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Short communication

LC–MS profiling of *N*-alkylamides in *Spilanthes acmella* extract and the transmucosal behaviour of its main bio-active spilanthol

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ABSTRACT

N-Alkylamides are a promising group of naturally occurring bio-actives, with evidence for immune stimulating properties, which find applications *i.a.* in buccal preparations. In *Spilanthes* extracts, these properties are mainly ascribed to the most abundant *N*-isobutylamide, spilanthol. Yet, other *N*-alkylamides present in these extracts may contribute to this effect, as well as to its potential toxicity and physiologic interactions. Therefore, *N*-alkylamide profiling of an ethanolic *Spilanthes* extract was performed using a gradient reversed phase high performance liquid chromatography/electrospray ionization ion trap mass spectrometry (HPLC/ESI-MS) method on an embedded polar column. MS^1 and MS^2 fragmentation data were used for identification purposes. Moreover, the transmucosal behaviour of spilanthol, formulated in this ethanolic extract and in two commercially available oral gels, was evaluated using porcine buccal mucosa in a Franz diffusion cell experimental set-up. A high-throughput HPLC-UV method was used for the quantification of spilanthol in the receptor phase. Fundamental permeation characteristics of spilanthol in a solvent-independent way (100% aqueous dose solution) were obtained using different propylene glycol/water ratios.

Eight *N*-isobutylamides, two 2-methylbutylamides and one 2-phenylethylamide were detected, with spilanthol as most abundant *N*-alkylamide (88.8%). Up till now, two of these *N*-isobutylamides were not yet described in *Spilanthes* extracts. We demonstrated for the first time that spilanthol permeates the buccal mucosa. Depending on the formulation, a more pronounced local or systemic effect is achieved, which is important for the regulatory product classification. The purely aqueous permeation coefficient $K_{p,aq}$ (±SEM) was found to be 11.3 (±0.403) 10^{-3} cm/h.

1. Introduction

N-Alkylamides, a group of natural secondary metabolites found in different plant genera (*Echinacea*, *Zanthoxylum*, *Heliopsis* and *Spilanthes*), are new and highly promising bio-actives, still to be developed. Although the used plant extracts are not always well-characterized, the use of *Echinacea* as a food supplement stimulating the immune system is growing rapidly. Compared to *Echinacea*, mainstream interest in *Spilanthes* is only recently fuelled by pre-clinical test results, which are mainly ascribed to the most prominent *N*-alkylamide, the *N*-isobutylamide spilanthol [1]. Besides acting as a flavoring agent and its topical use against fungal infections, *Spilanthes* extracts (and spilanthol) are formulated in local buccal mucosa preparations indicated for painful mouth issues and minor mouth ulcers. In addition to local effects, the buccal mucosa is a good alternative way to reach effective systemic concentrations, thus bypassing different disadvantages of oral administration. The rich microcirculation with direct drainage of blood into the internal jugular vein permits systemic effects of permeated molecules [2]. However, little transbuccal research on bio-active compounds of plant extracts has been performed up till now [3]. Only plant-derived lectins, Salvia *desoleana* essential oils and the mucilaginous properties of polysaccharide-containing plant extracts were investigated. Although several spilanthol-containing preparations for buccal use are commercially available, their intrinsic pharmacokinetics has not been investigated yet.

Depending on the manner and place of growth/cultivation of these plants, the use of dry or fresh plant parts and the extraction method, different yields of *N*-alkylamide constituents were found in *Echinacea* [4,5] and *Spilanthes* [6,7] extracts. It can be assumed that the different plant preparations possibly imply alterations in biological effect, toxicity and interaction characteristics due to

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a different *N*-alkylamide profile. Therefore, analytical profiling of bio-actives in plant preparations is a prerequisite in functional food and cosmetics, drug pharmacology as well as in toxicology. HPLC/ESI-MS is considered an excellent technique for comprehensive analysis of alkylamides in plant extracts [5,8–10]. Recently, Cech et al. [4] developed such a method for simultaneous analysis of caffeic acid derivatives and alkamides from *Echinacea purpurea* extracts. In contrast to previous methods, where identification relied solely on the retention time and m/z values of the protonated molecular ion, the proposed HPLC/ESI-MS method used MS^2 with collision induced dissociation (CID) for structural elucidation of the constituents. This method is less prone to misidentification. Two types of alkylamides are identified in *Echinacea*: *N*-isobutylamides and 2-methylbutylamides. Both are characterized by typical CID-fragments from fragmentation of the amide bond as well as from the main alkyl chain. This latter contains many sites of unsaturation, resulting in multiple stable cations. Another HPLC/ESI-MS method, aiming to evaluate the quantity, identity and stability of alkylamides in an ethanolic extract of *Spilanthes acmella*, was developed by Bae [6]. Here, alkylamides were tentatively identified upon molecular weight and fragmentation patterns published for previously identified alkylamides whose structure was elucidated by NMR and LC–MS. Eleven alkylamides were detected: seven *N*-isobutylamides, two 2-methylethylamides and two *N*-phenylethylamides.

