in HBSS were tested.

Final apical volumes of 0.4 ml and basolateral volumes of 1.2 ml were used for 12-mm filter supports during the transport experiment. After 15, 30, 60, 90 and 120 min, samples were taken from the receiver compartment (basolateral to apical transport: 100 µl; apical to basolateral transport: 300 µl) and immediately replaced by fresh HBSS. After 120 min, a sample from the donor compartment was taken as well and a mass balance was constructed for pellitorine, which ranged between 103.6 and 132.4% total recovery. To confirm the validity of the test, atenolol (50 µM) and propranolol (20 µM) were used as the negative (low permeability) and positive (high permeability) control, respectively [19]. Pellitorine was quantified using an ultra high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS^2) method as described below.

The apparent permeability coefficient (P_{app} in cm/s) of pellitorine in DS2 was calculated using a sink condition equation, while the P_{app} of pellitorine in DS1 was calculated using a non-sink equation. From Fick's first law: $J = -D \times [dC(x)]/dx$ (J is the flux or transfer rate along the donor-to-receptor side, D is the diffusion coefficient, x is the distance from the donor compartment and C(x) is the concentration in the barrier at the coordinate x in the barrier), the following differential equation is derived: $dM_r(t)/dt = P_{app} \times A \times [C_d(t) - C_r(t)]$, in which P_{app} is the apparent permeability coefficient (a product of distribution coefficient with diffusion coefficient divided by the barrier thickness), M_r is the amount of substance in the receiver chamber, A is the cross-sectional area of the barrier, C_d is the donor concentration and C_r is the receiver concentration. This differential equation can be solved to calculate P_{app} using different initial conditions depending on sink or non-sink conditions. In case of sink conditions, the following equation was obtained from the amount of compound transported per time $dM_r(t)/dt$ with the receiver concentration set to zero [18, 20]:

$$\frac{M_r(t)}{C_{d,0}} = P_{app} \times A \times t + c$$

where P_{app} is the apparent permeability coefficient (cm/s), A is the surface area of the filter (1.12 cm^2), $C_{d,0}$ is the initial concentration in the donor chamber (µg/ml), t is the time (s) and c is a constant term which vanishes, since M_r is initially zero at t = 0.

The donor concentration at each time point was calculated by taking into account the amount of compound which already permeated through the cell monolayer during that time interval. In this way, the reduction in donor concentration after every sampling was also taken into account.

The cumulative fraction transported FA_{cum} (cm) is defined as

$$FA_{cum} = \frac{1}{A} \times \sum_{k=1}^{i} \frac{[C_r(t_k) - f \times C_r(t_{k-1})] \times V_r}{[C_d(t_{k-1}) + C_d(t_k)]/2} = P_{app} \times t_i$$

in which V_r is the volume of the receiver compartment (ml), f is the sample replacement dilution factor (1-V_s/V_r with V_s being the sample volume), t_i (s) is the time at sampling i, t_k (s) is the time at sampling k, $C_r(t_k)$ (µg/ml) is the experimentally determined concentration of pellitorine in the receiver compartment at sampling k and $C_d(t_k)$ (µg/ml) is the experimentally determined concentration of pellitorine in the donor compartment at sampling k. C_d is considered as constant and is obtained by taking the average of C_d of the start and the end of the time interval. P_{app} was calculated from the slope of the cumulative fraction versus time curve using a linear fit.

In case of non-sink conditions, the differential equation resulted in the following solution [18, 20]:

$$C_r(t) = \left[\frac{M}{V_d + V_r}\right] + \left(C_{r,t-1} \times f - \left[\frac{M}{V_d + V_r}\right]\right) \times e^{-P_{app} \times A \times (\frac{1}{V_d} + \frac{1}{V_r}) \times \Delta t}$$

in which M (µg) is the total amount of pellitorine in the system at time t, V_d (ml) is the volume of the donor compartment, $C_{r,t-1}$ (µg/ml) the concentration of pellitorine in the receiver compartment at the previous time point, t the time (s), Δt (s) is the time at time t minus the previous time point and $C_r(t)$ (µg/ml) the concentration of pellitorine in the receiver compartment at time t. Minimisation of the sum of squared residuals (SSR) was used on the non-linear curve fitting to obtain the P_{app}. The uptake and efflux ratio are calculated as $P_{app,ab}/P_{app,ba}$ and $P_{app,ba}/P_{app,ab}$, respectively.

In vivo pharmacokinetic experiment with rats

Animals

Male and female Wistar rats weighing approximately 220 g were obtained from Bharat Serum and Vaccines pvt. Ltd, Thane, India (registration number 103/99/CPCSEA) and were housed at C. U. Shah College of Pharmacy, SNDT Women's University, Santacruz, Mumbai, India (registration number 39/99/CPCSEA) where the experiments were carried out. Rats of 7-8 weeks old were fasted overnight and used for the experiments.

Pharmacokinetic experiment

A pellitorine dose solution was prepared using the *Anacyclus pyrethrum* extract. To the rats, 1.5 ml of a 0.73 mg pellitorine/g dose solution in 10:20:30:40 (w/w/w/w) EtOH:PG:Vit E-TPGS:PEG 400 was administered using a gavage needle (stainless steel, length 3 inches and 2.5 mm internal diameter). As blank, a solution containing EtOH, PG, Vit E-TPGS and PEG without pellitorine was used. Each dose solution was orally administered to three female and three male rats. 1.5 ml blood was collected

from the retro orbital vein at 1, 2, 3, 4, 6, and 8h after administration of the dose solution and was centrifuged for 20 min at room temperature. Thereafter, the serum samples were immediately frozen at -80°C until bio-analysis. The animals were sacrificed at the end of the experiment by CO_2 inhalation. Using GraphPad software (La Jolla, USA), the elimination rate constant k_e (h^{-1}) is calculated using the following equation of a one compartmental model: $C(t) = C_0 \times e^{-k_e \times t}$, in which C(t) (ng/ml) is the concentration of pellitorine at time t (h) and C_0 the concentration of pellitorine at time t=0 (ng/ml). The elimination half-life $t_{1/2,e}$ (h) is calculated as $\ln(2)/k_e$.

In vivo blood-brain barrier experiment with mice

Animals

Seven to ten weeks old female, Institute for Cancer Research, Caesarean Derived-1 (ICR-CD-1) mice (Harlan Laboratories, Venray, Netherlands) weighing 29-32 g, were used for the BBB transport experiments. The experiments were performed according to the Ethical Committee principles of laboratory animal welfare as approved by Ghent University (Faculty of Veterinary Medicine, no. EC2014/128).

Blood-to-brain transport

An *in vivo* multiple time regression (MTR) analysis was performed in order to determine whether pellitorine could enter the brain. A dose solution of 1.8 mg/ml pure pellitorine dissolved in 6.9% EtOH, 2.5% dimethylacetamide, 0.5% tween 80 (all w/w) diluted in lactated Ringer's solution containing 1% BSA was prepared. After anesthetizing the mice intraperitoneally with a 40% (w/V) urethane solution (3 g/kg), the jugular internalis vein and carotid artery were isolated and 20 µl of the pellitorine dose solution was injected into the jugular vein. After 1, 3, 5, 10, 12.5 and 15 min post injection (with start and end in duplicate), blood was collected from the carotid artery. Immediately thereafter, the mice were decapitated and the brains were isolated. Serum was obtained by centrifuging the collected blood at 10 000 *g* for 15 min at 21°C. As negative and positive control, I^{125}-BSA and dermorphin were used, respectively, to assure the validity of the experiment [21-23]. The serum concentrations of pellitorine were plotted against the time (expressed in min). The curve was fitted using a first order kinetic, two-compartmental model: $C(t) = C_1 \times e^{-\alpha \times t} + C_2 \times e^{-\beta \times t}$, in which C is the concentration of pellitorine in serum at time t, α and β are hybrid constants, dependent on the rate constants k_e (elimination rate constant), $k_{1,2}$ (rate constant between central and peripheral tissue compartment), $k_{2,1}$ (rate constant between peripheral tissue and central compartment), C_1 is the concentration obtained after extrapolation of the distribution phase α to the Y axis and C_2 is the concentration obtained after extrapolation of the elimination phase β to the Y

axis. The ratio of the pellitorine brain and serum concentration (µl/g) was plotted versus the exposure time (θ), which is a derived time variable of the Gjedde-Patlak plot, to determine the BBB influx kinetics of pellitorine [24, 25].

The exposure time is computed as $\theta = \int_0^T \frac{C_s(t)\cdot dt}{C_s(T)}$ and is defined as the integral of the concentration of pellitorine in serum from start (t=0 min) to time T, divided by the concentration of pellitorine in serum at time T. The integral of the concentration of pellitorine in serum from zero to time T is the area under the curve until time T.

The brain uptake of pellitorine was fitted using a biphasic model, as elaborated by Wong *et al.* (1986) [26]:

$$\frac{C_{brain}(T)}{C_s(T)} = K\cdot\theta + V_g\cdot\left(1 - e^{\left(-\theta\cdot\left(\frac{K_1 - K}{V_g}\right)\right)}\right) + V_0 \quad \overset{K=0}{\cong} \quad V_g\cdot\left(1 - e^{\left(-\theta\cdot\left(\frac{K_1}{V_g}\right)\right)}\right) + V_0$$

in which $C_{brain}(T)$ is the concentration of pellitorine in the brain at time T (ng/g), $C_s(T)$ the concentration of pellitorine in serum at time T (ng/µl), K the net clearance (µl/(g·min)), V_g the tissue brain distribution volume (µl/g), K_1 is the unidirectional clearance from pellitorine from blood to brain (*i.e.* the unidirectional brain transfer coefficient, also referred to as influx rate) (µl/(g·min)) and V_0 the vascular brain distribution volume (µl/g). The vascular distribution volume of the negative control BSA (14.8 µl/g) was used as V_0 to calculate the brain kinetic parameters of pellitorine.

Capillary depletion

A capillary depletion experiment was performed to distinguish the transport of pellitorine into the brain, represented by the parenchyma, and part of pellitorine which is trapped by the endothelial cells of the brain, represented by the capillaries. A method of Triguero *et al*. (1990) was used, which was modified by Gutierrez *et al* (1993) [27-29]. The mice were anesthetized intraperitoneally with 40% (w/V) urethane solution (3 g/kg). Thereafter, 20 µl of the 1.8 mg/ml pellitorine dose solution was injected into the jugular vein. Ten min after injection, blood was collected from the abdominal aorta and serum was obtained by centrifuging the blood at 10 000 *g* during 15 min at 21°C. Then, after removing the skin of the mice's chest, the aorta is clamped, the jugular veins are severed and the brain is immediately perfused manually with 20 ml of Lactated Ringer's solution. The mice are decapitated, the brains are isolated, weighed and put into (LoBind Eppendorf) tubes, to which 525 µl ice-cold capillary buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 and 10 mM D-glucose adjusted to pH 7.4) was added. The (LoBind Eppendorf) tubes were homogenized. Next, 1000 µl of 26% ice-cold dextrane solution in capillary buffer was added, vortexed and the tubes were subsequently centrifuged at 20 000 *g* for 60 min at 4°C. Parenchyma and fat tissue (supernatant) were transferred into separate (LoBind Eppendorf) tubes and weighed. The

capillaries (pellet) were kept in the original tube. The sample preparation of the pellet and the supernatant is the same sample preparation method as used for the mice brains and mice serum, respectively, as further described in the 'bio-analytics' section.

The distribution in the brains was calculated as follows:

$$\text{Fraction (\%)} = \frac{\text{M}_{\text{tissue}}}{\text{M}_{\text{capillaries}} + \text{M}_{\text{parenchyma}}} \times 100$$

in which M_{tissue} is the amount of pellitorine in the capillaries and parenchyma; $M_{capillaries}$ the amount of pellitorine in the capillaries and $M_{parenchyma}$ the amount of pellitorine in the parenchyma. The capillary depletion experiment was performed in duplicate and mean values are reported.

Brain-to-blood transport

To evaluate the efflux of pellitorine out of the brain into the blood, a previously described *in vivo* method was used [28]. Briefly, the ICR-CD-1 mice were anesthetized with 40% (w/V) urethane solution (3 g/kg). Then, the skin of the skull was removed and a hole was made into the lateral ventricle using a 22 G needle marked with tape at 2 mm at the following coordinates: 1 mm lateral and 0.34 mm posterior to the bregma. Using a syringe pump (KDS100, KR analytical, Cheshire, UK), 1 µl of the 1.8 mg/ml pellitorine dose solution as used for the blood-to-brain influx experiment was injected intracerebroventricularly (ICV) at a speed of 360 µl/h for 10 s. The mice were decapitated after 1, 3, 5, 10, 12.5 and 15 min post-injection. Just before decapitation, blood was collected from the abdominal aorta and serum was obtained by centrifuging the blood at 10 000 *g* during 15 min at 21°C. Thereafter, brains were collected. The natural logarithm of the pellitorine concentration in brain (ng/g) was plotted versus time. The efflux rate constant k_{out} (min^{-1}) is obtained from the negative value of the slope of the linear regression, applying first order kinetics. From the k_{out}, the efflux brain half-life ($t_{1/2,brain}$) (min) is calculated as follows: $t_{1/2,brain} = \ln(2)/k_{out}$.

In vitro metabolic stability of pellitorine

The *in vitro* metabolic stability of pellitorine was evaluated in mouse brain and liver homogenates and mouse serum according to previously described protocols [22, 30]. Using the Pierce Modified Lowry Protein Assay method (Thermo Scientific), the protein content of each tissue homogenate was determined to prepare a stock solution containing a 0.6 mg/ml protein concentration in Krebs-Henseleit buffer (pH 7.4). Briefly, 75 µl of a 0.25 mg/ml pellitorine solution dissolved in 2% (V/V) EtOH in Krebs-Henseleit buffer pH 7.4 containing 3% (m/V) BSA was incubated in 375 µl of serum/organ homogenate and 300 µl of Krebs-Henseleit buffer pH 7.4 while shaking at 750 rpm at 37°C. After 0, 5, 7.5, 15, 60 and 120 min, aliquots of 100 µl were taken and transferred into 0.5 ml (LoBind Eppendorf) tubes containing 100 µl of 1% (V/V) TFA in water. Next, the samples were heated

at 95°C for 5 min and subsequently cooled for 30 min in ice. Thereafter, the samples were centrifuged (20 000 *g* for 30 min at 5°C) and 25 µl of the clear supernatant was injected and analysed using a HPLC-UV/MS method of 46 min which was previously described [15]. The HPLC–MS analysis was done on a HPLC system which consisted of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a Spectra System P1000XR pump, a Spectra System AS3000 autosampler, and a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA) equipped with a Waters 2487 Dual Absorbance detector and Xcalibur 2.0 software (Thermo) for data acquisition. Control solutions were also prepared but without pellitorine. Furthermore, control reference solutions were prepared as well with a prior heat inactivation or without tissue homogenate.

Bio-analytics

Serum sample preparation

To 60 µl of 4% (V/V) aqueous H_3PO_4 solution and 30 µl of the IS solution, 60 µl of the serum samples was added into a 0.5 ml (LoBind Eppendorf) tube and vortexed. By means of solid phase extraction using a positive pressure-96 processor (Waters, Zellik, Belgium), interfering compounds were removed. The sorbent in each well of the HLB Oasis® µelution 96 well plate (Waters, Zellik, Belgium) was preconditioned with 200 µl of MeOH and equilibrated using 200 µl of ultrapure water. Thereafter, 100 µl of the previous serum sample solution was loaded, followed by two washing steps using 200 µl of 5% MeOH in H_2O and 200 µl of 20% MeOH in H_2O, respectively. Two times 25 µl ACN was used to elute pellitorine. 25 µl of a 80:20 (V/V) H_2O:MeOH solution was added to the eluate and analysed with a UHPLC-MS^2 method, as described further in the section 'UHPLC-MS^2 method'.

The bio-analytical method for the quantification of pellitorine in serum was limited validated using rat serum, based upon the European Medicines Agency (EMA) guideline on bio-analytical method validation (EMEA/CHMP/EWP/192217/2009), with limited replicates and concentration levels [31]. The limit of detection (LoD) (S/N=3) and limit of quantification (LoQ) (S/N=10) of pellitorine, determined on the reference standard, were calculated as 0.19 ng/ml and 0.62 ng/ml, respectively, which correspond to 0.36 ng/ml and 1.16 ng/ml in serum, respectively. A matrix factor of 0.97 was observed for pellitorine in serum. The quantification of pellitorine in the mouse serum samples was performed using a calibration curve with pellitorine standards in 40:33.33:26.67 (V/V/V) MeOH:ACN:H_2O. The quantification of pellitorine in the rat serum samples was performed using a pre-extracted spiked matrix calibration curve. Linearity was assured in a working range from 0.62 ng/ml up to 101 ng/ml, corresponding to 1.16 ng/ml to 189 ng/ml in serum (R^2=0.9997).

The accuracy of the references used for the calibration curves with pellitorine standards in 40:33.33:26.67 (V/V/V) MeOH:ACN:H_2O, the pre-extracted spiked matrix samples and the quality control (QC) samples conformed to the specifications, *i.e.* < 15% of the nominal value and for the lower limit of quantification (LLoQ) < 20% of the nominal value. Precision was expressed as the coefficient of variation (CV). The within-run CV value did not exceed 15% for the pre-extracted spiked matrix samples. Also the CV for the LLoQ sample did not exceed 20% and are thus all conform to the specification limits. The recovery in serum was 90.65% (18.8 ng/ml to 189 ng/ml in serum concentration range), calculated from the slopes of the pre-extracted and post-extracted spiked matrix samples. No significant carry-over was observed (< 20% of LLoQ pellitorine and < 5% for IS). The selectivity was conform to the specifications (placebo serum < 20% of LLoQ pellitorine and < 5% for IS).

