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Original Research Article

N-alkylamide profiling of *Achillea ptarmica* and *Achillea millefolium* extracts by liquid and gas chromatography–mass spectrometry

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ABSTRACT

Achillea millefolium and *Achillea ptarmica* are both plants belonging to the Asteracea family and are traditionally used for their medicinal properties. It has already been shown that some *N*-alkylamides (NAAs) are responsible for these pharmacological actions. Therefore, in the present study, the NAA content of the two plants was analytically characterised. Different extracts were prepared from the roots, the leaves, the stems and the flowers. The structures of NAAs have been assigned in ethanolic extracts of *Achillea millefolium* and *Achillea ptarmica* using high performance liquid chromatography – electrospray ionisation – mass spectrometry (HPLC–ESI–MS) and gas chromatography – electron impact – mass spectrometry (GC–EI–MS). Using both analytical techniques, the structures of 14 and 15 NAAs have been assigned in *Achillea ptarmica* and *Achillea millefolium*, respectively. Structures of 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.

1. Introduction

The genus *Achillea*, mainly distributed in the Northern Hemisphere, consists of more than 120 species worldwide. *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* [1–3]. Two species of the genus *Achillea* (*millefolium* and *ptarmica*) will be discussed in detail, i.e., *Achillea millefolium* L. (*A. millefolium*) and *Achillea ptarmica* L. (*A. ptarmica*), both belonging to the Anthemideae tribe and Asteraceae plant family. *A. millefolium* and *A. ptarmica* plants are both ethnopharmacologically used to treat stomach disorders [4,5].

A. millefolium, also known as yarrow, consists of several closely related species, named a species complex or aggregate. A diversity of pharmacological properties is ascribed to this plant, such as spasmolytic, anti-inflammatory, analgesic, haemostatic, antidiabetic, cholagogue, antitumor, antioxidant, antifungal, antiseptic and liver protective effects. Furthermore, tea from *A. millefolium* is used to treat diseases of the gastrointestinal tract, like dyspepsia, flatulence, abdominal pain, diarrhea, stomachache and digestive complaints. In a double-blind randomized clinical trial, it has been shown that tea prepared from powder of the flowers of *A. millefolium* relieved the severity of pain in primary dysmenorrhea. *A. millefolium* can be consumed as essential oil, infusion or alcohol extract, decoction, hydroalcoholic, methanolic or aqueous extract [1,2,6–12].

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. [13] reported that the antioxidant properties are at least partly responsible for the gastroprotective effects of the extract. Furthermore, an *A. millefolium* extract of the aerial parts (hexane: ether:methanol (1:1:1, v/v/v)) 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].

A. millefolium is used in cosmetics as it has been proven in vivo that 2% *A. millefolium* extract has a rejuvenating effect on the appearance

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and feeling of the skin surface [14]. The extract is a biological ingredient in 65 cosmetic product formulations and creams used to accelerate the wound healing rate consisting of 2%, 5% or 10% *A. millefolium* extract [15,16]. Furthermore, there exist European national pharmacopoeia monographs about *A. millefolium*, such as the Hungarian, German, Austrian, Czech, French, and Romanian pharmacopoeias, extra pharmacopoeia Martindale, British herbal pharmacopoeia and the Polish herbal compendium [17].

Another plant belonging to the Asteracea family is *Achillea ptarmica* L. (sneezewort yarrow). Althaus et al. [18] 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) [18].

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 [19]. *Achillea* NAAs consist of the more widespread isobutylamides and phenethyl amides, and especially saturated and unsaturated 5- and 6-ring alkylamides (piperidides, pyrrolidides, piperideides, and 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 [2,3,20–22].

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 NAAs identified using gas chromatography–mass spectrometry (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 *A. millefolium* aggregate [2]. Greger and Hofer [23] identified 17 NAAs in *A. millefolium*, while Greger and Werner [24] identified two additional NAAs, namely undeca-2E,4E-diene-8,10-diynoic acid piperidide and tetradeca-2E,4E,12Z-triene-8,10-diynoic acid isobutylamide. 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 [17].

The main components of *A. ptarmica* are flavonoids, some essential oils and NAAs (carboxamides of olefinic and polyynic carboxylic acids with various amines) [18]. Kuropka et al. [25] and Althaus et al. [18] identified five and six NAAs in *A. ptarmica*, respectively.