In this study, an *N*-alkylamide profiling is performed in a commercially available ethanolic *S. acmella* extract using HPLC/ESI-MS. In order to obtain transmucosal parameters, the transbuccal behaviour of spilanthol in this extract, as well as that of two spilanthol-containing mouth gels and different propylene glycol (PG)/water formulations is evaluated.

2. Materials and methods

2.1. Products examined

The ethanolic *Spilanthes* extract (A. Vogel *Spilanthes*, Biohorma, Belgium) and the two mouth gels, gel 1 (Indolphar®, I.D.Phar, Haaltert, Belgium) and gel 2 (Buccaldol®, A.B.S., Brugge, Belgium), were obtained through a pharmacy. The composition of both gels, in decreasing weight order, was indicated on their packaging. Gel 1 (Indolphar®) contained water, glycerine, PVP, polyacrylic acid, propyleneglycol (PG), hydroxypropyl methyl cellulose, benzyl alcohol, sodium hydroxide, sodium benzoate, xanthan gum, aroma-parfum (cinnamon-mint), potassium sorbate, aroma (spilanthol), sodium hyaluronate and sucralose. Gel 2 (Buccaldol®) consisted of water, PG, alcohol, *Spilanthes*, sodium carbomer, methyl paraben and disodium EDTA. According to information by A.Vogel, the fresh *S. acmella* plant was used for the preparation of the extract. The plant/solvent ratio stated on the leaflet was 1/10 and the final extract contained 65% ethanol (EtOH). This extract was also used as starting material to evaluate the transmucosal behaviour of spilanthol in different PG/H_2O ratios. The solvent from A.Vogel *Spilanthes* was evaporated and the remaining substance was redissolved in a similar volume of 10% PG in water (v/v) or 30% PG in water (v/v), respectively.

2.2. Reagents

Phosphate buffered saline (PBS) (pH 7.4; 0.01 M) was purchased from Sigma (St. Louis, MO, USA). Propylene glycol (PG) and acetic acid were bought from Riedel-de Haën (Seelze, Germany). Hydroxypropyl-β-cyclodextrin (HPβCD) was obtained from Cerestar (Sas van Gent, Netherlands). HPLC gradient grade methanol (MeOH) came from Fisher Scientific (Leicestershire, UK). HPLC gradient grade acetonitrile (ACN) was obtained from Panreac (Barcelona, Spain). Pro analysis formic acid was purchased from Acros Organics (Geel, Belgium). EtOH, denatured with up to 5% ether, was bought from Chem Lab (Zedelgem, Belgium). Water was purified using an Arium 611 purification system (Sartorius, Göttingen, Germany), resulting in ultrapure water of 18.2 MΩ cm quality.

2.3. HPLC/ESI-MS analytical N-alkylamide profiling

Based upon the analytical method for *N*-alkylamides from *E. purpurea* extracts [4], an *N*-alkylamide profiling from the ethanolic *Spilanthes* extract was performed. The LC-UV/MS apparatus consisted of a Spectra System SN4000 interface, SCM1000 degasser, P1000XR pump, AS3000 autosampler and was equipped with a Finnigan LCQ Classic ion trap mass spectrometer in positive ion mode (all Thermo, San José, CA, USA). Data were acquired using Xcalibur 2.0 software (Thermo, SanJosé, CA, USA). A Prevail RP C_{18} column (250 mm × 4.6 mm, 5 μm) attached to a Prevail all-guard C_{18} cartridge (4.6 mm × 7.5 mm, 5 μm) (both from Grace, Lokeren, Belgium) was used. The injection volume was 25 μL. The flow rate was set to 1.0 mL/min and the linear gradient used was as follows (where A = 1% acetic acid in ultrapure water and B = HPLC grade acetonitrile): t = 0 min, A:B (80:20, v/v); t = 0–150 min, A:B (10:90, v/v); t = 150–151 min, A:B (80:20, v/v); t = 151–166 min, A:B (80:20, v/v). UV detection was done at 237 nm, which is the absorption maximum of spilanthol as determined using a photodiode array detector (DAD). ESI was conducted using a capillary voltage of 3 kV. Nitrogen was used as the sheath and auxiliary gas with the heated capillary set at 275 °C. For MS^2 spectra, the mass spectrometer was tuned optimizing the relative collision energy.