Brain sample preparation

The weighed mice brains were transferred into test tubes, 1.0 ml of the IS solution in ACN was added and the brains were crushed. The brains were shaken for 4 h at 110 rpm at room temperature using an Eppendorf centrifuge 5810 (Eppendorf, Rotselaar, Belgium). Thereafter, the test tube was centrifuged at 250 *g* for 5 min and 800 µl of the supernatant was transferred into other (LoBind Eppendorf) tubes. Then, these test tubes were centrifuged again at 20 000 *g* during 5 min at room temperature and 750 µl of the supernatant was evaporated to dryness under nitrogen. Pellitorine was redissolved in 175 µl 90:10 (V/V) H_2O:ACN and 160 µl of this solution was loaded on the HLB Oasis® µelution 96 well plate. The same washing and elution steps were applied as for the serum samples.

Again, a limited validation of the bio-analytical method for the quantification of pellitorine in brains was performed. The LoD and LoQ of pellitorine were 41.2 pg/g and 135 pg/g in brain, respectively. A matrix factor of 0.98 was observed for pellitorine in mice brains. Quantification of pellitorine in the brain samples was performed using the pre-extracted spiked matrix calibration curve (R^2=0.9982). Linearity was demonstrated in a working range of 0.69 ng/ml to 100 ng/ml pellitorine, corresponding to 135 pg/g to 19.6 ng/g in brain. The accuracy of the references used for the calibration curves and the QC samples with the pre-extracted spiked matrix samples conformed to the specification limits (< 15% of the nominal value; LLoQ < 20% of the nominal value), except for one QC reference pre-extracted spiked matrix sample (17% of the nominal value instead of < 15%), but was still acceptable for our purposes. The within-run CV value did not exceed 15% for the pre-extracted spiked samples. Also the LLoQ did not exceed 20% and are thus all conform to the limits. The recovery in mouse brains was 115.5% (0.98 to 19.6 ng/g brain concentration range).

UHPLC-MS² method

A UHPLC-MS² method using an Acquity UPLC RP C18 column (50 x 2.1 mm, 1.7 µm, Waters, Zellik, Belgium) with a suitable guard column was used for the quantification of pellitorine, developed on an Acquity UPLC chromatograph coupled to a Xevo™ TQ-S mass spectrometer (MS) (Waters, Zellik, Belgium) equipped with a triple quadrupole mass analyser and an electrospray ionisation (ESI) source. The column temperature was kept at 30°C, while the sample compartment remained constant at 5°C. The injection volume was 2 µl and the flow rate was set to 0.5 ml/min. A mobile phase was applied consisting of solvent A (0.1% FA in 30:70 (V/V) H_2O:MeOH) and solvent B (0.1% FA in MeOH) in gradient mode as follows: 0-1.6 min 100:0 (V/V) A:B, 1.6-2 min going from 100:0 (V/V) A:B to 0:100 (V/V) A:B, 2-3 min 0:100 (V/V) A:B, 3-3.4 min going from 0:100 (V/V) A:B to 100:0 (V/V) A:B, 3.4-5 min 100:0 (V/V) A:B. A needle wash solvent of 60:40 (V/V) DMSO:ACN was used. The MS operated in ESI^+ mode with an optimised cone voltage of 50 V, a capillary voltage of 3.0 kV and a source offset of 60 V. Cone and desolvation gas (N_2) flows were 180 and 1000 l/h, respectively, while desolvation and source temperatures were set at 500°C and 150°C and, respectively. Acquisition was performed in the multiple reaction monitoring (MRM) mode with *m/z* 224.11 to *m/z* 80.84 transition. The collision gas was argon and the applied collision energy was 26 eV. By means of MassLynx® software (V4.1 SCN 843, Waters, Zellik, Belgium), data were acquired and analysed.

3. RESULTS

Caco-2 cell permeability

Our results demonstrated that pellitorine was able to permeate the Caco-2 cell monolayer in both directions, *i.e.* from the apical-to-basolateral side, as well as from the basolateral-to-apical side. The percentages of pellitorine from the applied dose solutions (DS1 and DS2) which permeated through the Caco-2 cells in the course of time are shown in Figure 1. After 120 min, 56.7% (or 0.31 µg pellitorine) of the applied pellitorine with DS1 and 9.98% (or 2.24 µg pellitorine) of the applied pellitorine with DS2 permeated the Caco-2 cell monolayer in the apical-to-basolateral direction. In the basolateral-to-apical direction, the percentage of pellitorine which permeated through the Caco-2 cells after 120 min is 23.7% (or 0.39 µg pellitorine) with DS1 and 1.84% (or 1.24 µg pellitorine) with DS2. The $P_{app,ab}$ of the positive control propranolol resulted in $19.1 \cdot 10^{-6}$ cm/s and similar values are documented in literature [32]. The negative control atenolol showed a lower permeability compared to propranolol.

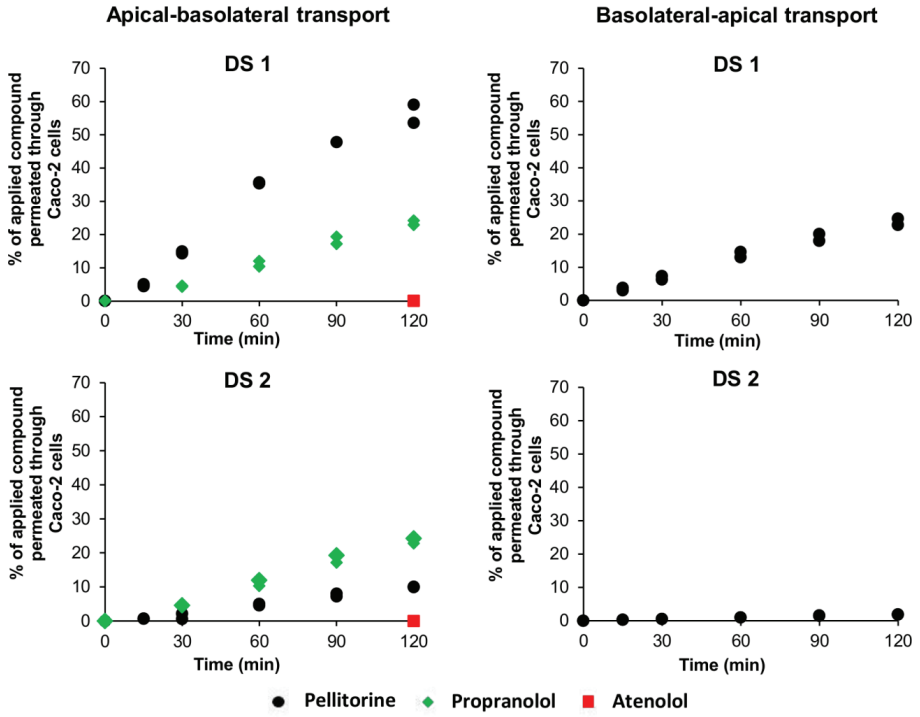

Figure 1. The percentages of pellitorine from the applied dose solutions using dose solution 1 (DS1) and dose solution 2 (DS2) which permeated through the Caco-2 cells monolayer in the course of time. Apical-to-basolateral transport and basolateral-to-apical transport experiments of pellitorine were performed in duplicate (individual results in the same figure).

Apparent permeability coefficients of pellitorine of 48.08 ± 0.08·10^{-6} cm/s (mean ± SD, n=2) and 5.50 ± 0.06·10^{-6} cm/s (mean ± SD, n=2) in case of DS1 and DS2, respectively, were calculated for the apical-to-basolateral transport. For the opposite direction (basolateral-to-apical), P_{app} values of pellitorine obtained with DS1 and DS2 are 57.49 ± 6.20·10^{-6} cm/s (mean ± SD, n=2) and 2.80 ± 0.09·10^{-6} cm/s (mean ± SD, n=2), respectively. Uptake ratios of 0.84 and 1.96 using DS1 respectively DS2 are obtained.

Oral gavage experiment

The pellitorine serum concentrations after oral gavage are plotted against time (Figure 2). Pellitorine was able to permeate the intestinal barrier *in vivo*.

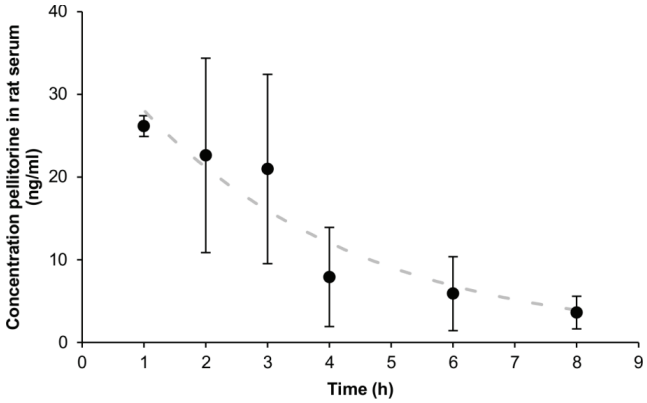

Figure 2. Concentration of pellitorine in rat serum (ng/ml) as a function of time (h) after oral gavage of pellitorine (5 mg pellitorine/kg body weight). Data are fitted according to a one compartmental model (n=2-3, mean, error bars: SEM).

The curve was fitted according to a one compartmental model. An elimination rate constant and half-life of 0.28 h^{-1} and 2.46 h were obtained.

Blood-brain barrier transport kinetics of pellitorine

Using the MTR method, pellitorine showed a significant influx into the mouse brain. In Figure 3, the ratio of the concentration of pellitorine in brain and serum is plotted versus the exposure time. The data were fitted using a biphasic model, based on the modified Gjedde-Patlak equation according to Wong *et al.*(1986) [26]. Pellitorine showed a very high and rapid influx into the brains and a K_1 of 153 µl/(g·min) was determined. Moreover, the tissue brain distribution volume (V_g) is 792 µl/g. After about 10 min exposure time, a plateau was observed, which is consistent with the efflux of pellitorine out of the brain.

Dermorphin (positive control) clearly showed an influx with a calculated unidirectional influx rate of 0.26 µl/(g·min). The negative control BSA gave a very small, almost negligible brain influx, with a K_1 of 0.12 µl/(g·min). Both controls thus indicated a good performance of the test and are in compliance with those previously reported [28, 33].

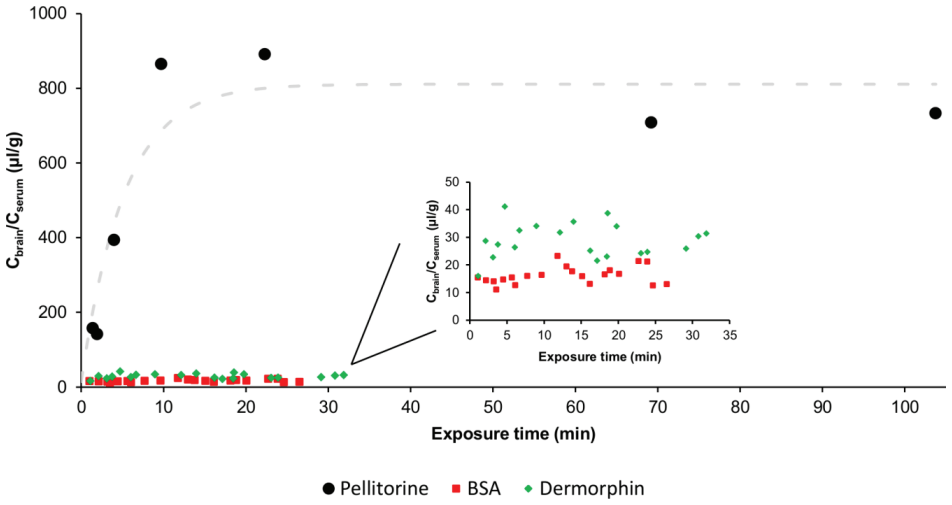

Figure 3. Blood-to-brain transport (multiple time regression experiment) results of pellitorine in mice. The ratio of pellitorine concentration in brain versus serum (µl serum/g brain) is plotted versus the exposure time (min). Start and end point were performed in duplicate. The data were fitted using a biphasic model.

The MTR data allowed us to evaluate the elimination kinetics of pellitorine in serum as well. The serum profile of pellitorine followed a two compartmental model. C_1 was 7.02 µg/ml and C_2 was 0.44 µg/ml. The distribution rate constant α was 1.56 min^{-1}, while the elimination rate constant β was 0.15 min^{-1} and an elimination half-life of 4.48 min was obtained.

Capillary depletion

The fraction of pellitorine that was taken up by the brain parenchyma and the fraction of pellitorine which was trapped in the endothelial cells of the brain capillaries was investigated. 97% of pellitorine (corresponding to 347.3 µl/g) transferred effectively into the brain parenchyma, versus a low percentage of pellitorine remaining in the brain capillaries: 3% (corresponding to 10.0 µl/g). The high absolute values in the brain parenchyma are consistent with the MTR results.

Brain-to-blood transport kinetics of pellitorine

A significant efflux of pellitorine out of the brain into the blood was observed after intracerebroventricular injection of the dose solution (Figure 4). The efflux behaviour can explain the rapid plateauing observed during the brain influx experiment. The efflux transfer constant k_{out} was calculated as 0.05 min^{-1}, equal to a $t_{1/2,brain}$ of 13.8 min.

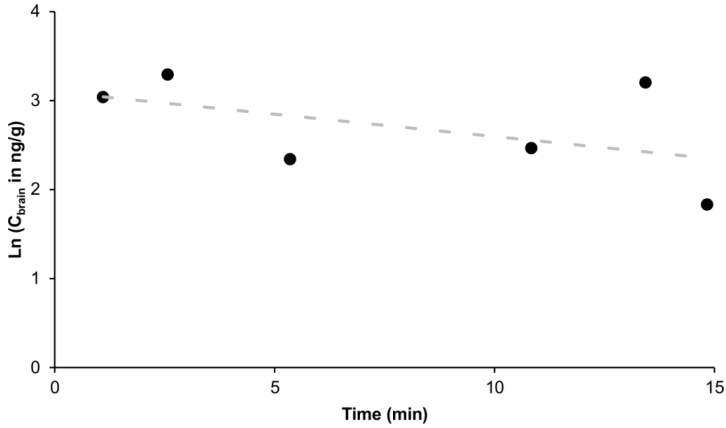

Figure 4. Brain-to-blood transport results of pellitorine in mice. The natural logarithm of the concentration of pellitorine in the brain (ng/g) in function of the time (min) is given.

In vitro metabolic stability of pellitorine

The stability of pellitorine was determined in mouse serum, mouse brain homogenate and mouse liver homogenate. The results of this study indicate that pellitorine was stable during one hour in serum, in brain as well as in liver (91% - 105% recovery).

4. DISCUSSION

To characterize the intestinal absorption of pellitorine, the permeability of pellitorine through Caco-2 cells was investigated using two different dose solutions. The ethanol percentage in both dose solutions was less than 0.5%. The permeation level of pellitorine through Caco-2 cells was higher from the absorptive apical-to-basolateral side (10-57%), compared to the permeation from the basolateral-to-apical side (2-24%). Pellitorine, applied as DS1 better permeated the Caco-2 cells monolayer compared to pellitorine applied as DS2 in both directions, indicating that co-solvents (vitamin E-TPGS, PEG 400, PG) did not influence the permeation level through the cells in a positive way. However, the esterified vitamin E derivative, vitamin E-TPGS, was added to increase the solubility and facilitate the permeation of pellitorine [34, 35]. The P_{app} values obtained for pellitorine ($0.3 \cdot 10^{-5}$ to $5.8 \cdot 10^{-5}$ cm/s) were all above $1 \cdot 10^{-6}$ cm/s, suggesting an almost complete intestinal absorption and are similar to values reported by Matthias *et al.* (2004) of the mono- and diene-NAAs from *Echinacea* plant species [36]. Given the physicochemical properties of pellitorine (logP=3.65, MW=223.36 g/mol), transcellular passive diffusion seems favourable for pellitorine [37]. This was confirmed in our study, as for both dose solutions containing pellitorine, the ratio of P_{app} values between the apical-to-basolateral and the basolateral-to-apical direction or vice versa was ≤ 2,

indicating that no active transport was involved [38]. As the efflux ratios were not greater than two, there is no efflux of pellitorine. Next to this *in vitro* evaluation of the intestinal barrier properties of pellitorine, a confirmatory *in vivo* oral gavage experiment in rats was performed. A liquid dose solution of pellitorine also containing EtOH, PG, Vit E-TPGS and PEG 400, was orally administered to rats. Pellitorine diffused through the gut barrier and was observable in rat serum. These results confirm the outcome of the initial *in vitro* Caco-2 cell permeability study. The elimination half-life of pellitorine was 2.46 h, which is in the same range as the elimination half-life of 1.20 h, obtained by Woelkart *et al.* (2009), who investigated the plasma concentration of dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamides from *Echinacea* via oral gavage in rats [39].