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

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water 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 and HPLC gradient grade acetonitrile (ACN) were purchased from Fisher Scientific (Erembodegem, Belgium). Pellitorine was purchased from Adipogen Life Sciences (99.8% purity determined by HPLC).

2.2. Plant material and extraction

A. millefolium (type: Wesersandstein) and *A. ptarmica* (type: The Pearl) were bought at the 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 (m/v), ranging from 1/12 to 1/64 for *A. millefolium* and from 1/12 to 1/34 for *A. ptarmica*. As extraction solvent, denaturated ethanol: H_2O (90:10, v/v) was used. After maceration in darkness for approximately 48 h at room temperature (20–27 °C, 230 rpm), the plant parts were removed by filtration (Whatman, UK). Thereafter, the extraction solvent was removed using a rotavapor (Heidolph and Büchi), and protected from light. The extraction yield of *A. millefolium* and *A. ptarmica* was between 3%–8% (m/m) and 3%–11% (m/m), respectively. The extract was kept in the dark at 4 °C until analysis.

2.3. HPLC–UV/ESI–MS analysis

The HPLC–MS analyses were done on an 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 ACN:H_2O (50:50, 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 C_{18} column (250mm × 4.6 mm, 5 μm) using a Waters HPLC equipped with a Waters 2487 Dual Absorbance detector set at 260 nm. A linear gradient with a flow rate of 1.0 mL/min was applied as follows: 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), and t=151–166 min: A: B (80:20, 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 was estimated as 0.6% (m/m) in *A. ptarmica* and 0.2% (m/m) in *A. millefolium*.

2.4. 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). The mass detecter was operated in EI mode (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×wash post injection). An HP-5MS column (30 m × 0.25 mm, 0.25 μm) (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,

Table 1
Characteristic fragment ions of NAAs after CID fragmentation.

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

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 min. The total run time was 41 min. The split ratio was set at 10:1 (v/v). 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 *m/z* 40 and 550.

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 [26–28]. The typical fragment losses including the loss of the alkyl group directly attached to the amine; the loss of the entire amine functional group, resulting in an acylium ion; the loss of the amide portion of the molecule and saturation of one of the double bonds on the alkyl chain; and the loss of the amide portion, resulted 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 [29]. Furthermore, also with electron impact, characteristic product ions of spilanthol, as a prototypical NAA, are formed [30–32].

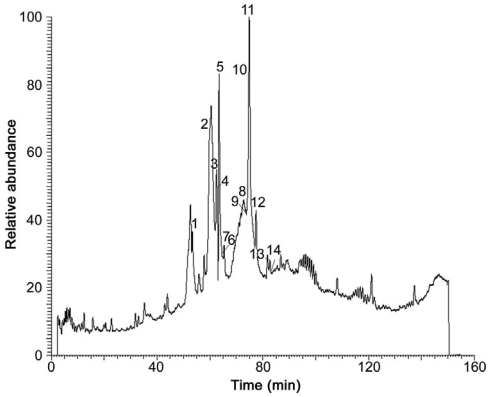


Fig. 1. TIC of *Achillea ptarmica* obtained using HPLC–ESI–MS.

3.1. A. ptarmica

3.1.1. N-alkylamide profiling using HPLC–ESI–MS

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

The major ions observed in the MS^1 spectra correspond to the protonated forms of NAAs. The MS^2 spectra are shown in Fig. 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 *A. ptarmica* extract: 6 NAAs having an isobutylamide function (compounds **P1**, **P5**, **P7**, **P10**, **P11** and **P12**), 3 NAAs with a piperidide function (saturated 6-ring $C_5H_{10}N$, compounds **P2**, **P9**, and **P13**), 1 NAA with a piperideide function (unsaturated 6-ring C_5H_8N, compound **P6**), 2 NAAs with a 2-methylbutylamide function (compounds **P3** and **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 NAAs varies in length (C10, C11, and 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 [26]. As can be seen in Table 2, NAAs with terminal alkynes elute early with reversed phase HPLC [29].