2.4. Franz diffusion cell experiments using porcine buccal mucosa

The permeation of spilanthol through buccal mucosa was determined using 5 mL static Franz diffusion cells (FDC, Logan Instruments Corp., NJ, USA). Diffusion experiments with the commercial available pharmaceutical formulations were performed in sixfold, experiments with spilanthol in 10 and 30% PG in quadruplicate. For all conditions, a randomized blocked design was used. Porcine buccal mucosa was obtained after excising the tissue from the vestibular area of freshly slaughtered pigs. After the removal, the specimens were immediately placed on ice in an expanded polystyrene box and transported to our laboratory within half an hour. Excesses of connective and adipose tissue were trimmed away using a scalpel. The remaining mucosa samples were wrapped in aluminum foil and stored at −35 °C until use (max. 1 month). Before the start of the FDC experiments, the tissue was thawed, mounted on a template and dermatomed. The experimentally obtained thickness of the mucosa, determined using a micrometer (Mitutoyo, Tokyo, Japan), was 426 μm ± 10 μm (mean ± SEM, n = 50). Subsequently, the dermatomed mucosa was inspected for damage and cut into smaller pieces that were clamped between the donor and the receptor chambers. This latter were filled with PBS + 0.5% HPβCD as receptor medium. The whole assembly was fixed on a magnetic stirrer and the solution in the receptor compartment was continuously mixed using a Teflon coated magnetic stirring bar (400 rpm).

For the gels, 0.5 g and for the liquid extracts, 500 μL were applied upon the surface of the mucosa.

The spilanthol content in the different formulations, as used in the FDC experiments, was assayed using a high-throughput LC-UV method, and was found to be (mean ± SEM) 1221.621 ± 9.795 μg/mL in the ethanolic extract, 0.012 ± 0.003% (m/m) in gel 1 and 0.003 ± 0.000% (m/m) in gel 2. In the 10 and 30% PG solutions, prepared from the ethanolic extract, the

spilanthol content did not differ and was determined to be 1105.381 ± 8.118 μg/mL (mean ± SEM).

The donor compartment was then covered with parafilm (American National Can™, Chicago, USA) and the temperature of the receptor compartment was kept at 37 ± 1 °C by a water jacket. The available diffusion area was 0.64 cm^2. FDC samples of the receptor fluid (200 μL) were drawn from the sample port at 30 min intervals between 0.5 and 5 h and immediately replaced by 200 μL fresh receptor solution.

Spilanthol concentrations in the receptor fluid were also determined by the high-throughput LC-UV method. The spilanthol assay values in the FDC samples were correspondingly corrected for the receptor replenishments. At the end of the experiment (*i.e.* after 5 h), the mucosa surfaces were swabbed with cotton wool and spilanthol was extracted from the mucosa to construct a mass balance. The overall percentage spilanthol recovered was 91.53 ± 1.64% (mean ±SEM).

The high-throughput LC-UV method applied in the FDC experiments used a Waters Alliance 2695 separation module and 2996 DAD controlled by Empower 2 software (all Waters, Millford, USA). LC separations were performed using a Symmetry 100 RP C_{18} (75 mm × 4.6 mm, 3.5 μm particle size) column (Waters, Brussels, Belgium) maintained at 30 °C and protected with a guard column. A degassed isocratic mobile phase consisting of 1% (v/v) formic acid in MeOH/H_2O (70:30, v/v) at a flow rate of 1.5 mL/min was used. UV detection was done at 237 nm. Under these circumstances, spilanthol eluted at approximately 2.6 min. Quantification was performed using a laboratory reference material ("lanol 10% in PG", Cosmade, Ranst, Belgium) with a spilanthol content of 10.80% m/m as determined using UV at 228.5 nm (ε = 33,700).

2.5. Kinetic analysis of Franz diffusion cell data

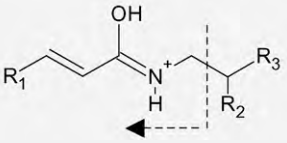
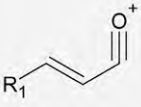
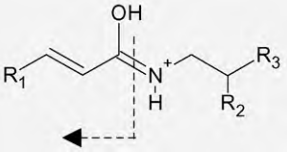
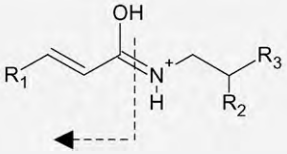
The permeation parameters were calculated from the plot of cumulative amount spilanthol permeated (in μg) as function of time (in h). Steady-state flux (J_{ss}) was obtained from the slope of the linear portion of the curve, divided by 0.64 to correct for the exposed mucosa area. The lag time (t_{lag}) was estimated by extrapolating the linear portion of the curve to the time-axis. The cumulative quantity, expressed as % of the effective dose applied, obtained after 5 h is the Q_{5h}. From the secondary flux parameter, the apparent primary permeability coefficient K_p was calculated according to ECETOC, CEFIC Workshop. This formula (steady-state flux divided by the spilanthol concentration in the dose solution) is used to determine the permeation across a homogeneous membrane. Although Kulkarni et al. noted that the buccal mucosa is a bilayer diffusion membrane [11] consisting of two independent layers (the epithelial and connective tissue layer), most studies still use the steady-state flux and permeability coefficient calculated for a homogeneous membrane [12]. One-way ANOVA and Tukey post hoc tests were performed for statistical conclusion confirmation ($p < 0.05$).