Once in the blood, pellitorine can distribute to extravascular compartments in order to exert a biological function. In the present study, the blood-brain barrier transport characteristics of pellitorine were explored in an *in vivo* mice experiment. Pellitorine was intravenously injected to the mice in a solution containing EtOH and dimethylacetamide, which are both frequently used as co-solvents in injectable pharmaceutical formulations; and tween 80, which is a traditional surfactant, and diluted in lactated Ringer's solution containing BSA [40, 41]. In our study, no toxicity was observed when performing the experiment and it is likely that the solvents did not have an influence on the BBB integrity, as lower doses are used than doses reported in literature which can cause blood-brain barrier disruption. A dose of tween 80 up to 30 mg/kg and 1-4 g/kg ethanol can disrupt the BBB, while in this study only 3 mg/kg tween 80 and 0.04 g EtOH/kg body weight were administered [42]. In the current study, a high initial unidirectional influx rate of 153 µl/(g·min) was observed, indicating that pellitorine rapidly penetrated the BBB after administration, which is consistent with the lipophilicity of pellitorine. In addition, pellitorine was distributed in the brains as follows: 97% in the parenchyma and only 3% in the capillaries, making it plausible to exert CNS effects. Badhe *et al.* (2010) already showed antidepressant activity of a hydroalcoholic *Anacyclus pyrethrum* root extract (50, 100, 200 mg/kg, p.o.) in albino mice [43]. Another study demonstrated the anticonvulsant and myorelaxation activity of an ethanolic AP root extract in albino mice. A dose dependent effect was observed against maximum electroshock test and rotarod test after intraperitoneal administration of the extract in doses of 200, 400 and 600 mg/kg [44]. Sujith *et al.* (2011) evaluated neuropharmacological activity of an ethanolic AP root extract (50, 100, 200 mg/kg, p.o.) in albino Wistar rats. Nootropic activity was observed in rats in a dose dependent way using the elevated plus maze (EPM) test. Furthermore, the extract also showed antidepressant activity in a forced swim test [14]. Another study showed the anticonvulsant activity against pentylenetetrazole (PTZ) of a hydroalcoholic root extract of AP after oral administration of 50, 100, 250 and 500 mg/kg to Wistar rats. The observed effect was dose dependent. In addition, protection against MES induced

seizures was observed in a dose dependent way after administration of the extract in doses of 250, 500 and 1000 mg/kg. Furthermore, the extract prevented seizure induced oxidative stress and showed protective effects against cognitive impairment in rats in a dose dependent manner [45]. Sujith *et al*. (2012) showed a dose dependent cognitive improvement of an ethanolic root extract of AP (50, 100, 200 mg/kg) after oral administration to albino Wistar rats in an EPM test and passive avoidance paradigms. Moreover, the extract enhanced short-term social memory in a social recognition task [46]. Zaidi *et al.* (2013) showed anticonvulsant activity against PTZ in a dose dependent manner in mice after administration of a chloroform AP root extract in a concentration of 100, 200, 400 and 800 mg/kg. Also against bicuculline, the 800 mg/kg AP extract showed anticonvulsant activity. Furthermore, anxiolytic behaviour was also observed in an EPM model after administration of 800 mg/kg AP extract. Administration of 1600 mg/kg AP extract showed impaired motor coordination in a rotarod test [47]. Another study showed that pretreatment of albino mice with 100, 250 and 500 mg/kg (p.o.) hydroalcoholic root AP extract increased myoclonic jerk latency and delay in the development of kindling. Protection against memory deficit was observed after pretreatment of the AP extract in doses of 100, 250, 500 mg/kg (p.o.) in PTZ kindled mice by decreasing oxidative stress and ROCK II expression [48]. Other research demonstrated the anticonvulsant activity of an ethanolic root extract of *Anacyclus pyrethrum* (200, 400 mg/kg, p.o.) in a MES model in albino mice. 400 mg/kg AP extract was protective against PTZ model [13].

In the previously described effect-studies, the AP extract was administered to rats or mice in doses ranging between 50 and 1600 mg extract/kg body weight. Assuming that the concentration of pellitorine is 1.55% w/w (determined on the ethanolic AP extract used in this study), the pellitorine dose ranges between 0.8-24 mg/kg. Since our study showed a maximum serum concentration of 26 ng/ml pellitorine after oral administration of 5 mg pellitorine/kg to rats (Figure 2), and assuming pellitorine follows a linear kinetic, the maximum serum concentration of pellitorine in the previously described effect-studies is between 4.0 and 129.0 ng/ml. Our BBB data (Figure 3) showed a brain/serum concentration ratio of 792 µl/g at equilibrium, so that the brain concentrations of pellitorine in the previous effect-studies correspond to 3.2 to 102.1 ng pellitorine/g brain. Looking at compounds used for the treatment of CNS diseases (*e.g.* multiple sclerosis, experimental autoimmune encephalomyelitis, epilepsy), administered at efficacious doses, drug concentrations in the brain ranging between 15 and 620 ng/g brain were found [49-51]. Hence, our BBB study corroborates well with the previously described effect studies using the AP extract and the brain concentrations of other CNS-active drugs.

5. CONCLUSION

In the current study, the intestinal barrier properties of pellitorine were investigated *in vitro* and *in vivo*. It was shown that pellitorine was able to cross the Caco-2 cell monolayer and the rat gut after oral administration. Furthermore, it was demonstrated that after intravenously administration of pellitorine to mice, pellitorine significantly and rapidly penetrated the BBB reaching the brain parenchyma. The biphasic course of the influx can partly be explained by an observed significant efflux of pellitorine out of the brain. These pharmacokinetic findings support the observed activities using the *Anacyclus pyrethrum* extract in the treatment of CNS diseases.

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CHAPTER VII

REGULATORY STATUS OF *N*-ALKYLAMIDE CONTAINING HEALTH PRODUCTS

"You can avoid reality, but you cannot avoid the consequences of avoiding reality."

Ayn Rand
(°1905-†1982, American novelist, philosopher)

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ABSTRACT

N-alkylamides (NAAs) are secondary metabolites occurring in more than 25 plant families. Plants containing NAAs are traditionally used in food for flavouring, tingling, pungent and saliva-enhancing properties but also to treat various diseases. NAA containing products are abundantly available on the market as food, cosmetics, medical devices and medicinal products. However, no unambiguous legal product classification is applied for these products. In this study, the different health product classes from a European viewpoint are discussed in relation to the pharmacokinetic and pharmacodynamic properties of the NAAs, their applied dosage and claimed usage.

CHAPTER VII

REGULATORY STATUS OF *N*-ALKYLAMIDE CONTAINING HEALTH PRODUCTS

Main focus in this chapter:

- To give an overview of the classification of health products containing plant(extract)s.
- To discuss the regulatory status of health products containing *N*-alkylamides on the market.

1. *N*-ALKYLAMIDES

Introduction

N-alkylamides (NAAs) are an important group of secondary metabolites in plants, attracting attention because of their numerous reported bioactivities. NAAs, available in more than 25 plant families (*e.g.* Asteraceae, Piperaceae, Lauraceae, Solanaceae), possess *i.a*. antimicrobial, insecticidal, antifungal, anti-inflammatory, immune-modulating and analgesic effects. Well-known, traditionally and medically important plants containing NAAs are *Anacyclus pyrethrum*, *Spilanthes acmella*, *Achillea millefolium* and *Echinacea* species, all belonging to the Asteraceae family. Furthermore, *Capsicum annuum* (Solanaceae) and *Piper* species (Piperaceae) are also plants rich in NAAs.

NAAs consist of a fatty acid chain which is linked to an amide part through a peptide bond. Because of the structural diversity in these two parts, they can be classified based on the combination of the fatty acid chain (F, from 1 to 13) and amide part (M, from 1 to 13). More than 400 NAAs are already discovered and their chemical name, occurrence, physicochemical properties and reported functionalities are gathered in the online database Alkamid® [1].

NAAs are mainly found in plants and their related plant extracts, purified to different extent, or chemically synthesised. Examples of synthesised molecules are the geometric isomers of the plant NAA *N*-(2-methylpropyl)-2,6,8-decatrienamide ('synthetic spilanthol') and the mammalian palmitoylethanolamide (PEA) [2]. PEA is a mammalian signaling molecule originating from *N*-palmitoylethanolamine-phospholipids in the lipid bilayer [3].

In most countries, NAA containing plants have not only been used in food, but also for therapeutic purposes. Ethnopharmacological studies investigate these plant medicines in particular ethnic

populations. This is generally grouped in the term 'traditional medicine' which is defined by the World Health Organisation (WHO) as "the total of knowledge, skills, and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness" [4]. Sometimes, traditional medicines are considered synonyms for complementary alternative medicines (CAM). Well-known traditional medicines include the Ayurveda and traditional Chinese medicine (TCM), both encompassing NAA containing plants.
Products containing NAAs are widely available on the market as *i.a.* food supplements, medicinal products, cosmetics and medical devices. Using the European regulatory-legal frame, the legal status of these NAA containing health products will be discussed.

Traditional use of plants containing *N*-alkylamides

Food

Plants are traditionally used in food, as spices and for their fragrance properties. For example, the fruits of *Piper nigrum* L. (black pepper) are well-known used for their flavouring properties [5]. Moreover, plants containing NAAs often give additional tingling and pungent sensations and/or increased salivation [6, 7]. Due to these remarkable and directly observable characteristics, NAA containing plants and extracts are globally used as tonic, to stimulate the appetite, or for its local mouth-cooling properties. In Iran, fruits of *Piper longum* L., *Piper nigrum* L. and *Capsicum annuum* L. are traditionally used as tonic, while in Cameroon, the fruits of *Capsicum annuum* are traditionally added to food [5, 8]. In Japan, Serbia, and Iran, *Spilanthes acmella* L., *Achillea millefolium* L. and the fruits of *Piper nigrum* L., respectively, have been used to stimulate the appetite [5, 9, 10].

The possibilities of this particular use are also reflected in patents, demonstrating the industrial-commercial interest (patents and patent applications: Ley *et al.* (US 2007/0202188), Hatasa *et al.* (US 3,720,762), Lombardo *et al.* (US 8,741,958), Langer *et al.* (US 8,828,469), Gatfield *et al.* (US 8,063,107), Kenichi *et al.* (WO/2016/088638)) [11-16]. Several use-patents related to *N*-alkylamides (alkene carboxylic acid *N*-alkylamides, spilanthol and isomers, trans-pellitorine) exist, covering the aromatic and flavouring properties (piquant, tingling, pungent, saltiness enhancing properties). In the patent claims, these NAAs are used in food and nutrition (*e.g.* bread, cereal products, chocolate, meat products, alcoholic and non-alcoholic drinks, eggs, salad dressings) and also to mask the unpleasant flavour of other components/products. Furthermore, applications are given in products for oral hygiene (*e.g.* tooth paste/gel, tooth powder, mouth washes) for their cooling and local anesthetic effect and their relief of pain in case of toothache. Next to these claimed properties, they

are also added to food for their saliva stimulating effects which in turn stimulates the appetite. Another purpose of these saliva-enhancing components is to improve oral hygiene for the treatment of a dry mouth and their usages are described in products like breath fresheners, chewing gum, mentholated cigarettes, food, cough drops, therapeutic compositions, pharmaceuticals and nutraceuticals. Use protection in these patents is also described for their heat-generated effects on the mucosa and skin. Some patents also include a synthesis process to produce these *N*-alkylamides.

Ethnopharmacological use

A selection of plant examples of the Solanaceae (genus *Capsicum*), Piperaceae (genus *Piper*) and Asteraceae (genus *Spilanthes*, *Anacyclus*, *Echineacea*, *Achillea*) plant families, frequently used as traditional medicine, exemplifies the traditional healing properties (Tables 1-4). The treatment of toothache is a common ethnopharmacological indication of *Spilanthes acmella*, *Anacyclus pyrethrum*, *Echinacea purpurea* and *Achillea millefolium* [17-23].

Fruits of *Capsicum annuum* L. (common name: chilli pepper) are used as a stimulant, aphrodisiac, to treat and prevent malaria, to treat osteoarthritis and female infertility [5, 24-26]. Another plant frequently used is *Achillea millefolium* L., known to treat toothache, stomach disorders, menstrual complaints, cough and cramps, fever, lung cancer, migraine, gynaecological disorders, rheumatism, cardiopathy, headache and dizziness [10, 22, 27]. *Piper longum* L. has been used to treat asthma and tuberculosis, to improve food digestion and hence to relieve flatulence, while the fruits of *Piper nigrum* L. are also used as a stimulant [5, 19].

Especially in India, *Spilanthes acmella* is traditionally applied in the Ayurveda system to treat toothache, oral lesions, gum inflammation, throat problems, cough, paralysis, epilepsy, leucorrhoea and a poisonous sting [18, 19, 28-33]. An overview of the plants, local names, the country where the survey was conducted, the used plant parts, the form and medicinal uses, is given in Table 3. Because of its frequent use in the treatment of toothache, the plant is also called the toothache plant [34].

Table 1: Ethnopharmacological use of *Piper longum*, *Piper nigrum*, *Capsicum annuum* and *Achillea millefolium*.

Country	Plant name	Local name	Family	Plant part	Form	Medicinal use	Reference
Iran	*Piper longum* L.	Darfelfel	Piperaceae	Fruits	n/a	Food digestion, carminative properties	[5]
India		Pipli		n/a	n/a	Asthma, tuberculosis	[19]
Iran	*Piper nigrum* L.	Felfel Siah		Fruits	n/a	Stimulant, appetizer	[5]
Iran	*Capsicum annuum* L.	Felfel Ghermez	Solanaceaee	Fruits	n/a	Stimulant, aphrodisiac, osteoarthritis	[5]
Zimbabwe		Mhiripiri		Fruits	n/a	Prevention of malaria	[24]
Nigeria		Atawewe		Fruits	n/a	Female infertility	[25]
Nigeria		Ata-jije		Fruits	Decoction	Malaria	[26]
India	*Achillea millefolium* L.	Bergeur		Fresh leaves	n/a	Mouth-freshener, toothache	[22]
Serbia		Hajducka trava, stolisnik, sporis	Asteraceae	n/a	Infusion	Loss of appetite, stomach disorders, menstrual complaints, cough and cramps	[10]
Pakistan		Ratti boti/Sultani Booti		Leaves	Decoction	Fever, colds, lung cancer, migraine, gynaecological disorders, rheumatism, cardiopathy, headache, dizziness and menstrual regulatory	[27]

n/a: not available

Table 2: Ethnopharmacological use of *Echinacea* (Asteracea family).

Country	Plant name	Plant part	Form	Medicinal use	Reference
Cameroon	*Echinacea purpurea*	Leaves and stem	Paste	Oral cancer, toothache, sinusitis	[17]
Serbia	*Echinacea angustifolia*	n/a	Tincture	Weakened immune system	[10]
Ethiopia	*Echinacea* spp.	Roots	Chopped and soaked in warm water	Diarrhea	[35]

n/a: not available

Table 3: Ethnopharmacological use of *Spilanthes acmella* (Asteracea family).

Country	Local plant name	Plant part	Form	Medicinal use	Reference
Bangladesh	Jhummosak	Whole plant	Paste	Poisonous sting	[28]
India	Akarkara	n/a	n/a	Cough	[29]
India	Akarkara	n/a	n/a	Throat problems	[32]
India	Manjal Poo Chedi	Flowers	Crushed	Toothache	[18]
India	Akarkara	Inflorescence	n/a	Gum inflammation	[30]
India	Akarkara	n/a	n/a	Paralysis, toothache, epilepsy	[19]
Bangladesh	Gha-gota gach	Leaves	Crushed	Toothache, oral lesions	[23]
Bangladesh	Vhadalika	Leaves, flowers	n/a	Leucorrhoea	[33]

n/a: not available

Table 4: Ethnopharmacological use of *Anacyclus pyrethrum* (Asteracea family).

Country	Local plant name	Plant part	Form	Medicinal use	Reference
Morocco	Tigendist	Roots	n/a	Chest pain, stomach problems, rheumatism	[36]
Morocco	Tiguenditz	n/a	n/a	Stomach problems, chest pain, rheumatism	[37]
Morocco	Tigendaste	n/a	n/a	Toothache, respiratory, urinary, genital diseases, anaesthetic, pesticide, astringent agent	[20]
Morocco	Tigentest	Roots, stems and sheets, flowers	n/a	Hair, diabetic, dermatological, bio-pesticide, aphrodisiac, blood anemia, rheumatism, neuralgia, tooth infection, sore throat	[38]
Morocco	Tigendast	Roots	Powder	Toothache, bad breath, aphrodisiac	[21]
Iran	Aghergherka	Roots	n/a	Stuttering	[5]
India	n/a	n/a	n/a	Together with leaf extract of Centilla asiatica seed powder of *Centrtherumant helminticum* L. and honey: unconsciousness	[39]
India	Akki rakaram	Roots	Powder	Dental problems	[40]

n/a: not available

Another member of the Asteracea family with strong ethnopharmacological properties is *Anacyclus pyrethrum* (Table 4). This plant is employed not only in the treatment of chest pain, stomach problems, dental problems (tooth infection, toothache), bad breath, rheumatism, blood anemia, neuralgia, sore throat, respiratory, urinary and genital diseases, hair, diabetic and dermatological disorders, but also as an anaesthetic, aphrodisiac, pesticide and astringent agent [5, 20, 21, 36-40]. Ethnopharmacological uses of *Echinacea*, also belonging to the Asteraceae family, are summarised in Table 2. Field surveys revealed that the roots of *Echinacea* spp. are used for diarrhea, while a tincture of *Echinacea angustifolia* DC. is used to strength a weakened immune system. Furthermore, the leaves and the stem of *Echinacea purpurea* are used in a paste to treat oral cancer, toothache and sinusitis [10, 17, 35].