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 and 91 have been reported previously for undeca-2E,4E-diene-8,10-diynoic acid isobutylamide [26,28,33]. 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 were consistent with values reported in literature [28,34]. 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 [28]. 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 contained doubly allylic carbon atoms, there was 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) [34]. There were cleavages between C1-C2 (*m/z* 169),

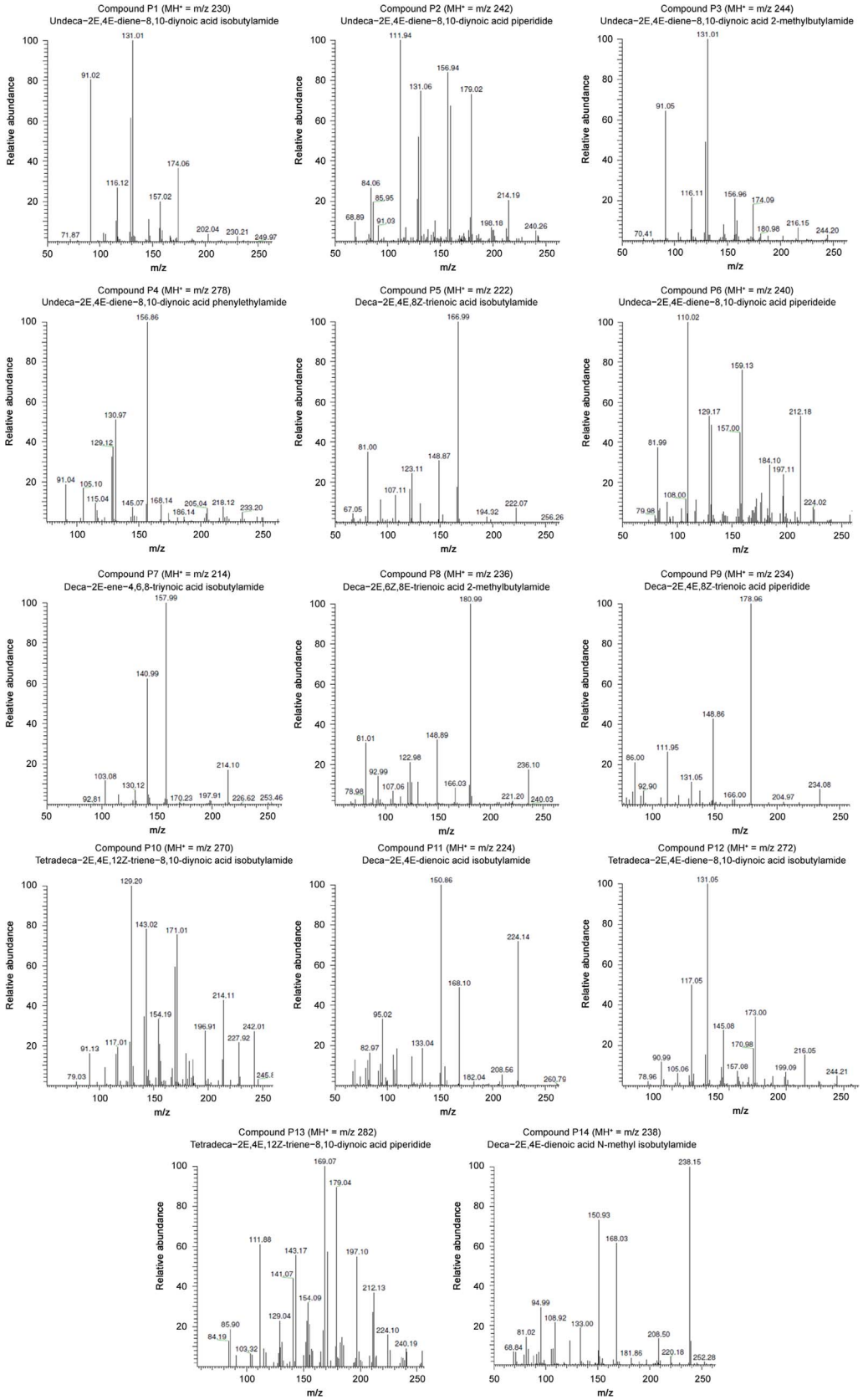


Fig. 2. MS^2 fragmentation spectra (CID) of *N*-alkylamides in the *A. ptarmica* extract.

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).