3. Results and discussion

3.1. N-Alkylamide profiling in ethanolic Spilanthes extract

Identification of *N*-alkylamides in the *S. acmella* extract consists of two steps. First, it needs to be demonstrated that the detected compounds are in fact *N*-alkylamides. Secondly, it should be determined which type of *N*-alkylamide is dealt with. Various *N*-isobutylamides, 2-methylbutylamides and 2-phenylethylamides have been identified [6], revealing different characteristic *N*-isobutylamide and 2-methylbutylamide fragmentation ions formed by CID [4,5,13]. For 2-phenylethylamides, similar fragmentation sites are present. Table 1 illustrates these sites and lists the respective losses of the formed characteristic fragments. A major alkylamide fragment often formed by CID is the acyllium ion, with a *m*/*z* value indicative for the amount of carbon atoms in the alkyl chain. HPLC/ESI-MS detected eleven *N*-

Table 1
Characteristics fragment ions of *N*-alkylamides formed by CID.

$M+H^+$ fragments formed by the loss of the	*N*-Isobutylamides (IBA)	2-Methylbutylamides (MBA)	2-Phenylethylamides (PEA)	CID fragments of the precursor ion[a,b]
Alkyl group directly attached to the amine	−56	−70	−103	
Entire amine functional group	−73	−87	−120	
Amide portion of the molecule and saturation of one of the double bonds on the alkyl chain	−99	−113	−147	
Amide portion	−101	−115	−149	

[a] R_1 = alkyl chain (most are 2*E* isomers); R_2 = CH_3 for IBA and MBA and H for PEA; R_3 = CH_3 for IBA, C_2H_5 for MBA and C_6H_5 for PEA.
[b] Acyllium ion.

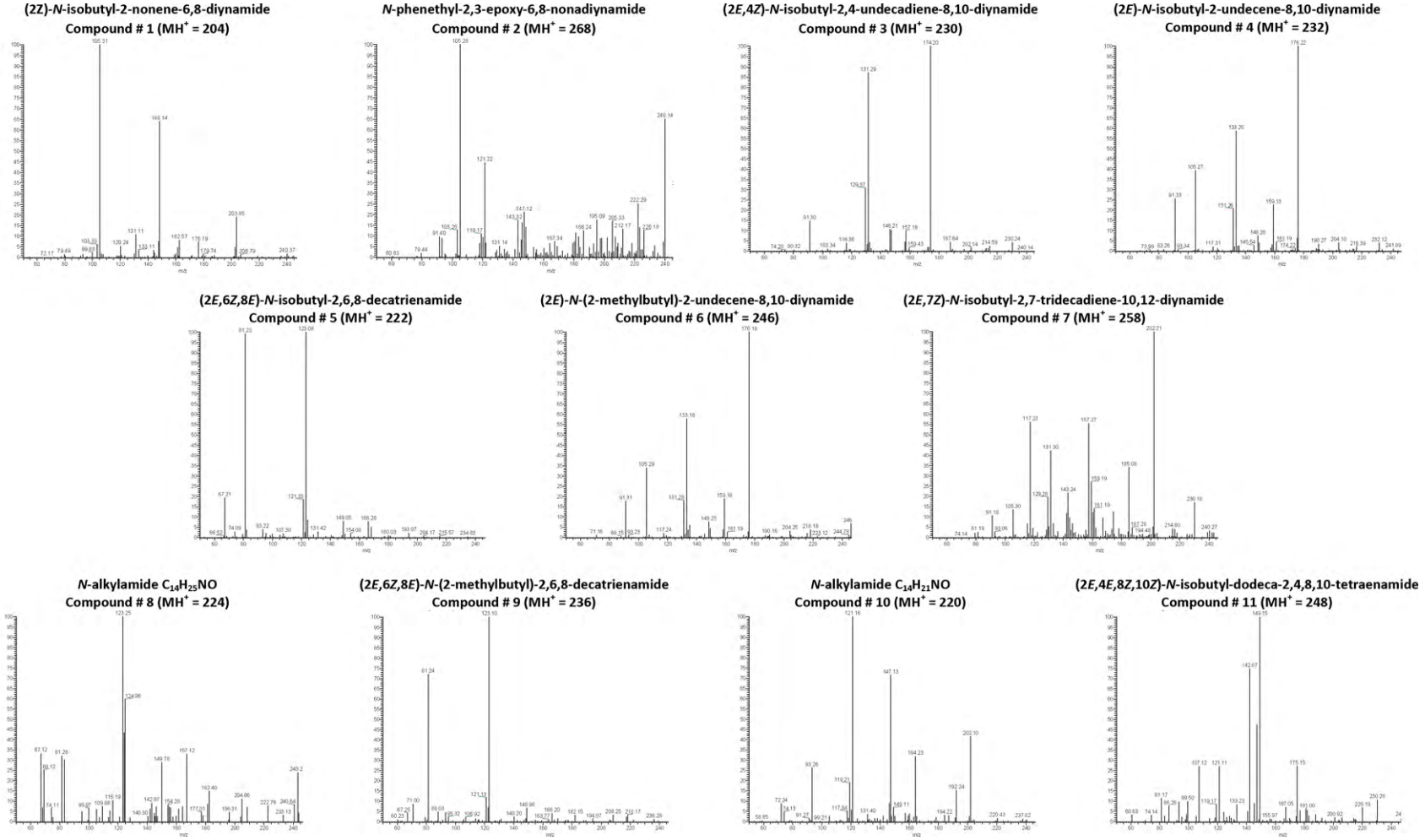
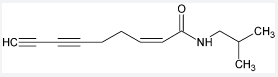
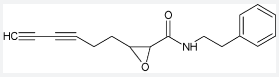
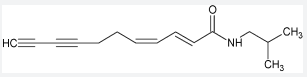
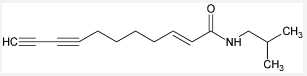
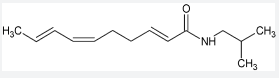
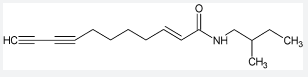
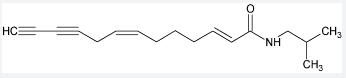
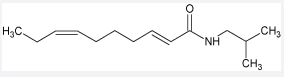
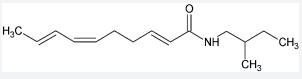
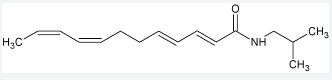