Pharmacological effects of *N*-alkylamides

NAAs obtained from synthesis or plants after extraction and/or purification were studied towards their pharmacodynamic (PD) and pharmacokinetic (PK) properties. Due to their high/ubiquitous abundance in frequently used medicinal plants, the pharmacological properties of typical NAAs such as spilanthol, pellitorine, NAAs isolated from *Echinacea* species and synthetic derivatives like palmitoylethanolamide, are being explored in more depth.

Spilanthol is known to cause a very strong tingling and saliva stimulation effect [7]. It appears that mechanosensitive neurons are involved in this tingling effect by blocking two-pore potassium channels [41, 42]. Furthermore, spilanthol induced the GABA release in mice cortical brain slices, indicative for an analgesic activity [43]. Déciga-Campos *et al.* (2010) also studied the analgesic properties of spilanthol in pain models and dose-dependent analgesic effects were observed after intraperitoneal administration of spilanthol to capsaicin and acetic acid treated mice. Capsaicin activates transient receptor potential vanilloid 1 (TRPV1). There was a reduction of the number of abdominal writhes and the time of the mouse licking its injured paw, respectively [44]. In a formalin induced rat pain model, the analgesic effect of spilanthol was demonstrated. Spilanthol also showed anti-anxiety behaviour, anti-convulsant effects and hypnotic potention in mice [45]. Furthermore, spilanthol inhibited the nitric oxide (NO) production in a dose dependent manner (20% NO production at 360 μM spilanthol, compared to 100% of the control LPS) in murine macrophage-like RAW 264.7 cells. This anti-inflammatory effect was due to suppression of the production of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes at transcriptional and translational levels. In LPS-treated RAW 264.7 cells, spilanthol was able to reduce the production of the proinflammatory cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α).

Furthermore, it was shown that spilanthol inactivated nuclear factor-κB (NF-κB) and hence reduced the transcription of proinflammatory mediators [46]. Anti-inflammatory effects were also *in vivo* demonstrated by Hernandez *et al.* (2009) for spilanthol on arachidonic acid - and phorbol myristate acetate-induced oedema in mouse ears [47]. Furthermore, antimutagenic properties of spilanthol were shown by reducing oxidative DNA damage induced by norfloxacin and reducing mutations caused by 2-aminoanthracene [48].

Molina-Torres *et al.* (1994, 2004) demonstrated the fungistatic (100% growth inhibition relative to control against *Sclerotium rolfsii*, *Sclerotium cepivorum*, *Phytophthora infestans*, *Rhizoctonia solani* at 150 µg/ml) and bacteriostatic (100% growth inhibition relative to control against *Escherichia coli* at 75 µg/ml and *Bacillus subtilis* at 150 µg/ml and *Pseudomonas solanacearum* at 150 µg/ml and 95% growth inhibition against *Saccharomyces cerevisiae* at 75 µg/ml) activities of spilanthol [49, 50].

In addition, a 1:1 mixture of spilanthol and undeca-2E,7Z,9E-trienoic acid isobutylamide is also active against the larvae of the yellow fever mosquito *Aedes aegypti* (50% mortality with 6.25 µg/ml) and the neonates of the moth *Helicoverpa zea* (corn earworm) (66% weight reduction with 250 µg/ml after 6 days) [51]. Another study demonstrated that spilanthol had ovicidal, larvicidal and pupicidal activity against the mosquitos *Anopheles culicifacies*, *Culex quinquefaciatus* and *Aedes aegypti* [52]. Spelman *et al.* (2011) demonstrated antimalarial activity of spilanthol. IC_{50} values of 16.5 µg/ml and 5.8 µg/ml were obtained against *Plasmodium falciparum* strain PFB (compared to positive control chloroquine: IC_{50}=0.009 µg/ml) and the chloroquine resistant *P. falciparum* K1 strain (compared to positive control chloroquine: IC_{50}=0.032 ng/ml), respectively [53]. Furthermore, insecticidal activity of spilanthol against the moth *Tuta absoluta* was shown, with a LD_{50} value of 0.13 µg/mg compared to 0.71 µg/mg of the positive control permethrin [54].

Pharmacokinetically, spilanthol is able to permeate oral mucosa and human skin. In Chapter V, it was demonstrated that spilanthol crossed the intestinal barrier and via the blood circulation penetrated the blood-brain barrier. Hence, spilanthol is rapidly and extensively absorbed into the systemic circulation via different exposure routes, and distributed towards other organs including the relatively impermeable brain [55-57].

The saliva-enhancing properties of spilanthol were also observed with pellitorine [7]. Ku *et al.* (2013) demonstrated the antithrombotic properties of pellitorine, *i.e.* 10 µM pellitorine increased the activated partial thromboplastin time (aPTT) (41 s) compared to the negative control saline (31 s) in human plasma; with 10 µM pellitorine, the prothrombin time (PT) was higher (23 s) compared to saline (14 s) and, after administration of 4.5 µg pellitorine to mice, there was a higher tail bleeding time (58 s) compared to the control saline (42 s) [58]. Furthermore, pellitorine showed antiseptic properties. The release of high mobility group box 1 (HMGB1) was inhibited, which is a late mediator

of septic conditions in LPS-induced human umbilical vein endothelial cells (HUVECs) and cecal ligation and puncture (CLP)-treated mice. Furthermore, pellitorine down-regulated HMGB1-dependent inflammatory responses in HUVECs. Pellitorine was also able to inhibit LPS-induced barrier disruption of HUVECs and the expression of cell adhesion molecules (CAMs). In addition, the production of the proinflammatory cytokines TNF-α and IL-6 was reduced in LPS-treated HUVECs after incubation with pellitorine. Leukocytes migration was also inhibited by pellitorine in mice [59, 60].

In another study, carried out by Ee *et al.* (2010), anticancer properties of pellitorine were demonstrated. It was found that pellitorine had an IC_{50} value of 1.8 µg/ml against the MCF-7 cell line (breast cancer) and an IC_{50} of 13.0 µg/ml against HL60 cell-line (human promyelocytic leukemia cells), meaning that pellitorine is toxic against these cell lines [61]. At a concentration of 100 µM, trans-pellitorine was also able to reduce fatty acid uptake for 14% in Caco-2 cells, compared to non-treated control cells [62]. Furthermore, pellitorine is shown to be an inhibitor of acyl-CoA: cholesterol acyltransferase (ACAT) and, by preventing cholesterol esterification, it can potentially be used in pharmaceutical compositions or health food to prevent and treat hypercholesterolemia vascular diseases [63].

Pellitorine possesses antifungal properties against *Cladosporium cladosporioides* and the minimum amount required for the inhibition of fungal growth was 5 µg, compared to 0.5 µg of the positive controls nystatin and miconazole [64]. Insecticidal properties of pellitorine against *Sitophilus oryzae* and *Rhyzopertha dominica* have been observed: 80% insect mortality at 200 mg/ml pellitorine [65]. In addition, pellitorine showed antiprotozoal properties against *Plasmodium falciparum, Leishmania donovani*, *Trypanosoma cruzi*, *Trypanosoma brucei rhodesiense* with IC_{50} values of 3.26 µg/ml (positive control chloroquine K1 strain: 0.080 µg/ml), 5.96 µg/ml (positive control miltefosine: 0.127 µg/ml), 8.45 µg/ml (positive control benznidazole: 0.439 µg/ml) and 5.35 µg/ml (positive control melarsoprol: 0.003 µg/ml), respectively [66]. No experiments were carried out with purified pellitorine regarding the systemic effects.

Furthermore, in Chapters III and VI, the pharmacokinetic properties of pellitorine were investigated and it was demonstrated that pellitorine was able to penetrate human skin, thereby reaching the blood circulation. Moreover, pellitorine was able to cross Caco-2 cell monolayers *in vitro*, which was *in vivo* confirmed in an oral gavage experiment using rats. Once present in the blood, pellitorine rapidly crossed the blood-brain barrier [67, 68]. These mucosal/skin and BBB permeation studies of NAAs corroborate well with the traditional use of NAA containing plants, *e.g.* the Asari Radix and Rhizoma use in TCM to treat brain diseases [69].

Echinacea plants are well-known for their anti-inflammatory and immune-modulating properties. NAAs isolated from different species of *Echinacea* have shown to increase the phagocytic activity of

alveolar macrophages in rats and to potently modulate the expression of TNF-α in human monocytes [70, 71]. Furthermore, alkylamides were able to suppress the production of TNF-α in LPS and H1N1 influenza A stimulated RAW 264.7 macrophage-like cells. NO and NF-κB concentrations were also decreased after NAA treatment in LPS stimulated RAW 264.7 cells, while prostaglandin E2 (PGE2) production was reduced in H1N1 influenza A infected RAW 264.7 cells [72, 73]. Other studies showed that *N*-alkylamides from *Echinacea* modulated the NF-κB expression in LPS stimulated Jurkat cells (a human T cell line) and that the NAAs dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide and dodeca-2E,4E-dienoic acid isobutylamide were able to decrease the expression of IL-1β, IL12p70 and TNF-α in human blood stimulated with LPS [74, 75]. Some anti-inflammatory effects of NAAs are indicated in Figure 1. In addition, NAAs from *Echinacea* showed binding affinity for the mouse as well as for the human cannabinoid (CB)2-receptor [75-77]. Other NAAs also interact with the endocannabinoid system. Anandamide or *N*-arachidonoylethanolamine (AEA) is an endocannabinoid, produced in mammalians and metabolically degraded by fatty acid amide hydrolase (FAAH). *N*-benzyl-(9Z,12Z)-octadecadienamide, isolated from *Lepidium meyenii*, showed binding affinity for the CB1-receptor, inhibited FAAH and inhibited anandamide cellular uptake [78].

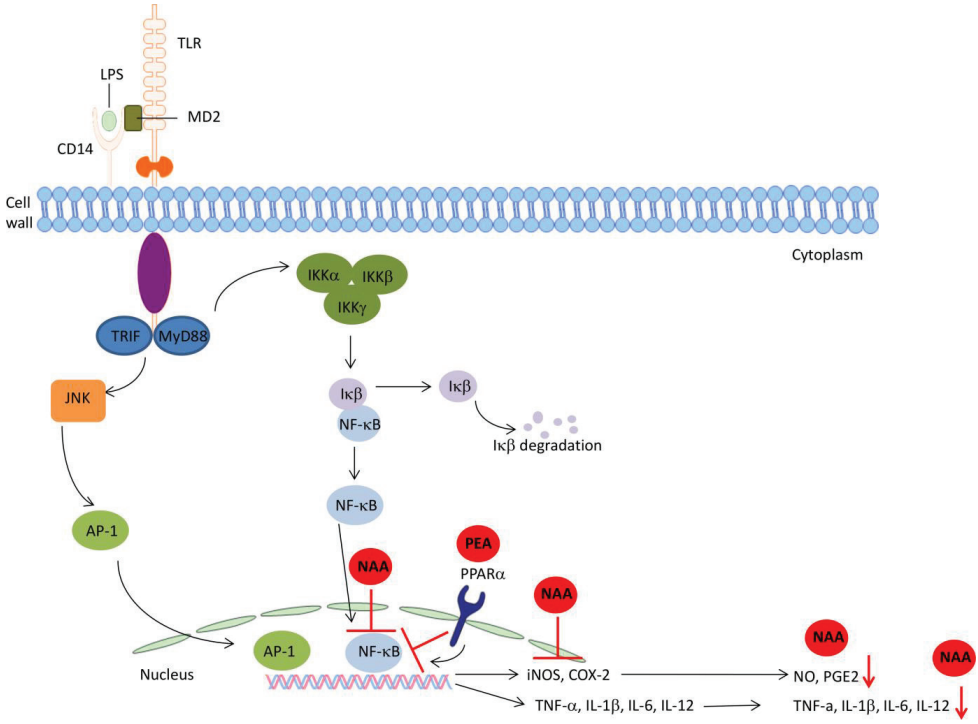

Figure 1. Anti-inflammatory effects of *N*-alkylamides.

(Abbreviations= NAA: *N*-alkylamides like 2E-*N*-isobutylundeca-2-ene-8,10-diynamide, PEA: palmitoylethanolamide, LPS: lipopolysaccharide, TLR: Toll-like receptor, PPARα: peroxisome proliferator-activated receptor α, NF-κB: nuclear factor-κB, IκB: inhibitor of κB, MD2: myeloid differentiation protein-2, TRIF: TIR domain-containing adapter-inducing interferon-β, MyD88: myeloid differentiation factor 88, CD14: cluster of differentiation antigen 14, JNK: c-Jun-*N*-terminal kinase, AP-1: activator protein 1, IKK: IκB kinase, iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase 2, IL: interleukin, TNF-α: tumor necrosis factor α, NO: nitric oxide, PGE2: prostaglandin E2).

Pharmacological effects such as immune-modulating, anti-inflammatory and analgesic properties are also reported for PEA. In models of neuropathic and chronic inflammation, it has been shown that PEA was able to reduce pain and endoneural oedema. Furthermore, at the injury site, there was a reduction of mast cells and proinflammatory mediators [3]. Many molecular mechanisms have been suggested for the pleiotropic effects of PEA. It has been shown that pharmacological effects of PEA are mediated by activation of the main molecular target peroxisome proliferator-activated receptor (PPAR)-α. This type of receptors switch off the NF-κB signalling cascade which results in an anti-inflammatory effect [79]. Other mechanisms of action are also reported such as the interaction with uncharacterised CB2-like receptors. Furthermore, PEA displays an 'entourage effect': it enhances the

analgesic effect of anandamide by inhibition of FAAH resulting in higher tissue levels of the endogenous anandamide [80].

2. CLASSIFICATION OF HEALTH PRODUCTS

The legal, regulatory classification, and hence the requirements for placing these NAA products on the market, is an important aspect for the patient/consumer, the manufacturer/distributor and for the competent authorities in an attempt to acquire correct information, create added value and protect the different stakeholders of the society at large. This 'classification' system should be considered as a tempo-spatial characteristic, *i.e.* changing in time and dependent on the country/region. Currently, however, the guiding principles in product classification are globally relatively similar. For the sake of transparency and focus, we will use the European legal and regulatory frame to elaborate the different health product classifications possibly applicable to NAAs. A schematic overview is given in Figure 2.

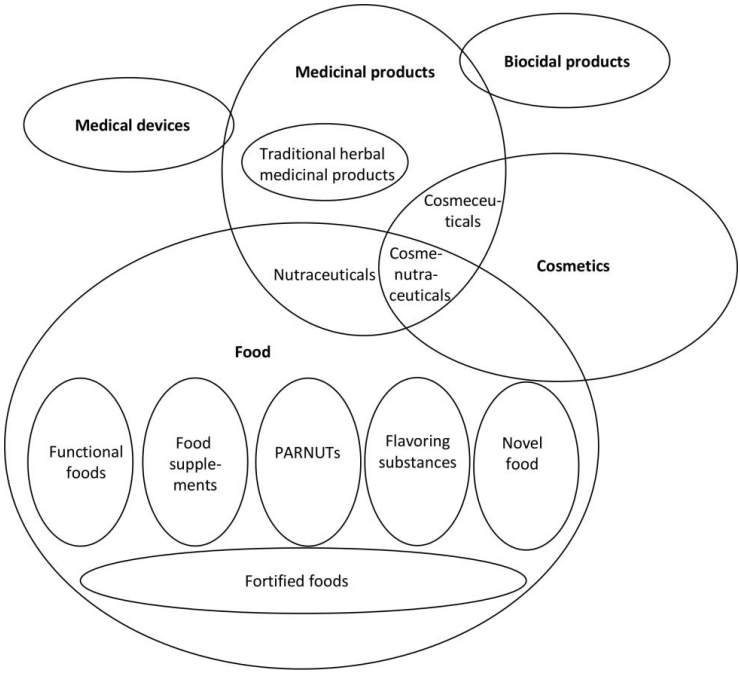

Figure 2. Product classification.

Medicinal products

To clarify the terms used in the text below, some terms will first be defined. A medicinal product (MP) is defined as "(a) any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or (b) any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis." Hence, there are two "classes" of medicinal products: (a) by presentation or (b) by function. Herbal medicinal products (HMP) are defined as "any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations". Furthermore, herbal substances are defined as " whole, fragmented or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried form, but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal substances. Herbal substances are precisely defined by the plant part used and the botanical name according to the binomial system (genus, species, variety and author)", while herbal preparations are "preparations obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices and processed exudates" [81]. Next to the conventional medicines, also non-conventional medicines exist, *i.e.* complementary and alternative medicines (CAM), of which homeopathic medicines are part of it, but will not be further discussed in this work.