NAAs having a phenylethylamide alkyl group showed similarly formed fragments as the isobutylamide NAAs, i.e., the typical losses, which are presented in Table 1. The typical *m/z* values of these losses

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

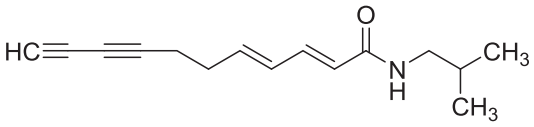
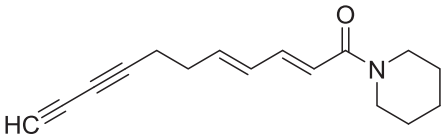
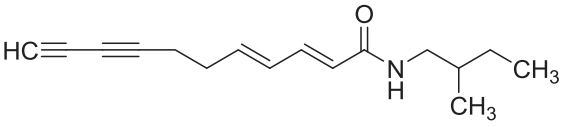
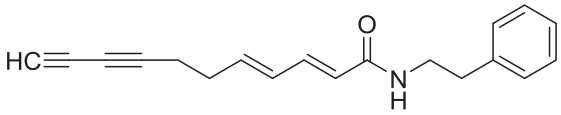
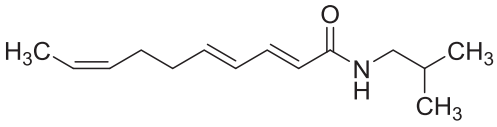
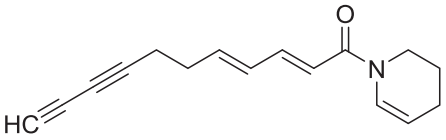
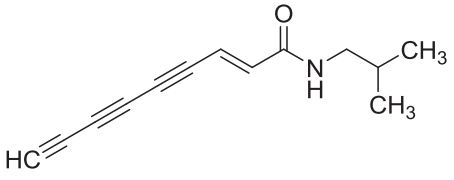
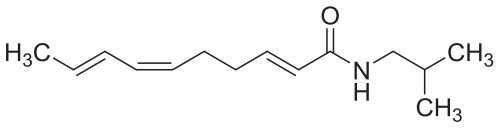
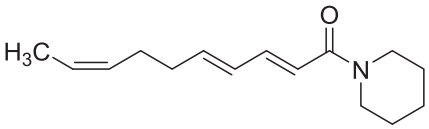
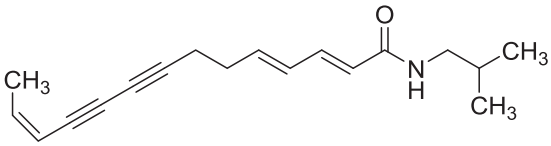
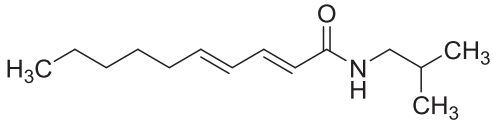
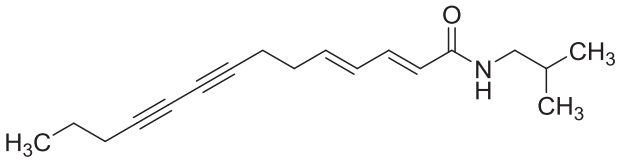
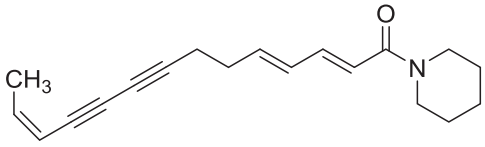
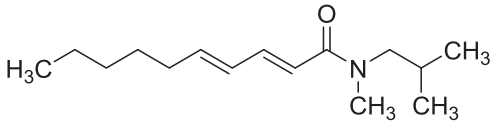
Compound	R_t (HPLC) (min)[a]	R_t (GC) (min)	Structure	Chemical name	MW (g/mol)	Classification
P1 (=M1)	53.4 [17.7%]	23.3		Undeca-2E,4E-diene-8,10-diynoic acid isobutylamide	229.32	F3M1
P2	59.7 [14.3%]	–		Undeca-2E,4E-diene-8,10-diynoic acid piperidide	241.33	F3M5
P3	62.4 [3.4%]	–		Undeca-2E,4E-diene-8,10-diynoic 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 *(continued)*

Compound	R_t (HPLC) (min)[a]	R_t (GC) (min)	Structure	Chemical name	MW (g/mol)	Classification
P10 (=M17)	74.5 [11.0%]	34.5		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.