Fig. 1. Detailed TIC-MS chromatogram of the identified *N*-alkylamides, together with their isomers (indicated with the number of the *N*-alkylamide accompanied with letters b–e).

Table 2
Detected *N*-alkylamides in the ethanolic *Spilanthes* extract.

#	R_t (min)	Precursor ion, *m*/*z*	Product ions, *m*/*z*[a]	Resp. loss[b]	Identity	Tentative identification	Structure	%Relative to spilanthol
1	46.02	204	99; 103; 105: *131*; 148; 176	−105; −**101**; −**99**; −**73**; −**56**; −28	Isobutylamide	(2*Z*)-*N*-isobutyl-2-nonene-6,8-diynamide[c]		1.63
2	48.42	268	91; 103; 105; 121; 148; 195; 222; 240	−177; −165; −163; −**147**; −**120**; −73; −46; −28	2-Phenylethyl-amide	*N*-phenethyl-2,3-epoxy-6,8-nonadiynamide[c]		0.77
3	54.18	230	91; 129; 131; 146; *157*; 174; 188; 202	−139; −**101**; −**99**; −84; −**73**; −**56**; −42; −28	Isobutylamide	(2*E*,4*Z*)-*N*-isobutyl-2,4-undecadiene-8,10-diynamide[c]		0.62
4	58.59	232	91; 105; 131; 133; *159*; 176; 204	−141; −127; −**101**; −**99**; −**73**; −**56**; −28	Isobutylamide	(2*E*)-*N*-isobutyl-2-undecene-8,10-diynamide[c]		1.15
5	62.37	222	81; 121; 123; *149*; 166	−141; −**101**; −**99**; −**73**; −**56**	Isobutylamide	(2*E*,6*Z*,8*E*)-*N*-isobutyl-2,6,8-decatrienamide[c]		100
6	67.02	246	91; 105; 131; 133; *159*; 176; 218	−155; −141; −**115**; −**113**; −**87**; −**70**; −28	2-Methylbutyl-amide	(2*E*)-*N*-(2-methylbutyl)-2-undecene-8,10-diynamide[c]		0.77
7	69.11	258	91; 105; 117; 131; 157; *185*; 202; 230	−167; −153; −141; −127; −**101**; −**73**; −**56**; −28	Isobutylamide	(2*E*,7*Z*)-*N*-isobutyl-2,7-tridecadiene-10,12-diynamide[c]		0.70
8	70.54	224	81; 83; 123; 125; 149; 167	−143; −141; −**101**; −**99**; −75; −57	Isobutylamide	(2*E*,7*Z*)-*N*-isobutyl-2,7-decadienamide[d]		0.78
9	71.21	236	81; 121; 123; 149; 166	−155; −**115**; −**113**; −**87**; −**70**	2-Methylbutyl-amide	(2*E*,6*Z*,8*E*)-*N*-(2-methylbutyl)-2,6,8-decatrienamide[c]		7.27
10	74.32	220	72; 93; 119; 121; *147*; 164; 192	−148; −127; −**101**; −**99**; −**73**; −**56**; −28	Isobutylamide	–[d,e]		0.83
11	75.96	248	107; 121; 142; 147; 149; *175*; 220	−141; −127; −106; −**101**; −**99**; −**73**, −28	Isobutylamide	(2*E*,4*E*,8*Z*,10*Z*)-*N*-isobutyl-dodeca-2,4,8,10-tetraenamide[c]		1.52