Herbal medicinal products are licensed and marketed in Europe according to Directive 2004/24/EC, which amends Directive 2001/83/EC related to medicinal products for human use [82]. Individual herbal medicinal products are nationally licensed by Member States (MS). Due to Directive 2004/24/EC, the licence of herbal substances and preparations became more harmonised across the EU. Furthermore, in Directive 2004/24/EC, a subcategory of HMPs is introduced, namely the traditional herbal medicinal products (THMPs). Herbal medicinal products can reach the market in the European Union (EU) as (a) a traditional use HMP (simplified registration procedure by a Member State) and (b) a well-established use (WE) HMP (marketing authorisation by MS or European Medicines Agency (EMA)). In case of WE HMP, the active substances are in use in the EU for at least 10 years and enough safety and efficacy data must be available. No proof of clinical efficacy is needed for THMPs as this efficacy is plausible due to their traditional long-standing use. Registration as THMP is only possible if the product has been used for more than 30 years of which at least 15

years in at least one country of the EU [83-85]. Independent of the type of registration procedure, the quality of the HMP must always be guaranteed. The Committee on Herbal Medicinal Products (HMPC), one of the seven scientific committees of the EMA prepares the EU herbal monographs, consisting of the scientific opinion of the HMPC on efficacy and safety data of a herbal substance or preparations for medicinal use. These monographs consist of two parts: a well-established use and a traditional use part. Monographs include important information about the composition, pharmaceutical form, therapeutic indications, posology and method of administration, contraindications, special warnings and precautions for use, interactions with other medicinal products, information in case of pregnancy and lactation, ability to drive and use machines, undesirable effects, overdose information, pharmacokinetic (PK) and pharmacodynamic (PD) properties and preclinical safety data. The final monographs are published by the EMA and are used as a reference by a traditional use registration applicant (traditional use part) or by a marketing authorisation applicant (WE use part). The MS can consult these monographs for the examination of an application, however monographs have no legally binding character. But to favour the harmonisation, and avoid discrimination and unfair competition, if there is a deviation from the monographs, an appropriate justification is needed. Besides the monographs, there exist also list entries: "the EU list of herbal substances, preparations and combinations thereof for use in THMP", which is legally binding and is published by the European Commission (EC) [83, 85].

Food

The purpose of the implementation of the general food law, Regulation (EC) No. 178/2002, was to provide assurances of high level protection for consumers of all kinds of foods *e.g*. dietetic foods, food supplements, 'functional foods' and 'nutraceuticals' [86]. Food (or foodstuff) is defined as "any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be ingested by humans. 'Food' includes drink, chewing gum and any substance, including water, intentionally incorporated into the food during its manufacture, preparation or treatment." Food does not include "feed; live animals unless they are prepared for placing on the market for human consumption; plants prior to harvesting; medicinal products within the meaning of Council Directives 65/65EEC and 92/73/EEC; cosmetics within the meaning of Council Directive 76/768/EEC" [87]. The European Food Safety Authority (EFSA) was implemented in Regulation (EC) No. 178/2002 and provides scientific advice for the Community's legislation. MS collaborate with EFSA to follow up the missions of the EFSA. Herbal products can be seen as food as they comply with the applicable food law Regulation (EC) No. 178/2002 and if they do not comply with the definition of medicinal products. EFSA discusses which type of botanical ingredients may be

used and which health claims are allowed [84]. Several subtypes of food are legally defined, which are discussed hereafter.

Food supplements

Since Directive 2002/46/EC, food supplements have been harmonised in the EU. Food supplements are defined as: "foodstuffs with the purpose to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities". Food supplements thus contain substances with a nutritional or physiological effect such as nutrients (minerals and vitamins), essential fatty acids, amino acids and fibers. Specific rules are set up for vitamins and minerals and there is a positive list of vitamins and minerals that may be used in food supplements [88]. For other substances than vitamins and minerals with a nutritional or physiological character, there is little harmonisation across the EU and national rules of the MS are applicable. Belgium has its own unique Royal Decree on the manufacture and trade of foods composed of or containing plants or plant preparations. This Royal Decree can be an example for other countries in Europe how to deal with food composed of plants. In this Royal Decree, three lists are included with information about (1) the dangerous plants that may not be used in food, (2) edible mushrooms and (3) plants that needs to be notified if in dose form [89]. Food supplements must meet Directive 2000/13/EC in which rules for food stuffs are described considering the labelling, presentation and advertising [90].

At the moment, no EU legislation for botanicals and derived preparations as food ingredients exists and therefore the general food law Regulation (EC) No. 178/2002 is applicable. These products must also comply with Regulation 1924/2006 on nutrition and health claims made on foods [87]. Nothing is mentioned in the Regulation about the assessment of the safety of botanicals and botanical preparations in food. Therefore, the scientific committee of the EFSA issued a living document, "the Compendium of botanicals reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements" [91]. In the current list, *Anacyclus pyrethrum* is included, with *N*-alkylamides (for example pellitorine) indicated as chemicals of concern in the plant.

Functional foods

There is currently no single legislative definition of 'functional foods'. First used in the 1980's in Japan, it means that this food can improve the health status of the body and decrease the risk of diseases. Products with a FOod for Specified Health Uses (FOSHU) symbol were seen in Japan as food where the function is superior to the taste. In contrast to Japan, in Europe, functional food is not considered as a separate food category, but it is more seen as a concept. The following definition of functional foods was given by the European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE): "a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism, thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes. Therefore, it could not be in the form of pill or capsule" [92]. The USA-based Functional Food Center (Dallas, TX) suggested that a functional food definition should be harmonised and proposed a new definition: "natural or processed foods that contain known or unknown biologically-active compounds, which, in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management or treatment of chronic disease" [93]. Different types of functional foods have been defined: probiotics (live microorganisms if consumed in adequate numbers conferring a health benefit on the host), prebiotics (non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health), functional drinks, functional cereals, bakery products, spreads, functional meat and functional eggs [92]. To regulate some aspects of functional foods, the general food law Regulation (EC) No. 178/2002 and Regulation (EC) No. 1924/2006 on nutrition and health claims made on foods are applicable [87, 94].

Products on the market must be safe and appropriately labeled and Regulation (EC) No. 1924/2006 came into force to harmonise rules between MS on the use of nutrition and health claims on foods. Prior to the authorisation of health claims or foods in the Community, EFSA carries out a scientific assessment. Different kind of claims can be used for functional foods: (1) nutrition claim, (2) health claim and (3) reduction of disease risk claim. A nutrient claim is defined as "any claim which states, suggests or implies that a food has particular beneficial nutritional properties due to (a) the energy (calorific value) it provides/provides at a reduced or increased rate /does not provide, and/or (b) the nutrients or other substance it contains/contains in reduced or increased proportions/does not contain" (*e.g.* low energy, low sugar, fat-free), while a health claim means "any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its

constituents and health" (*e.g.* helps maintaining acceptable cholesterol levels). A reduction of disease risk claim is defined as "any health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease" (*e.g.* reduces cholesterol) [94]. It is due to this Regulation that debatably, foods can have claims with the name of a disease in it. Only nutrition claims mentioned in Annex of the Regulation may be used and they must comply to the conditions of the Regulation. Foods with claims must comply to Directive 90/496/EC considering general nutrition labelling for foodstuffs [84, 95, 96].

'Nutraceuticals' is another term frequently used which has no regulatory framework and is not considered as a specific food category [86]. The term nutraceutical is a combination of 'nutrition' and 'pharmaceutical'. It is often defined as a food or parts of food providing medical or health benefits, including the prevention and treatment of a disease [97, 98]. Nutraceuticals can be functional foods or food supplements [99].

Novel foods

Foods and food ingredients belong to 'novel foods' if they have not been used to a significant degree for human consumption in the EU before 15 May 1997. Different categories of novel foods exist: "(1) foods and food ingredients with a new or intentionally modified primary molecular structure; (2) foods and food ingredients consisting of or isolated from micro-organisms, fungi or algae; (3) foods and food ingredients consisting of or isolated from plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use; (4) foods and food ingredients to which has been applied a production process not currently used, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances" [100]. The use of genetically modified organisms (GMOs) in food or feed is separately regulated by Regulation (EC) 1829/2003 [101].

PARNUTS foods

Foodstuffs for particular nutritional uses (PARNUTS or dietetic foods) are defined as "foodstuffs which, owing to their special composition or manufacturing process, are clearly distinguishable from foodstuffs for normal consumption, which are suitable for their claimed nutritional purpose and which are marketed in such a way as to indicate such suitability" [102]. 'Particular nutritional use' means that particular nutritional requirements must be fulfilled for infants or young children in good health, people with disturbed digestive processes or disturbed metabolism or people having special

physiological conditions. These products are intended to be consumed by a small part of the population. Specific directives are described covering infant formulae and follow-on formulae (Directive 2006/141/EC), processed cereal-based foods and baby foods for infants and young children (Directive 2006/125/EC), food intended for use in energy-restricted diets for weight reduction (Directive 96/8/EC), dietary foods for special medical purposes (Directive 1999/21/EC) and foods intended to meet the expenditure of intense muscular effort, especially for sportsmen (Directive 89/398/EEC) [103-107]. In Commission Regulation (EC) No. 953/2009 of 13 October 2009 on substances that may be added for specific nutritional purposes in foods for particular nutritional uses, a list of substances (vitamins, minerals, amino acids, carnitine and taurine, nucleotides, choline and inositol) allowed to be added for specific nutritional purposes in foodstuffs for particular nutritional uses, is described [108]. For other substances which are not listed like botanicals and fatty acids, there are no harmonised EU rules and their use as PARNUTS food is considered at a national level [98].

Fortified foods

A list of vitamins and minerals which may be added to foods are described and specific rules are given [109]. There is a harmonisation between MS considering the addition of vitamins and minerals and of certain other substances. The rules in Regulation (EC) No. 1925/2006 for vitamins and minerals are not applicable for food supplements covered by Directive 2002/46/EC. This Regulation is applicable for foods for particular nutritional use, novel foods and novel foods ingredients, food additives and flavourings, genetically modified food and authorised oenological practices and processes.

Flavouring substances in food

Flavourings and food ingredients with flavouring properties must fulfill the criteria laid down in Regulation (EC) No. 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in foods and amending Council Regulation (EEC) No. 1604, Regulations (EC) No. 2232/96 and (EC) No. 110/2008 and Directive 2000/13/EC. Flavouring products are “not intended to be consumed as such, which are added to food in order to impart or modify odour and/or taste; made or consisting of the following categories: flavouring substances, flavouring preparations, thermal process flavourings, smoke flavourings, flavour precursors or other flavourings or mixtures thereof.” However, this Regulation does not apply to “non-compound foods and mixtures such as, but not exclusively, fresh, dried or frozen spices and/or herbs, mixtures of tea and mixtures for infusion as such as long as they have not been used as food ingredients” [110].

Cosmetics

A cosmetic product is defined as "any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours" [111]. In annex II of Directive 76/768/EEC, a list is summarised of substances prohibited in cosmetic products, while annex III contains a list of substances which may not be included in cosmetic products, except if they are subject to the restrictions mentioned in annex III [112]. Commission Regulation (EU) No. 655/2013 is about common criteria for the justification of claims used in relation to cosmetic products [113].

A term frequently used in the cosmetic industry for marketing purposes is 'cosmeceuticals' and is a combination of 'cosmetics' and 'pharmaceuticals'. It bears no legally defined definition. These are cosmetics having medicinal product-like properties, but are not considered as a separate product class. Ingredients of these products can affect the biological skin function [114]. A product in the borderline section between food (nutrient), cosmetics and medicinal products can be classified as 'cosmenutraceuticals', which is again not legally defined. These are products intended to be placed in contact with the external parts, of the human body or with the teeth and the mucous membranes of the oral cavity, providing systemic health benefits, including the prevention and treatment of a disease.

Medical devices

A medical device is "any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, including the software intended by its manufacturer to be used specifically for diagnostic and/or therapeutic purposes and necessary for its proper application, intended by the manufacturer to be used for human beings for the purpose of: diagnosis, prevention, monitoring, treatment or alleviation of disease; diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap; investigation, replacement or modification of the anatomy or of a physiological process; control of conception; and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means" [115]. Some medical devices look like medicinal products or cosmetics. Examples of medical devices in this area are Kamillosan ocean nose spray (moistens and cleans the nasal cavity), Sensodyne Rapid toothpaste (rapid pain relief in case of

sensitive teeth), Flamigel (to treat minor wounds), Bepanthen cream (against itching) and Angifyt Naturactive throat spray (softens and cleanses the throat).

Biocidal products

Currently, biocides are regulated by the biocidal product Regulation (EC) No. 528/2012. Seen the ethnopharmacological use and insecticidal pharmacological properties of NAAs, a classification as biocide would not be unexpected. A biocidal product is defined as "any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action; any substance or mixture, generated from substances or mixtures which do not themselves fall under the first indent, to be used with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action". Germs and pests are examples of such harmful organisms. Biocidal products include preservatives, disinfectants and pest control products. Product-type 18 and 19, insecticides and repellents, respectively, are both product types belonging to the pest control products [116].

3. *N*-ALKYLAMIDE CONTAINING PRODUCTS ON THE MARKET

Currently, NAA containing products are abundantly available on the market. However, the composition and quality of these products are often not well defined or uncontrolled. NAA containing products are presented under different forms by different companies with different health-related claims: gel (*e.g.* Buccaldol by A.B.S, contains *i.a. Spilanthes*, analgesic and prevents inflammation of the mouth mucus membrane), cream (*e.g.* Pea by RScience, contains palmitoylethanolamide, for sensitive skin and based on nutrient and protecting ingredients), ointment (*e.g.* Musculus Liniment by The Herbs, contains *i.a. Achillea millefolium* extract*, Capsicum annuum* extract *and Echinacea angustifolia* extract, cooling for the skin around the muscles), capsules (*e.g.* Cayenne, *Capsicum annuum* by Nature's answer, contains *i.a.* fruits of *Capsicum annuum*), chewable tablet (*e.g.* Echinaforce chewable tablets forte + vit C by A. Vogel, contains *i.a. Echinacea purpurea*, supports immune system), powder (*e.g.* Natural herb powder by Evaidyaiji, contains *Anacyclus pyrethrum*, aphrodisiac and improves physical strength and supports the immune system), liquid (*e.g.* Dentaforce by A. Vogel, containing *i.a. Spilanthes oleracea* extract, supports oral hygiene and freshens breath), tincture (*e.g. Spilanthes paracress* drops by A. Vogel, contains *Spilanthes oleracea*), syrup (*e.g.* Thyme and *Echinacea* syrop by Unipharma, containing *i.a. Echinacea*

tincture, beneficial and soothing for the throat), pastilles (*e.g. Echinacea* bonbons by A. Vogel, contains *i.a. Echinacea*, refreshing and soothing for the throat), effervescent tablets (*e.g.* EchinaCold by Schwabe, contains dried juice of *Echinacea purpurea* herb, relieves symptoms of a common cold and influenza), tea (*e.g.* Echinacea Plus tea by Traditional Medicinals, contains *Echinacea purpurea* and *Echinacea angustifolia*, supports the immune system), toothpaste (*e.g.* Parodontax gel fluor + Echinacea by Paradontax, contains *i.a. Echinacea purpurea*, for a complete oral hygiene, *Echinacea* supports the immune system), throat spray (*e.g. Echinacea* throat spray by A. Vogel, contains *i.a. Echinacea purpurea*, relieves discomfort of the throat) and lip balm (*e.g.* Echinaforce lip balm by A. Vogel, contains *i.a. Echinacea* concentrate, caring and protective). A non-exhaustive list of typical products are exemplified in Table 5.

The health-related claims of these products are very diverse, while no unambiguous and consistent product classification is applied. While some products explicitly claim the NAA (containing plant) as active ingredient, others present it as inactive excipient. They are sold as medicinal products (*e.g*. Echinacin liquid by Madaus, contains *i.a. Echinacea purpurea*, antiseptic properties in case of upper respiratory infections), food supplements (*e.g*. Anabeta by PES, containing *i.a. Anacyclus pyrethrum* DC root extract, muscle transformation agent), medical devices (*e.g*. Indolphar gel by I.D.Phar, contains *i.a.* spilanthol as aroma, reduces pain in case of mouth ulcers) or cosmetics (*e.g.* Parodontax gel fluor + *Echinacea* by Paradontax).

Table 5: NAA containing products on the market.

Trade name (Company)	NAA containing plant or NAA ingredients	Intended use	Product classification
Spilanthes drops (A. Vogel)	*Spilanthes*	Strengthens sensitive skin of the feet (after swimming)	n/a
Spilanthes paracress drops (A. Vogel)	*Spilanthes oleracea* plant and leaves tincture	For fungal skin infections	n/a
Herbal extract made with Aloha (Hawaiipharm)	*Acmella oleracea* extract	n/a	n/a
Fungus fighter™ liquid (Herb Pharm)	*Spilanthes acmella* flowers	Cleanse and detoxify	Food supplement
Indolphar gel	Spilanthol	Forms a protective film that softens the pain of ulcers and small mouth disease	Medical device
Buccaldol mouth gel	*Spilanthes*	Analgesic, prevents inflammation of mouth mucus membrane	Cosmetic
Anti-wrinkle serum (Dermaviduals)	*Acmella oleracea* extract	Anti-wrinkle	n/a
Dentaforce mouthwash (A. Vogel)	*Spilanthes acmella*	Freshens breath, supports oral hygiene	n/a
Carrot & cranberry radiance face cream (EE's cosmetics)	*Spilanthes acmella* flower extract	Helps to restructure, firm and smooth the face	n/a
Organic pomegranate anti-wrinkle care night cream (Dr. Scheller)	*Spilanthes acmella* flower extract	A conditioning, anti-wrinkle product to use at night to stimulate cell renewal for firmer facial contours	n/a
Intensive smoothing serum enriched with *Para cress* extract (Organic surge)	*Spilanthes acmella* flower extract	Rejuvenate, condition, protect	n/a
Relax-o-Firm Mask (QMS medicosmetics)	*Acmella oleracea* extract	Helps minimize facial expression lines and fine wrinkles. Herbal oils calm irritated and stressed skin, leaving it smooth and supple. Plant extracts target dryness and expression lines with visibly improved long-term results.	n/a
Natural herb tea (Best tea online)	*Spilanthes acmella*	Malignancy, bronchitis, tonsillitis, acute appendicitis, hepatitis, urinary tract infection.	Food

Table 5: NAA containing products on the market (continued).

Trade name (Company)	NAA containing plant or NAA ingredients	Intended use	Product classification
Echinacea Plus tea (Traditional Medicinals)	*Echinacea purpurea* herb, *Echinacea purpurea* root dry extract	Supports the immune system	Food
Echinaforce drops (A. Vogel)	*Echinacea purpurea*	Use in case of insufficient resistance to colds and flu	Traditional herbal medicinal product
Echinaforce tablets (A. Vogel)	*Echinacea purpurea*	Use in case of insufficient resistance, flu, colds	Traditional herbal medicinal product
Echinaforce hot drink forte + elderberry syrup (A. Vogel)	*Echinacea purpurea*	Resistance, accelerates recovery after illness	Food supplement
Echinaforce chewable tablets forte + vit C (A. Vogel)	*Echinacea purpurea*	Resistance	Food supplement
Echinaforce lip balm (A. Vogel)	*Echinacea* concentrate	Nourishes and protects	n/a
Echinaforce cream (A. Vogel)	n/a	Supports healing ability of the skin	n/a
Echinacea bonbons (A. Vogel)	*Echinacea*	Refreshing and soothing for the throat	n/a
Echinacin juice (Madaus)	*Echinacea purpurea*	Herbal medicine for colds, immune activated	Medicinal product
EchinaCold effervescent tablets (Schwabe)	*Echinacea purpurea* dried juice	Relief of symptoms of common cold and influenza type infections	Homeopathic medicine
Echinacea throat spray (A. Vogel)	*Echinacea purpurea*	Natural relief for the discomfort of the throat	n/a
Parodontax gel fluor + Echinacea toothpaste (Parodontax)	*Echinacea purpurea*	For a complete oral care. *Echinacea* increases resistance	Cosmetic
Thyme + *Echinacea* syrup (Unipharma)	*Echinacea* tincture	Beneficial soothing for the throat	n/a
Echinacea angustifolia 1x tablets (Schwabe)	*Echinacea angustifolia*	Immune stimulant	Homeopathic medicine
Echinacea & Goldenseal root capsules (Now)	*Echinacea purpurea* extract, *Echinacea angustifolia* extract	Immune system support	Food supplement
Echinacea pallida flower & herb powder (Terravitta)	*Echinacea pallida*	To support colds and chills, coughs, infections, inflammations, sore throat and much more	Food supplement

Table 5: NAA containing products on the market (continued).

Trade name (Company)	NAA containing plant or NAA ingredients	Intended use	Product classification
Herbal Akarkara capsules (Evaidyaiji)	*Anacyclus pyrethrum*	Improves physical strength of a fellow, aphrodisiac, corrects the metabolism and helps in expelling the unnecessary fluids out of the body, strengthens the immune system	n/a
Natural herbal Akarkara powder (Evaidyaiji)	*Anacyclus pyrethrum*	Improves physical strength of a fellow, aphrodisiac, corrects the metabolism and helps in expelling the unnecessary fluids out of the body, strengthens the immune system	n/a
Battle Fuel XT capsules (Muscle Pharm)	*Anacyclus pyrethrum*	Increase in strength, muscle growth & greater muscle hardness. To support estrogen regulation and anabolic processes	Food supplement
Pellitory extract (Making Cosmetics)	*Anacyclus pyrethrum*	Astringent	n/a
Pellitory cream (Bianca Rosa)	*Anacyclus pyrethrum*	n/a	n/a
Pellitory powder (Terra Vita)	*Anacyclus pyrethrum*	n/a	Food supplement
Anabeta capsules (PES)	*Anacyclus pyrethrum* root extract	Muscle transformation agent	Food supplement
Yarrow environmental solution herbal supplement spray (Flower Essence Services)	*Achillea millefolium* tincture and flowers, *Echinacea purpurea* tincture and flowers	n/a	n/a
Yarrow Flowers capsules (Nature's Way)	*Achillea millefolium* flowers	Ancient curative	Food supplement
Yarrow Oil (*Achillea millefolium*) (Health Aid)	*Achillea millefolium*	Sweet spicy aroma. Anti-inflammatory or for colds and influenza	n/a
Musculus Liniment ointment (The Herbs)	*Achillea millefolium* extract, *Capsicum annuum* extract, *Echinacea, angustifolia* extract	Cooling for the skin around muscles	n/a
Achillea millefolium 5 ch (Boiron)	*Achillea millefolium*	n/a	Homeopathic medicine

Table 5: NAA containing products on the market (continued).

Trade name (Company)	NAA containing plant or NAA ingredients	Intended use	Product classification
Maca *Lepidium Meyenii* capsules (Sanar Naturals)	*Lepidium meyenii*	Energy boost, stamina enhancer	Food supplement
Maca powder (*Lepidium Meyenii*) (Wilderness Poets)	*Lepidium meyenii*	Naturally sweet and malty. It is a potent, healing food. It is known to increase stamina, stimulate libido, regulate hormones, and increase mental focus.	n/a
Whole Root Maca (Herb Pharm)	*Lepidium meyenii* dried roots	System restoration, promotes libido, function and fertility	Food supplement
Pure Castor Oil (Dève herbes)	*Ricinus communis*	Skin care, hair care, purgative, anti-inflammatory, lactation, birth control, treats menstrual disorders, rheumatism, constipation, indigestion, cough, cold, used on burns, wounds	n/a
Trikatu capsules, three spices (Ayurvedic)	*Piper longum, Piper nigrum*	Stimulating to digestion, burns away toxins and fats, clears digestive and respiratory channels, warms the body	Food supplement
Cayenne, *Capsicum annuum* capsules (Nature's Answer)	*Capsicum annuum* fruits	n/a	Food supplement
PeaPure capsules (RScience)	Palmitoylethanolamide	n/a	Food supplement
Pea cream (RScience)	Palmitoylethanolamide	For sensitive skin	n/a

n/a: not available

4. DISCUSSION OF THE CLASSIFICATION OF *N*-ALKYLAMIDE CONTAINING PRODUCTS

Each health-related product class has its benefits and disadvantages for the manufacturers on the one hand and the consumers/patients on the other hand, with the competent authorities as the go-between arbitrator protecting the society. Manufactures often want to avoid a medicinal product classification, as this requires the most stringent, time-consuming and expensive development and authorisation process. On the other hand, public health concerns ask for sufficient safety and information measures being installed before certain products are launched.

The classification decision falls within the competence of individual countries, and hence differences may exist for example among different Member States in Europe. In case of doubt, the Court of Justice of the European Union (CJEU) clarifies the interpretation of the different product classifications. In article 2 of Directive 2004/27/EC, amending Directive 2001/83/EC, it is also explicitly mentioned that in cases of a borderline product, which can be considered as a medicinal product but also as another product type, the medicinal product classification shall be applicable [117]. Decisions must be taken for the whole product, taking into account all its characteristics and not for only one ingredient of it. The same ingredient can be used in *e.g.* food supplements as well as in medicinal products, depending on its use [95].

Most *N*-alkylamide containing plants are marketed as a food supplement or cosmetic, and only very seldomly as a medicinal product. For the plants used in the medicinal products, community herbal monographs exist *i.e.* for *Echinacea purpurea* (L.) Moench, *Echinacea pallida* (Nutt.) Nutt., radix, *Echinacea angustifolia* DC., radix and *Achillea millefolium* L., herba [118-121]. Echinacin liquid is an example of such herbal medicinal product on the market. For the marketing authorisation of this product, it was possible for the applicant to rely on the community herbal monograph of *Echinacea purpurea* (L.) Moench, radix [118]. The therapeutic indication mentioned in the community herbal monographs of *Echinacea pallida* (Nutt.) Nutt. and *Echinacea angustifolia*, is the supportive treatment of a common cold, while *Achillea millefolium* is used for temporary loss of appetite, the symptomatic treatment of mild, spasmodic gastro-intestinal complaints including bloating, and flatulence, the symptomatic treatment of minor spasm associated with menstrual periods and for the treatment of small superficial wounds [119-121].

Interactions with other drugs are increasingly becoming important due to *i.a.* polypharmacy in aging population [122]. Therefore, it is vital to consider the theoretically possible interactions from the mechanism of action and pursue further studies. Some studies have investigated the pharmacokinetic interactions of *Echinacea* preparations and other drugs [123]. However, for other

NAA containing products, no information is available. As an example, the well-known tingling effect of *N*-alkylamides is caused by blocking the two-pore potassium channels occurring in cells widespread over the body. The function of the two-pore potassium channels is to control the excitability of the cells by leaking potassium and these potassium channels are activated by some anesthetic drugs [124]. When two or more drugs are taken at the same time *e.g.* a medicinal product containing NAAs causing tingling effects and an anesthetic drug acting on the two-pore potassium channels, an interaction is theoretically not excluded, and its clinical relevance depends on dosing and pharmacokinetic considerations. Another example is related to the demonstrated interaction of NAAs with the endocannabinoid system. NAAs have shown to inhibit FAAH, thereby enhancing levels of the endogenous CB receptor agonist anandamide [78]. It has been demonstrated that FAAH inhibitors may impair the working memory in rats [125]. Hence, as FAAH inhibitors influence some regions in the brain, it is not without any risk to take such substances. However, more human derived data are needed to evaluate the clinical relevance of the information obtained from *in vitro* and animal studies.

Besides the possibility to regulate NAA or acylethanolamide (*e.g.* PEA) containing products as medicinal products, they can also be registered as food supplements, governed by the food law. Currently, the list of the EU Register on nutrition and health claims includes no *N*-alkylamides or NAA-containing products. Food supplements have physiological and nutritional effects with the function to maintain health, while medicinal products exert pharmacological, immunological or metabolic properties and treat or prevent a disease [81, 88]. The borderline between a medicinal product and a food supplement is sometimes subject to interpretation, confounded by the fact that the form of a food supplement closely resembles that of medicinal products, for example capsules, tablets or powder sachets. To comply with the definition of a food supplement, the nutrients or 'other substances' must have only a nutritional or physiological effect. While NAAs and PEA refer to 'other substances' in the food supplement definition, it has been demonstrated that these NAAs and PEA possess several pharmacological effects with different molecular targets already described. Even when a product is not presented as a medicinal product (presentation criterion), their mechanism of action applies to the second part of the definition of a medicinal product (functionality criterion), which is confirmed by the community herbal monographs of *Echinacea* species and *Achillea millefolium*, plants consisting of mixtures of NAAs. Often, a product is considered as a medicine if they potentially have an effect on the human body and affect the metabolism. However, this part of the definition alone cannot be the only reason to classify a product as a medicinal product. It is also important to consider the dose of the active substance(s). If the dose is below the dose able to restore, correct or modify a physiological function as established by the current state of scientific

knowledge, then it is not a medicinal product. This has been elaborated/explicated by CJEU judgments: the argument that there is a human health risk at a lower (but sub-therapeutic) dose is on itself not sufficient to consider a product as a medicine; hence remaining its status as a food supplement [126]. Furthermore, the claims and presentation of the substances are also important to take into account for classification issues. Products resembling medicinal products as well as food supplements are often named nutraceuticals, although this is not a legally defined term. Besides food supplements, it should also be possible to consider NAA containing plants as functional foods, since these plants contain bioactive NAAs and have already shown beneficial effects on human body; they are able to reduce the risk of the evolution of a disease as well [1]. To present substances as functional food, it is important that the appearance of the product looks like 'normal food' and not in the form of capsules or pills [92]. Although no NAA plant is currently included in the EU register on nutrition and health claims, this may change in the future [127].

The classification of products containing NAAs as a medical device should also be questioned. In some countries, medical devices which have the appearance of a medicinal product, are sold in selected distribution channels like the pharmacy [128]. Although medical devices may closely resemble medicinal products ('borderline products'), there is a difference between the two product classes [129]. The principal intended action of a medical device is obtained by physical means. For example, Virumed is a medical device as its main action is to block the UV rays, hence eliminating one of the factors eliciting cold sore caused by active Herpes simplex virus. The other components like *Echinacea purpurea* extract only assist in the functionality of the product by supporting the resistance [130]. Medical devices may thus contain medicinal substances having an ancillary action. The pharmacological action of these ancillary medicinal substances are subordinate to the physical action of the medical device [131]. Some of these medical devices are clearly very close to medicinal products.

There are also NAA containing products on the market as cosmetics. Often, cosmetic products are closely related to medicinal products and borderline issues are judged by the European Court. An example of such 'cosmeceutical' issue was the discussion about the meaning of 'pharmacological action' in the definition of a medicinal product of Directive 2001/83/EC [81]. The European Court decided that the definition explained in the Guidance Document on the demarcation between cosmetic products and medicinal products [132] can be used, which states that for a medicinal product, there must be "an interaction between the molecules of the substance in question and a cellular constituent, usually referred to as a receptor, which either results in a direct response or blocks the response of another agent". Furthermore, the Court ruled that the 'cellular constituent' in

the definition does not mean cellular constituents of the user's body, but it can also be cellular constituents of *e.g.* bacteria present in human body [133]. From the pharmacological effects of NAAs described previously, the interaction between NAAs and cellular constituents is beyond any doubt. The classification of NAA containing products as cosmetics can be similarly treated. Studies have shown that the *N*-alkylamides spilanthol and pellitorine, after topical administration were able to penetrate the different cell layers (stratum corneum and viable cell layers) and reach the systemic blood circulation. These compounds then exert a pharmacological effect, *i.e.* they interact with cells. As previously listed in Table 5, there are products on the market containing *Spilanthes acmella* (contains spilanthol) and *Anacyclus pyrethrum* (contains pellitorine) in the form of a cream or gel. This raises the question if such products, marketed as cosmetics, still can be considered as such, because when topically applied, the viable cells in the skin and the blood circulation below these cells are clearly reached, resulting in a systemic exposure with rapid tissue distribution. Depending on the amount that reaches the blood and the corresponding pharmacological effect, classification of such borderline products, informally called cosme(nutra)ceuticals, should be reconsidered.

In conclusion, from their pharmacokinetic properties, *i.e.* absorbed in the systemic circulation and distributed, combined with their pharmacological activities, *i.e.* different molecular targets have been identified, the NAAs and their originating plant materials plausibly show beneficial health effects, supported by the ethnopharmacodynamic use, but also possess inherent risks (side effects, overdose, interactions, contraindications). Therefore, their use, and its related dose-rate, influencing the biological positive as well as negative functionalities, is a critical determinant for their legal-regulatory classification.

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BROADER INTERNATIONAL CONTEXT, RELEVANCE & FUTURE PERSPECTIVES

"The secret of success is to know something nobody else knows."

Aristotle Onassis
(°1906-†1975, Greek shipping magnate)

BROADER INTERNATIONAL CONTEXT, RELEVANCE & FUTURE PERSPECTIVES

1. BROADER INTERNATIONAL CONTEXT AND RELEVANCE

Plant derived products are worldwide mainstream health preparations. For a full understanding of their health properties and a justified exploitation, analytical and biofunctional characterisations as well as regulatory positioning are required. This Ph.D. research is a contribution to our knowledge and current understanding in this field.

N-alkylamides as a new class of medicines

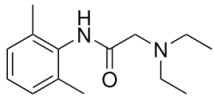
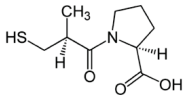
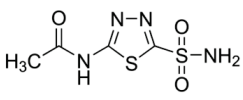
Medicinal plants containing *N*-alkylamides (NAAs) are widespread in **nature**. A well-known example of such plant is *Echinacea purpurea*. It is a popular flowering plant in gardens attracting bees and butterflies, but many people who have this easy-to-grow plant in their garden don't know that it has medicinal properties due to its NAAs. The same is also true for *Achillea* plants like *Achillea millefolium* (common name: yarrow).

Previously, it was demonstrated that spilanthol can penetrate human skin, and now a key finding of the presented study is that also pellitorine is able to penetrate human skin and reach the systemic blood circulation. Systemic concentrations of NAAs were found in a concentration range able to exert bioactive effects. In addition, high concentrations of NAAs were also achieved in the skin layers [1, 2]. Moreover, after oral administration, spilanthol and pellitorine reached the blood circulation. Another important observation of the presented study is that these NAAs were able to penetrate the blood-brain barrier (BBB) [3, 4]. In combination with the findings of Woelkart *et al.* (2009), who demonstrated that dodeca-2E,4E,8Z,10E/Z tetraenoic acid isobutylamides from *Echinacea* species rapidly crossed the BBB after oral administration to rats, our results suggest that the BBB is indeed permeable for certain NAAs [5]. This is a major observation, given that this barrier, which separates the blood from the brain, is characterised by tight junctions between the endothelial cells, preventing most molecules to penetrate this highly impermeable barrier [6]. It can be expected that next to spilanthol and pellitorine, also other NAAs can enter the brain as well. Due to their many pharmacological actions described in the literature and their good **bioavailability** from our research,

N-alkylamides are important **lead** compounds for **drugs** [7]. These findings stimulate further investigation towards the central nervous system (CNS) pharmacodynamic properties of these compounds and will be of interest in the search of new CNS drugs, where the BBB is usually a hampering factor. NAAs are also incorporated in topical formulations to exert not only local but also systemic functionalities. However, the pharmacological effects and good bioavailability not only offer perspectives, but also regulatory challenges.