[a] The produced acyllium ions are in italic.
[b] Losses of fragments which characterize the identity of the amide moiety are in bold.
[c] Bae et al. [6].
[d] Not available from literature.
[e] Structure is a tetraene, a diene-monoyne or a diyne.

alkylamides in our *Spilanthes* extract: eight *N*-isobutylamides, two 2-methylbutylamides and one 2-phenylethylamide. A typical total ion current (TIC)-MS chromatogram is presented in Supplementary Fig. S-1, while the MS^2 fragmentation spectrum of each detected *N*-alkylamide can be found in Fig. 1. Table 2 gives their retention time, precursor ion and fragmentation information (formed by CID), as well as the tentative identities and relative abundance to the main compound spilanthol.

Nine *N*-alkylamides structures could be identified based upon this fragmentation information combined with the molecular weight of previously identified *N*-alkylamides in *Spilanthes* extracts in literature [6]. LC–MS confirmed that the main peak (compound 5) in the detected series of *N*-alkylamides was spilanthol (*m*/*z* = 222). Its mass spectrum is given as insert in Supplementary Fig. S-1. Applying a reporting threshold of 0.5% and excluding the solvent and unrelated peaks (retention time < 40 min), 88.84% of the total amount of *N*-alkylamides in the extract is identified to be spilanthol. Its corresponding 2-methylbutylamide (compound 9 with *m*/*z* = 236) elutes after 71 min. This compound, also known as homospilanthol, is the second most abundant *N*-alkylamide in the ethanolic *Spilanthes* extract (9.04% of the total amount of *N*-alkylamides). Compound 2, an *N*-alkylamide with *m*/*z* = 268, is identified as *N*-phenethyl-2,3-epoxy-6,8-nonadiynamide. Seen the phenylethylamide structure and the epoxide attached to the amide function, another fragmentation mechanism for this compound is assumed, explaining the absence of the typical acyllium ion [6]. Compound 8 (*m*/*z* = 224) is a new compound, that has not yet been reported in *Spilanthes* extracts: due its mass fragmentation pattern [13], it is very likely that this *N*-isobutylamide with structure formula $C_{14}H_{25}NO$ corresponds to (2*E*,7*Z*)-*N*-isobutyl-

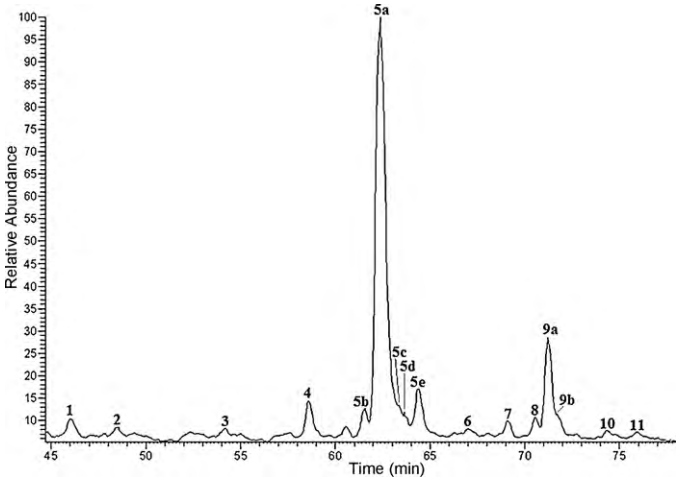


Fig. 2. MS^2 fragmentation spectra (CID) of the 11 *N*-alkylamides identified in the ethanolic *Spilanthes* extract.

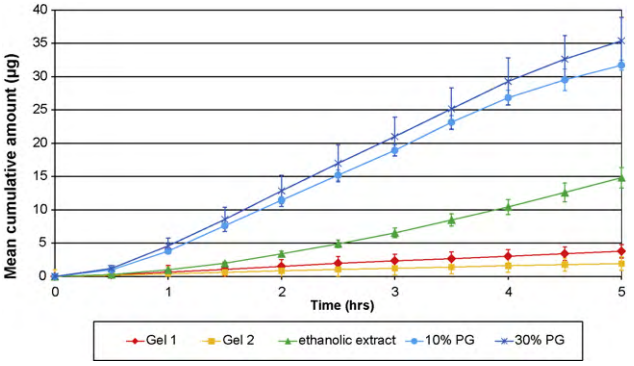


Fig. 3. Mean cumulative amount versus times curves of spilanthol applied in two mouth gels ($n=6$), the ethanolic *Spilanthes* extract ($n=6$) and the extract in PG based donor solutions (10 and 30%) ($n=4$). Error bars indicate ± SEM.