In Chapter VII, some medicinal products containing NAAs are already mentioned, such as Echinaforce (A. Vogel) or Echinacin (Madaus), derived from *Echinacea purpurea*. Not only in plants, also other **synthetic** compounds have an *N*-alkylamide-like structure and are registered as medicines, such as lidocaine (local anesthetic), captopril (angiotensin-converting enzyme (ACE) inhibitor) and acetazolamide (diuretic). They can also be classified according to the FxMy classification system and are shown in Table 1 [7-10].

Table 1: Structures of lidocaine, captopril, phenacemide and acetozalomide.

Name	Lidocaine	Captopril	Acetazolamide
Structure			
Classification	F2M12	F2M6	F1M8

N-alkylamides are structurally related to the endocannabinoid anandamide. Both NAAs and endocannabinoids consist of an amide bond, which resembles the peptide bond in peptides and proteins. Endogenous agonists for the cannabinoid receptors (CB1R and CB2R), like anandamide, are being explored as they are pleiotropic, also activating other receptors such as transient receptor potential cation channel subfamily V member 1 (TRPV1) and peroxisome proliferator-activated receptors (PPARs). **Pepcans** are such a family of peptides, shown to be ligands for the CB1R [11].

Awaiting novel, more efficient synthetic pathways for NAAs, **isolation** from plants is still required for further exploration of the medicinal use of these compounds. We succeeded in isolating spilanthol and pellitorine from the raw plant extracts *Spilanthes acmella* and *Anacyclus pyrethrum*, respectively, allowing further pharmacodynamic experiments being executed using these isolated NAAs. This is important, as at present most pharmacodynamic investigations are carried out with the whole plant extract, often uncharacterised, instead of the isolated NAAs.

Phytotherapy

Phytotherapy or herbal medicine is based on the use of plant derived medicines. Phytotherapy is a kind of complementary and alternative medicine (**CAM**) and is often not evidence-based according to our current paradigms. Other CAM systems include Ayurveda, Chinese traditional medicine, homeopathy and Unani [12]. The production of herbal preparations requires some additional steps compared to single substances, such as the collection and cultivation of the plants and extract characterisation [13]. Isolated active compounds from plants may be less active than the whole plant. This is the case if multiple compounds are responsible for the observed pharmacological effect of the plant or if the pharmacokinetics is mutually influenced [14-16].

In this research the practical use of medicinal plants was clearly demonstrated, suggesting that sufficient academic attention should be devoted to the medicinal properties of plants. After all, plants are an important source in the search for new medicines and still a major part of synthetic drugs have plant precursors. In this context, it is important to bear in mind that a substantial knowledge of medical properties of plants can be found in indigenous cultures. In these cultures, plant knowledge is of vital economic and health importance. This **ethnopharmacological knowledge** is traditionally passed on via (1) vertical transmission, *i.e.* from parents to children; (2) horizontal transmission, *i.e.* between individuals of the same generation and (3) oblique transmission, *i.e.* from adults (other than parents, *e.g.* teacher) to children. Traditional healers play a pivotal role in this transmission. A common method for the discovery of drugs derived from plants, is to study the use of plant preparations in the folk medicine of indigenous cultures. This way of discovering drugs is opposite to Western drug research, in which clinical data are the end point instead of the start point. Studying these cultures can result in information about pharmacologically active plants, their extraction method, routes of administration, formulations and the dosages used. Hence, less research must be spent on these aspects and the cost of research can be reduced. At present however, the described ethnopharmacological knowledge is on the verge of extinction, as the access to modern medicine, the globalisation of cultures and the ongoing deforestation are threatening traditional habits. It is in our view of utmost importance for current and future generations that the existing knowledge is studied and documented. This is especially important for African traditional medicine where ethnopharmacological knowledge is passed on only orally and is not documented. This is in contrast to the Chinese traditional medicine and the Ayurveda, Unani and Sithda, where this knowledge is documented [14, 15, 17-20]. An efficient way to document medicinal knowledge about plants is constructing databases. To document the knowledge of *N*-alkylamides, there is the Alkamid® database, containing 439 *N*-alkylamides [7]. It is important that databases are updated on a regular basis, in order that always the latest and newest information is available online.

There is the **belief** of many people that what is **natural** is **safe**. However, natural products cannot be regarded automatically as safe. The unsafety of plants can have different causes, *e.g.* insufficient knowledge of plants, plants containing toxic compounds or contaminated plants. In the past, there have been serious health problems after consuming Chinese herbals. It has been demonstrated that health problems related to the use of herbal products were mainly due to mislabeling of the package and not mentioning all plant species. Plants from the Aristolochia species caused nephropathy, also named "Chinese herbs nephropathy". Aristolochic acid was the toxic compound present in the plants [21]. If fungi grow on plants, this is the cause of a lack of quality control (QC). Quality control is a common problem with herbal medicines, but also their proper use information to the patient will influence the safety profile [22].

People who live in **developing countries** have not always access to Western medicine. Therefore, they still use plants in primary health care. And even when they have access to it, Western medicine (active compounds isolated and purified from plants or synthetised) is often too expensive for them [13]. If the knowledge of traditional medicines can be improved and their safety and effectiveness can be assured, then it should be possible to include herbal preparations in local healthcare systems in an affordable way. On the other side, phytotherapy will only be accepted in Western medicine if the safety, efficacy and quality of the plants or plant derived products can be properly controlled, using similar criteria as used for synthetic compounds. Efforts have been made for standardising herbal medicines, which is a considerable challenge due to plant composition variability [15, 23, 24].

Of the many herbal products available, only for few of them, **clinical trials** have been conducted. Often, there is a poor quality of the clinical studies, *e.g*. too small sample size, inadequate controls and the study duration is too short. Many herbal medicines are not evaluated for their adverse reactions in clinical trials, as this is much more complicated compared to Western medicine (no standardisation, lack of quality systems). In order to compare clinical studies, exactly the same extract must be used. For clinical trials, the extract must be well characterised. A lot of *in vitro* and *in vivo* studies were performed with herbal medicines, supporting the healing properties of the herbal medicines used in traditional medicine. However, this is not always that simple to translate these effects to humans. And there is still the question/discussion if costly clinical trials will actually deliver added value for herbal medicines based on traditional medicine, as they have shown their efficacy in indigenous cultures [25].

Improvement of analytical techniques

Identification of all active compounds present in plants or plant extracts is not always easy, as plants are often complex mixtures. Currently, sensitive and selective analyses can be performed using gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled to MS. These techniques can provide detailed information about the composition of multicomponent products [23]. In this work, we characterised the *N*-alkylamide content in two plant extracts belonging to the Asteracea family, *i.e. Achillea millefolium* and *Achillea ptarmica*. Two different analytical techniques were used: HPLC-MS and GC-MS. Especially using GC-MS, up till now, scarce analytical NAA profiling was performed. Furthermore, additional NAAs were identified in *Achillea millefolium* using GC-MS compared to HPLC-MS. Our data supplement the current existing information about the NAA content of the two plants, as we have elucidated new NAAs, never previously reported in *Achillea millefolium* and *Achillea ptarmica* [26-30]. In addition, these analytical methods can be further used for the identification of NAAs in other plants too. The **progress in analytical techniques** is very important as still a lot of plants have not yet been investigated for their medicinal effects or for a detailed compound profiling [15].

Another analytical technique that can be used in plant research is UHPLC coupled to a **single quadrupole MS detector**. It was shown that the use of a single quad mass detector has several **selectivity** and **sensitivity** advantages compared to traditional ultraviolet (UV) detectors. We have demonstrated the applicability of this detector in transdermal research, in which bioactive *N*-alkylamides that are present in complex plant extracts, were identified and quantified with UHPLC-single quad MS. Using this detector, no baseline separation between compounds was needed for their quantification. It was also possible to quantify low concentrations of NAAs. Moreover, this type of detector has other applications too, such as its use in the quality control of peptides [31]. It is to be expected that this type of detector will be used in the near future more often for *e.g.* routine analyses and QC of drugs, and that it will be incorporated as a standard technique in pharmacopoeial monographs.

Legal and regulatory framework of N-alkylamide containing plants

Lastly, *N*-alkylamides are used worldwide due to their various biofunctionalities and have a vast range of applications in the food industry, as well as in the pharmaceutical and cosmetic industries. The interest in these compounds is also reflected in a number of patents related to the synthesis and/or the bioactive properties of NAAs and their application in food, food supplements, cosmetics, nutraceuticals and medicinal products [32-38]. In Chapter VII, it was the purpose to draw the attention to the **legal status** of these NAA containing products. Products on the market containing

NAAs are poorly or not regulated and we conclude that the legal and regulatory framework of NAA containing products should be strengthened. A system of objective, preferably quantitative, efficacy-risk balance should be developed to allow a more consistent classification.

2. FUTURE PERSPECTIVES

In this work, some pharmacokinetic properties of spilanthol and pellitorine have been characterised. The next logical aspects to further investigate are the skin and brain pharmacodynamic properties of these NAAs. Plants containing NAAs are ethnopharmacologically used in topical applications, such as the plant *Anacyclus pyrethrum* in Morocco [39]. Nowadays, topical products containing these plants are available on the market, used to treat a variety of health problems. We have shown that spilanthol and pellitorine were able to penetrate the stratum corneum (the outerlayer of the skin) and NAA concentrations were found locally in the epidermis and dermis and in the systemic blood circulation [1, 2, 40]. However, the mechanism of action of NAAs regarding their dermatological use is not yet elucidated. As NAAs can penetrate the skin and since they have *in vitro* proven to possess anti-inflammatory properties, they can possibly play an important role in the treatment of various inflammatory related skin diseases [41-46]. In certain skin diseases, such as contact allergy and cutaneous inflammation, skin cancer, and pruritus, the endocannabinoid system is involved, in which the cannabinoid receptor 1 (CB1R) and CB2R are main receptors and are *i.a.* distributed in the skin [47]. Raduner *et al.* (2006) demonstrated already the ability of *Echinacea* NAAs to interact with both cannabinoid receptors [48]. However, no data are yet reported about the binding affinity of spilanthol and pellitorine towards the CBRs. Therefore, the investigation of more fundamental pharmacodynamic parameters including the binding affinity of different NAAs to various targets is a future research objective.

A second aspect in need of further research is the question which effects NAAs cause in the brain. This study demonstrated that spilanthol and pellitorine are able to cross not only the stratum corneum, but also the blood-brain barrier [3, 4]. Although this is a major finding, in order to be correlated with the ethnopharmacological use and drug development, the effects of these NAAs in the brain have to be assessed thoroughly. As previously mentioned in Chapter I, CB1 receptors are expressed in the CNS. Hence, investigating the binding affinity of NAAs towards the cannabinoid receptors, is not only of importance in the treatment for skin related diseases, but also for CNS related disorders. Initial *in vitro* pilot experiments were performed in our laboratory using BV2-cells, murine microglial cells, which play a role in immunological reactions and occur in the CNS (data not yet reported). Microglia produce proinflammatory mediators in response to *i.a.* neurodegenerative

diseases (*e.g.* Alzheimer disease and Parkinson's disease) and infection [49-51]. The influence of different concentrations of spilanthol and pellitorine on the production of proinflammatory cytokines was evaluated and compared to the positive control lipopolysaccharide (LPS). Our pilot data indicate that pellitorine and spilanthol have no influence on tumor necrosis factor-α (TNF-α) and nitric oxide (NO) production compared to the negative control, indicating that these NAAs cause no inflammatory reaction. However, also the anti-inflammatory properties of spilanthol and pellitorine are under investigation, with promising first results which should stimulate further investigations. In addition, initial tests are also on-going with spilanthol and pellitorine on a pheochromocytoma (PC12)-cell line, which is an *in vitro* model for studying neuronal functions. These cells respond to neurite outgrowth if the neurotrophin nerve growth factor (NGF) binds with the tropomyosin receptor kinase A (TrkA) receptor and cell protective properties are then obtained [52]. Neurite formation stimulants are important compounds in the treatment of neurodegenerative disorders, such as Alzheimer's disease in which neurotrophin levels are altered [53]. Two types of tests are being performed, namely the effect of the NAAs after stimulating the cells with NGF and the effect of NAAs on untreated cells. From the initial *in vitro* pilot tests, it appears that both spilanthol and pellitorine had no effect on neurite outgrowth. Further investigations, including other brain cell types, are required to confirm these results and validate these pilot conclusions. Besides the distribution of spilanthol and pellitorine into the brain, further research should ideally also focus on the distribution of the NAAs within the different brain regions. As the brain uptake might not be homogeneous, this might lead to different concentrations in the different brain regions. Next to the brain, the distribution of these NAAs towards other tissues deserves further investigation as well, as these compounds can exert functional effects in these tissues.

More academic attention should in the third place also be devoted to the structure-activity relationship (SAR) regarding the anti-inflammatory and immune-modulating activities described for NAAs [41-46]. Only few studies document the structure-activity relationship. A study of Moazami *et al.* (2015) investigated the SAR of selected NAAs on the suppression of TNF-α production in LPS stimulated RAW 264.7 cells (murine macrophage-like cell line). It appears that the double bounds in the fatty acid chain of the *N*-alkylamide are not critical to exert an effect. The suppression of TNF-α production was dependent on the fatty acid chain length *i.e.* minimum 11 carbons are required if there is no saturation in the fatty acid chain of the molecule to have an effect [54]. However, from the Alkamid® database, it appears that *N*-alkylamides have various structural diversities in the fatty acid part, as well as in the amide part [7]. SAR studies with NAAs using totally different structures are

currently lacking and should be further investigated in the future. Besides the TNF-α production, other immunological responses deserve further investigation as well.

As medicinal products consisting of NAA containing plants are registered as traditional use herbal medicinal products via a simplified registration procedure, no pharmacokinetic/pharmacodynamic (PK/PD) and preclinical safety data need to be reported [55]. However, it is still important to investigate the interactions between these NAAs or NAA containing plants/products and drugs. It is after all not unlikely that the efficacy of drugs can be affected by NAAs, when taken simultaneously due to potential interactions between them. Because of interactions between the bioactive NAAs and drugs, the action of a particular drug may be altered, *i.e.* the drug can be less or more effective or an antagonistic effect can take place or side effects can occur [56]. Furthermore, NAAs can enhance the bioavailability of other drugs. These bio-enhancing properties have already been demonstrated for piperine, the main bioactive NAA in *Piper longum* and *Piper nigrum*. Piperine combined with other drugs or nutrients increased the bioavailability of these drugs/nutrients *e.g*. rifampicin, vitamin B6, theophylline, nimesulfide, pentobarbitone, and beta-carotene [57]. As interactions with NAAs like spilanthol and pellitorine with other drugs are not yet established, it is therefore important to further explore this. If bio-enhancing properties can be proven, it would have clear benefits for the application of drugs, given that in this scenario the dose of drugs could be lowered in combination with the NAA.

Finally, also the relationship between the human microbiome and the intake of NAAs, under whatever form or via whatever route, is a hot topic for future investigations. It is known that diet, nutrition habits, age, health status and geographical location influence the human microbiome. Many studies have shown that the microbiome plays a role in the well-being of humans and the development of diseases such as the irritable bowel syndrome (IBS) [58, 59]. As NAAs or plants containing NAAs can be part of food, *e.g.* as spices, they can possibly alter the gut microbiome composition. Hence, it seems crucial to study whether manipulating the human microbiome – of which the composition can be determined by investigating the feces – by NAAs can have positive effects on the development of specific diseases.

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SUMMARY & GENERAL CONCLUSIONS

"As to diseases, make a habit of two things — to help, or at least, to do no harm."

Hippocrates
(°460 BC-†370 BC, Greek physician)

SUMMARY & GENERAL CONCLUSIONS

This research project aimed to analytically characterise two plant extracts and to demonstrate some pharmacokinetic properties of two *N*-alkylamides (NAAs) spilanthol and pellitorine. In **Chapter I**, NAAs were described as secondary metabolites occurring in more than 25 plant families. Their wide structural diversity is reflected in an online database Alkamid®. The database is kept up to date and gathers information about NAAs and their structural, botanical, physicochemical properties and functionalities. Plants containing NAAs have been used ethnopharmacologically for decades for their medicinal properties and are often used in food. In this thesis, two specific bioactive NAAs were focused on, namely the triene NAA spilanthol (deca-2E,6Z,8E-trienoic acid isobutylamide) and the diene NAA pellitorine (deca-2E,4E-dienoic acid isobutylamide). These NAAs are most abundantly present in respectively the plants *Spilanthes acmella* and *Anacyclus pyrethrum*, both belonging to the Asteracea family.

In **Chapter II**, two other plants of the Asteracea family were analytically characterised: *Achillea ptarmica* and *Achillea millefolium*. NAAs were assigned in ethanolic extracts of these plants (roots, leaves, stems, flowers) using two different analytical methods: HPLC-ESI-MS and GC-EI-MS, allowing tentative structural assignments. By using both techniques, 14 NAAs were reported in *Achillea ptarmica*: 6 NAAs with an isobutylamide group, 3 NAAs with a piperide group, 1 NAA having a piperideide group, 2 NAAs with a *N*-methyl isobutylamide group, 1 NAA with a phenylethylamide group and 1 NAA with a *N*-methyl isobutylamide group. In the *Achillea millefolium* extract, 15 NAAs were reported: 6 NAAs with an isobutylamide group, 1 NAA with a tyramide group, 1 NAA having a 2-methylbutylamide group, 2 NAAs having a piperidide group, 4 NAAs having a piperideide group and 1 NAA with a 4-methoxy phenylethylamide group. In both plant extracts, new NAAs were reported: deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) or a related isomeric compound was assigned for the first time in both extracts, while another new NAA was assigned in *Achillea ptarmica*: deca-2E,4E-dienoic acid *N*-methyl isobutylamide. In both extracts, the roots contained the highest number of NAAs.

In **Chapter III**, the transdermal properties of pellitorine were investigated for the first time. It was demonstrated that pellitorine was able to penetrate human skin in a Franz diffusion cell set-up. The permeability kinetics were investigated of (1) purified pellitorine (isolated from *Anacyclus pyrethrum*) and of (2) pellitorine, present in the *Anacyclus pyrethrum* extract. Samples were analysed using (U)HPLC-UV. The permeability coefficient (K_p) of purified pellitorine proved to be lower ($K_p = 1.1 \cdot 10^{-4}$ cm/h) than the K_p of pellitorine in the extract ($K_p = 2.3 \cdot 10^{-4}$ cm/h), which can be explained by the fact that other NAAs in the extract can enhance the penetration properties of pellitorine. Pellitorine

concentrations were calculated in the epidermis, dermis and the receptor fluid, indicating that pellitorine is able to reach the systemic blood circulation. Hence, after topical administration of pellitorine, local and/or systemic effects are possible. As it is difficult to accurately quantify not baseline separated compounds with UV, the transdermal kinetics of NAAs in complex plant extracts were investigated again using another type of detector, *i.e.* UHPLC coupled to a single quadrupole mass spectrometric detector. The applicability of this new type of single quad MS detector in transdermal research is described in **Chapter IV**. Several advantages were demonstrated compared to the UV detector, such as a gain in selectivity as well as a lower limit of detection. It was possible to quantify two *N*-alkylamides pellitorine and anacycline, present in the *Anacyclus pyrethrum* extract, in transdermal samples, without a baseline separation. The local skin pharmacokinetic properties of spilanthol, present in *Spilanthes acmella*, were also investigated and transdermal samples were quantified with UHPLC-single quad MS. An additional advantage of this type of detector is that the lag time of compounds can be more accurately and precisely determined in transdermal research. In conclusion, the "single quad MS" detector has proven to be useful for biomedical-pharmaceutical purposes in transdermal research and development.

In **Chapter V**, the intestinal barrier and blood-brain barrier (BBB) permeability properties of spilanthol were explored. In order to analyse all these biological samples with a bio-analytical UHPLC-MS^2 method, a sample preparation method was developed using solid phase extraction (SPE). In an *in vitro* Caco-2 cell monolayer experiment, spilanthol was able to penetrate the Caco-2 cell monolayer from the apical to basolateral direction (absorptive direction) and from the basolateral to apical direction. Apparent permeability coefficients P_{app} between $5.2 \cdot 10^{-5}$ and $10.2 \cdot 10^{-5}$ cm/h were obtained. These results were confirmed *in vivo* in Wistar rats. In an oral gavage experiment, spilanthol concentrations were detected and quantified in the blood. Moreover, for the first time the blood-brain barrier characteristics of spilanthol were studied in the gold-standard *in vivo* mice model. Spilanthol was able to rapidly penetrate the BBB after intravenous injection with a high unidirectional influx rate constant K_1 of 796 µl/(g·min). These results were further explored and 98% of spilanthol was found in the brain parenchyma with only remaining 2% in the brain capillaries. In addition, also efflux from the brain into the blood was observed with an efflux transfer constant k_{out} of 0.11 min^{-1}.

All the above pharmacokinetic properties were described for pellitorine as well (**Chapter VI**). In a Caco-2 cell monolayer experiment, pellitorine penetrated the cells from the apical to basolateral direction (P_{app} between $0.6 \cdot 10^{-5}$ and $4.8 \cdot 10^{-5}$ cm/h) and from the basolateral to apical direction (P_{app} between $0.3 \cdot 10^{-5}$ and $5.8 \cdot 10^{-5}$ cm/h). Also in the *in vivo* oral gavage experiments with Wistar rats, pellitorine was rapidly absorbed from the intestinal lumen into the blood. Furthermore, once in the blood, pellitorine rapidly penetrated the blood-brain barrier in mice with a unidirectional influx rate K_1 of 153 µl/(g·min). 97% of pellitorine was able to reach the brain parenchyma, while only 3% was

captured in the brain capillaries. Furthermore, efflux from the brain into the blood was also demonstrated (efflux transfer constant k_{out}: 0.05 min^{-1}).

Lastly, in **Chapter VII**, the regulatory status of *N*-alkylamide containing health products was discussed. First, the traditional use of *N*-alkylamide containing plants was described, *i.e.* their use in food and their use to treat diseases in an ethnopharmacological context. Beneficial health effects were attributed to the NAA containing plants. Moreover, an overview of the different product classes of health products was presented mainly from an European viewpoint, followed by a selection of products containing *N*-alkylamides available on the market. From this information, the legal-regulatory classification system of *N*-alkylamide containing products was discussed as currently, there is no unambiguous legal product classification for these products: their classification is dependent on the dose of the bioactive NAA compounds, their (claimed) use and their biological functionalities.

To conclude, the **relevance** of this research was discussed and some hot topics for **future** investigations were suggested.

SAMENVATTING & ALGEMENE CONCLUSIES

"La perfection est atteinte, non pas lorsqu'il n'y a plus rien à ajouter, mais lorsqu'il n'y a plus rien à retirer."

Antoine de Saint Exupéry
(°1900-†1944, French writer, poet)

SAMENVATTING & ALGEMENE CONCLUSIES

Het doel van dit onderzoeksproject was om twee plantextracten analytisch te karakteriseren en om de farmacokinetische eigenschappen aan te tonen van twee *N*-alkylamides (NAA's): spilanthol en pellitorine. In **Hoofdstuk I** werden NAA's beschreven als secondaire metabolieten die in meer dan 25 plantfamilies voorkomen. Van hun structurele diversiteit getuigt de online database Alkamid®, die regelmatig geüpdatet wordt en informatie bevat over NAA's en hun structuur, botanische en fysicochemische eigenschappen en de bijhorende functionaliteiten. Planten die NAA's bevatten worden al decennia lang ethnofarmacologisch gebruikt voor hun geneeskundige eigenschappen en komen vaak voor in voeding. In deze scriptie werd gefocust op twee bioactieve NAA's, namelijk de 'triene' NAA spilanthol (deca-2E,6Z,8E-trienoic acid isobutylamide) en de 'diene' NAA pellitorine (deca-2E,4E-dienoic acid isobutylamide). Die NAA's zijn de meest voorkomende NAA's in respectievelijk de planten *Spilanthes acmella* en *Anacyclus pyrethrum*, die beide behoren tot de Asteracea plantfamilie.

In **Hoofdstuk II** werden twee andere planten van de Asteracea familie analytisch gekarakteriseerd, namelijk *Achillea ptarmica* en *Achillea millefolium*. NAA's werden gerapporteerd in ethanolische extracten van deze planten (wortels, bladeren, stengels, bloemen) met twee verschillende analytische methodes: HPLC-ESI-MS en GC-EI-MS. Door gebruik te maken van beide technieken werden 14 NAA's structureel toegewezen in *Achillea ptarmica*: 6 NAA's met een isobutylamide functie, 3 NAA's met een piperide functie, 1 NAA die een piperideide functie bezit, 2 NAA's met een *N*-methyl isobutylamide functie, 1 NAA met een fenylethylamide functie en 1 NAA met een *N*-methyl isobutylamide functie. In het *Achillea millefolium* extract daarentegen, werden 15 NAA's gerapporteerd: 6 NAA's met een isobutylamide functie, 1 NAA met een tyramide functie, 1 NAA die een 2-methylbutylamide functie bezit, 2 NAA's met een piperidide functie, 4 NAA's die een piperideide functie bezitten en 1 NAA met een 4-methoxy fenylethylamide functie. In beide plantextracten werden nieuwe NAA's gerapporteerd: deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) of een isomeer hiervan werd voor de eerste keer gerapporteerd in beide extracten, terwijl er nog een nieuw NAA werd gevonden in *Achillea ptarmica*: deca-2E,4E-dienoic acid *N*-methyl isobutylamide. In beide extracten werd het hoogste aantal NAA's aangetroffen in de wortels.

In **Hoofdstuk III** werden voor het eerst de transdermale eigenschappen van pellitorine onderzocht. Er werd aangetoond dat pellitorine in staat is om de humane huid te penetreren in een Franz diffusiecel experiment. De permeabiliteitskinetieken werden onderzocht van (1) zuiver pellitorine (geïsoleerd uit *Anacyclus pyrethrum*) en van (2) pellitorine aanwezig in het *Anacyclus pyrethrum* extract. Stalen

werden geanalyseerd met (U)HPLC-UV. De permeabiliteitscoëfficiënt (K_p) van zuiver pellitorine was lager (K_p = 1.1·10^{-4} cm/h) vergeleken met de K_p van pellitorine in het extract (K_p = 2.3·10^{-4} cm/h), wat verklaard kan worden door het feit dat andere NAA's aanwezig in het extract de permeabiliteitseigenschappen van pellitorine kunnen verhogen. Pellitorine werd aangetroffen in de epidermis, dermis en de receptor vloeistof, wat aangeeft dat pellitorine in staat is om de bloedcirculatie te bereiken. Daardoor zijn lokale en/of systemische effecten mogelijk na topicaal toedienen van pellitorine. Aangezien het moeilijk is om componenten accuraat te kwantificeren die niet basislijn gescheiden zijn van elkaar met UV, werd de transdermale kinetiek van NAA's in complexe plantextracten opnieuw onderzocht met een ander type detector, namelijk UHPLC gekoppeld aan een enkelvoudige quadrupool massaspectrometrische detector. De toepasbaarheid van het beschreven nieuwe type enkelvoudige quadrupool massaspectrometrische detector in transdermaal onderzoek werd beschreven in **Hoofdstuk IV**. De detector bleek verschillende voordelen te hebben ten opzichte van een UV-detector, zoals een winst aan selectiviteit en een lagere detectielimiet. Het bleek bovendien mogelijk om de twee *N*-alkylamides pellitorine en anacycline, aanwezig in het *Anacyclus pyrethrum* extract, in transdermale stalen te kwantificeren zonder dat die NAA's basislijn gescheiden waren. De lokale huid farmacokinetische eigenschappen van spilanthol, aanwezig in *Spilanthes acmella*, werden ook onderzocht en transdermale stalen werden gekwantificeerd met een UHPLC-enkelvoudige quadrupole MS. Een voordeel van dit type detector is dat de *lag time* van componenten meer accuraat en precies bepaald kan worden in transdermaal onderzoek. Samenvattend, kunnen we stellen dat een 'enkelvoudige quadrupole MS' detector kan gebruikt worden voor biomedische-farmaceutische toepassingen in transdermaal onderzoek en ontwikkeling.

In **hoofdstuk V** werden de permeabiliteitseigenschappen van spilanthol doorheen de darmbarrière en de bloed-hersenbarrière (BBB) onderzocht. Om de biologische stalen te analyseren met een bio-analytische UHPLC-MS² methode, werd een staalvoorbereidingsmethode ontwikkeld, gebruik makend van vaste fase-extractie. In een *in vitro* Caco-2 cel monolaag experiment, werd aangetoond dat spilanthol in staat is om de Caco-2 cel monolaag te penetreren in de apicale tot basolaterale richting (absorberende richting) en in de basolaterale tot apicale richting. Permeabiliteitscoëfficiënten (P_{app}) tussen 5.2·10^{-5} en 10.2·10^{-5} cm/h werden verkregen. Die resultaten werden *in vivo* bevestigd in Wistar ratten. In een orale *gavage* experiment werden spilanthol concentraties gedetecteerd en gekwantificeerd in het bloed. Bovendien werden voor het eerst de BBB-karakteristieken van spilanthol bestudeerd in een gouden standaard *in vivo* muismodel. Spilanthol penetreerde snel de BBB na intraveneuze injectie met een hoge unidirectionele influx snelheidsconstante K_1 van 796 µl/(g·min). In een verdere studie van die resultaten werd 98% van spilanthol teruggevonden in het hersenparenchym en slechts 2% in de hersencapillairen. Daarnaast

werd ook de efflux van spilanthol uit de hersenen naar het bloed geobserveerd met een efflux transfer constante k_{out} van 0.11 min^{-1}.

De beschreven farmacokinetische eigenschappen werden ook onderzocht voor pellitorine (**Hoofdstuk VI**). In een Caco-2 cel monolaag experiment werd aangetoond dat pellitorine die cellen penetreerde van de apicale tot basolaterale richting (P_{app} tussen $0.6 \cdot 10^{-5}$ en $4.8 \cdot 10^{-5}$ cm/h) en van de basolaterale tot apicale richting (P_{app} tussen $0.3 \cdot 10^{-5}$ en $5.8 \cdot 10^{-5}$ cm/h). Ook in het *in vivo* orale *gavage* experiment met Wistar ratten, werd pellitorine vlug geabsorbeerd in het bloed vanuit het darmlumen. Bovendien werd aangetoond dat pellitorine, eens aanwezig in het bloed, de bloedhersen-barrière van muizen snel penetreerde met een unidirectionele influx snelheidsconstante K_1 van 153 µl/(g·min). 97% pellitorine was in staat om het hersenparenchym te bereiken en slechts 3% werd vastgehouden in de hersencapillairen. Daarnaast werd ook de efflux aangetoond van pellitorine van de hersenen naar het bloed (transfer constante k_{out}: 0.05 min^{-1}).

Tot slot werd in **Hoofdstuk VII** het wetgevend kader van gezondheidsproducten met *N*-alkylamides bediscussieerd. Eerst werd het traditionele gebruik van planten met *N*-alkylamides beschreven, met onder andere toepassingen in de voedingsindustrie en de ethnofarmacologische ziektebehandeling. Gunstige gezondheidseffecten worden immers toegeschreven aan planten die NAA's bevatten. Daarnaast werd ook vanuit een hoofdzakelijk Europees standpunt een overzicht geschetst van verschillende productklassen van gezondheidsproducten, gevolgd door een selectie van NAA-producten die aanwezig zijn op de markt. Op basis van die informatie werd het wettelijk-regelgevende classificatiesysteem van NAA-producten besproken. Daaruit bleek dat er momenteel geen eenduidige wettelijke productclassificatie voor die producten wordt toegepast. Hun classificatie is afhankelijk van de dosis van de bioactieve NAA's, hun (voorgesteld) gebruik en hun biologische functionaliteiten.

Op het einde van deze scriptie werd de **relevantie** van het onderzoek besproken en sommige interessante studies werden voorgesteld voor **verder onderzoek**.

CURRICULUM VITAE

"Each problem that I solved became a rule which served afterwards to solve other problems."

Rene Descartes
(°1596-†1650, a French philosopher, mathematician, and scientist)

CURRICULUM VITAE

LIESELOTTE VERYSER

PERSONALIA

Surname:	Veryser
First name:	Lieselotte
Nationality:	Belgian
Place & date of birth:	Ypres, 7 March 1988
Private address:	Tarbotstraat 66 9000 Ghent
Professional address:	Drug Quality & Registration (DruQuaR) laboratory Department of Pharmaceutical Analysis Faculty of Pharmaceutical Sciences Ghent University Ottergemstesteenweg 460 9000 Ghent Belgium
Phone:	(+32) 9 264 81 01
E-mail:	Lieselotte.Veryser@UGent.be
URL:	http://www.ugent.be/DruQuaR

EDUCATION

2009-2011:	Master of Pharmaceutical Care at Ghent University
2006-2009:	Bachelor of Pharmaceutical Sciences at Ghent University
2002-2006:	Science-Maths at Lyceum, Onze-Lieve-Vrouw-Ter-Nieuwe-Plant, Ypres
2000-2002:	Latin at Lyceum, Onze-Lieve-Vrouw-Ter-Nieuwe-Plant, Ypres

ADDITIONAL COURSES

2014-2015:	Assistant training (Liesje Liagre, Department of Educational Policy, Ghent University)
2013-2014:	Registration of drugs (Prof. B. De Spiegeleer, Faculty of Pharmaceutical Sciences, Ghent University)
2012-2013:	Effective slide design (Tom De Moor, Faculty of Languages, Ghent University)
2012-2013:	Multivariate data analysis (Prof. T. De Beer, Faculty of Pharmaceutical Sciences, Ghent University)
2012-2013:	Design of experiments (Prof. T. De Beer, Faculty of Pharmaceutical Sciences, Ghent University)
2012-2013:	Advanced drug analysis (Prof. B. De Spiegeleer, Faculty of Pharmaceutical Sciences, Ghent University)
2011-2012:	Research and valorisation: 'Your Research and Legal Rights: IP, contracts, copyright,…' (Ghent University)
2011-2012:	Optimisation techniques (Prof. Y. Vander Heyden, Faculty of Pharmaceutical Sciences, Brussels University)
2011-2012:	Immunology and pathology (Prof. J. Grooten, Faculty of Sciences, Ghent University)

PROFESSIONAL EXPERIENCE

February 2012 – present:	Staff scientist at DruQuaR, Ghent University Supporting master theses and practical courses, as well as industrial servicing activities.

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