2,7-decadienamide. Moreover, a second new compound has been detected: a *N*-isobutylamide with $m/z=220$ (compound 10). For this compound, an acyllium ion is present at $m/z=147$, which is indicative for a $C_{10}H_{11}$ alkyl chain. Consequently, this compound contains in total fourteen carbon atoms and 21 hydrogen atoms, resulting in a structure formula of $C_{14}H_{21}NO$. Because of the related structures in *Spilanthes*, the alkyl chain is probably a tetraene, but a diene-monoyne or diyne structure cannot be excluded as well. Further characterisation of these two new *N*-isobutylamides (compounds 8 and 10) in *Spilanthes* is needed.

Structural and geometric *N*-alkylamide isomers of the main components are likely to be present in these extracts [6]. Fig. 2 shows the presence of these isomers in the TIC-MS chromatogram in more detail. For spilanthol (5a), four isomers are detected (5b–5e), while for homospilanthol only one is observed (9b).

3.2. Transbuccal behaviour of spilanthol-containing formulations

The analytical validation of the high-throughput LC-UV method resulted in the following chromatographic method characteristics for spilanthol. The capacity factor k', with t_0 defined as the first change in baseline (*i.e.* 0.5 min), was 4.6. Asymmetry factor As and apparent number of theoretical plates N as defined in the European Pharmacopeia were 1.21 and 3756, respectively. LoD and LoQ, defined as the concentrations equivalent to a signal to noise value of 3 and 10, respectively, were determined to be 9.10 ng/mL and 30.32 ng/mL. Linearity, with correlation $R^2=0.9996$, is assured in a working range of 30.32 ng/mL (LoQ) up to 22.52 μg/mL. A typical chromatogram is displayed in Supplementary Fig. S-2.

For the first time, it is shown that spilanthol permeates the buccal mucosa when applied in three commercially available formulations and in PG/H_2O. All individual runs confirmed the steady-state conditions: after 5 h, only 1.71–14.56% of the applied dose was cumulatively found in the receptor chamber. Moreover, a linear relationship of all individual cumulative amounts versus time was observed between time points 1 and 4.5 (R^2 not less than 0.983). Fig. 3 shows the mean cumulative amount of spilanthol (in μg) versus time (in h) curves for all investigated conditions. Linear least-squares regression on the linear part of the individual cumulative amounts for each condition versus time curves was performed. The values of the transmucosal parameters thus calculated are given in Table 3. The more concentrated gel 1 (Indolphar®) has an expected higher spilanthol flux than gel 2 (Buccaldol®). Therefore, the permeability coefficient K_p was calculated, which is independent of the concentration applied. As presented in Supplementary Fig. S-3, the permeability of spilanthol in gel 2 is significantly higher than in all other conditions. This can be explained by the different quantitative and even qualitative compositions, including the presence of penetration enhancing additives. Indeed, in contrast to gel 1 and the organic solvent (EtOH and PG) based *Spilanthes* extracts, gel 2 contains an additional buccal penetration enhancer (*i.e.* disodium EDTA [14]).

The amount of spilanthol found in the mucosa at the end of the FDC experiments was (mean ± SEM) 0.72 ± 0.01 μg for gel 1, 0.32 ± 0.04 μg for gel 2, 18.66 ± 3.06 μg for the ethanolic *Spilanthes* extract and 5.59 ± 0.91 μg for the PG based *Spilanthes* extract dose solutions. This corresponds to a spilanthol concentration of respectively 2.64×10^4 ng/mL, 1.19×10^4 ng/mL, 68.43×10^4 ng/mL and 20.33×10^4 ng/mL (or 1.19×10^5 nM, 0.54×10^5 nM, 30.91×10^5 nM and 9.18×10^5 nM) in the buccal mucosal tissue. This local concentration spilanthol in the mucosa was thus approximately 10^5 times (mouth gels) and 10^6 times higher (ethanolic extract and PG based dose solutions) than another *N*-alkylamide from *Echinacea* in plasma, previously proven to possess bioactivity esp. anti-inflammatory effect [15]. Our findings are thus indicative that local mucosa effects with topical *Spilanthes* formulations can be expected.

Besides these local mucosa effects, systemic effects after topical administration on buccal mucosa can be expected as well. Compared to the oral application of similar *N*-alkylamides [15], comparable (gels) or higher (10-fold for the EtOH extract or 20-

Table 3
Transmucosal parameters for spilanthol in different formulations (mean values ± SEM; $n=4$–6).

Formulation ([spilanthol] in μg/mL)	J_{ss} (μg/cm²/h)	Q_{5h} (%dose applied)	Lag time (h)	K_p ($\times10^{-3}$ cm/h)
Indolphar®[a] (62.6)	1.291 ± 0.128	4.464 ± 0.318	0.279 ± 0.043	19.516 ± 1.752
Buccaldol®[a] (12.8)	0.616 ± 0.038	10.484 ± 1.623	0.277 ± 0.073	47.533 ± 2.900
EtOH extract[a] (1221.6)	6.418 ± 0.710	2.434 ± 0.256	1.422 ± 0.111	5.275 ± 0.595
10% PG[b] (1114.1)	12.134 ± 0.526	5.585 ± 0.172	0.525 ± 0.045	10.891 ± 0.418
30% PG[b] (1096.7)	12.830 ± 1.160	9.663 ± 1.794	0.472 ± 0.112	11.696 ± 0.941

[a] $n=6$.
[b] $n=4$.

fold for PG based solutions) plasma steady-state concentrations are obtained with the topical buccal applications. Consequently, a local formulation of an *N*-alkylamide on the buccal mucosa is an interesting route to administer immune modulating *N*-alkylamides for systemic use. It is clear from our results that a local and/or systemic functionality can be aimed for, depending on the formulation used, with consequences to the legal product classification, *i.e.* a pharmaceutical drug (if systemic effects are aimed for or are dominant present) versus a cosmeceutical product (if mainly local effects are expected) or even a medical device (if only as a functional ingredient like taste).

The influence of the organic solvent used in the extract was investigated by comparison of the 65% ethanolic *Spilanthes* extract with the PG based (10 and 30%) extracts. The permeability (mean ± SEM) of the ethanolic extract ($5.28 \pm 0.60 \times 10^{-3}$ cm/h) is two times lower than for the two PG dose solutions (10 and 30%) which gave similar results ($11.29 \pm 0.40 \times 10^{-3}$ cm/h). This can be explained by a pronounced better penetration enhancing effect of PG compared to EtOH and/or an influence of the percentage of the organic solvent used and/or (less probable) a confounding effect of other compounds present which maybe changed in the drying/resolubilisation process. Within skin, EtOH's modifying effect for different investigated compounds appears to be concentration dependent [16]. This was ascribed to the ability of higher EtOH levels to dehydrate biological membranes and/or to extract stratum corneum intercellular lipids, thereby reducing the skin permeability. Similar mechanisms with buccal intercellular lipids seems to be involved in the permeation of mucosa as well [17].

Seen the lipophilicity of spilanthol ($\log P_{ow} = 3.4$) and its practically water insolubility (18.63 mg/L, calculated according Tetko et al. [18]), an extrapolation of spilanthol in 10 and 30% PG is performed to give the purely aqueous flux, hereby neglecting the penetration modifying effect of the co-solvent PG. The extrapolated value of the apparent primary parameter K_p is considered the best estimate of $K_{p,aq}$ (±SEM) = 11.3 (±0.403) 10^{-3} cm/h.

4. Conclusions

An *N*-alkylamide profiling from a 65% ethanolic *S. acmella* extract was done using HPLC/ESI-MS. From the fragmentation information, 11 compounds were detected to be *N*-alkylamides: eight *N*-isobutylamides, two 2-methylbutylamides and one 2-phenylethylamide. Based upon previously characterized *N*-alkylamides in *Spilanthes* extracts, nine *N*-alkylamides structures could completely be identified, with spilanthol as most abundant alkylamide. Two new (*i.e.* not yet identified in *Spilanthes* extracts) *N*-isobutylamides were detected. Based upon the fragment ions, the alkyl portion for the $m/z = 224$ isobutylamide is suggested to be a $C_{10}H_{15}$ diene structure and has a structure formula of $C_{14}H_{25}NO$. The isobutylamide with $m/z = 220$ possesses a structure formula of $C_{14}H_{21}NO$, with an alkyl chain being most probably a tetraene, or less likely, a diene-monoyne or diyene structure.

Moreover, we demonstrated and quantified that spilanthol can permeate the mucosa in a formulation dependent way. Gel 1 (Indolphar®) is adequate to achieve local effects, while gel 2 (Buccaldol®) realizes more systemic effects. A solvent influence is seen with a two times lower permeability for spilanthol in the 65% ethanolic extract compared to 10 and 30% PG based extracts. No influence of the percentage PG (10–30%) was observed and the porcine buccal mucosa $K_{p,aq}$ (±SEM) was quantitatively estimated to be 11.3 (±0.403) 10^{-3} cm/h.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.02.010.

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