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 were consistent with values reported earlier [28]. Moreover, assignment of the NAA was also based upon retention time comparison [28].

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 (Fig. 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 [27].

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 of compound 14 is shown in Fig. 4. 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 [28].

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 were 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-

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.

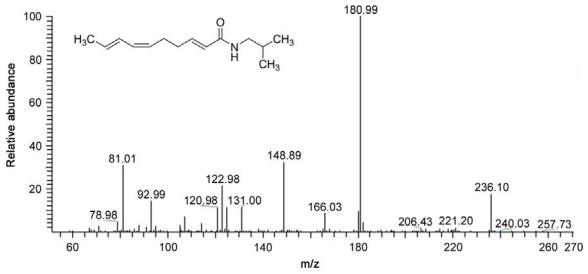


Fig. 3. MS^2 spectra of deca-2E,6Z,8E-trienoic acid 2-methylbutylamide or homospilanthol (compound P8) with $[M+H]^+$= *m/z* 236.

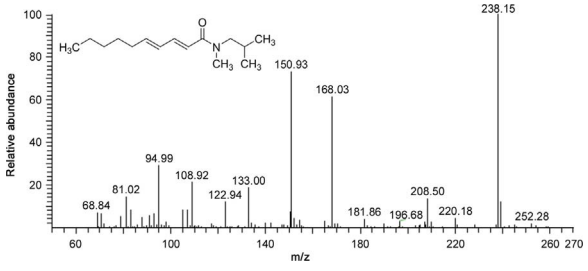


Fig. 4. MS^2 spectra of deca-2E,4E-dienoic acid *N*-methyl isobutylamide (compound P14) with $[M+H]^+$= *m/z* 238.

C7 (*m/z* 179) [34]. An acylium ion was also formed for 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) [34].

All the reported NAAs were found in the roots of *A. 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 the literature [35].

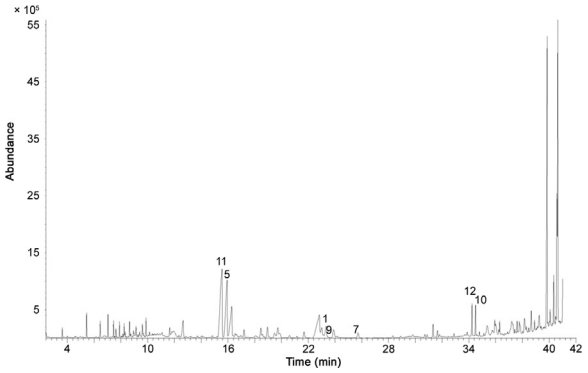


Fig. 5. TIC of *Achillea ptarmica* obtained using GC–EI–MS.

3.1.2. *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 Fig. 5, using the same numbers as indicated in HPLC-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

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: no % between brackets means intensity < 7% of base peak.
Product ions indicated in bold: characteristic product ions for the amide functional group of the NAA.

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. [2]. Compound **P1** was also recognized by the Nist library.

3.2. A. millefolium

3.2.1. N-alkylamide profiling using HPLC–ESI–MS

The TIC of the root extract of *A. millefolium* is shown in Fig. 6 in which peak labels correspond to NAA designations.

In the MS^1 spectra, the major ions are the protonated forms of the NAAs. MS^2 spectra are presented in Fig. 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

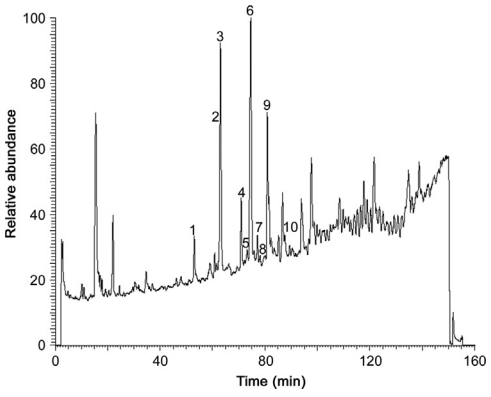


Fig. 6. TIC of *Achillea millefolium* obtained using HPLC–ESI–MS.

assigned in the *A. 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 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). For 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 were previously reported [26,28,33,34]. 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 [28]. 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) [34].

NAAs having a 4-hydroxyphenylethylamide also showed 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

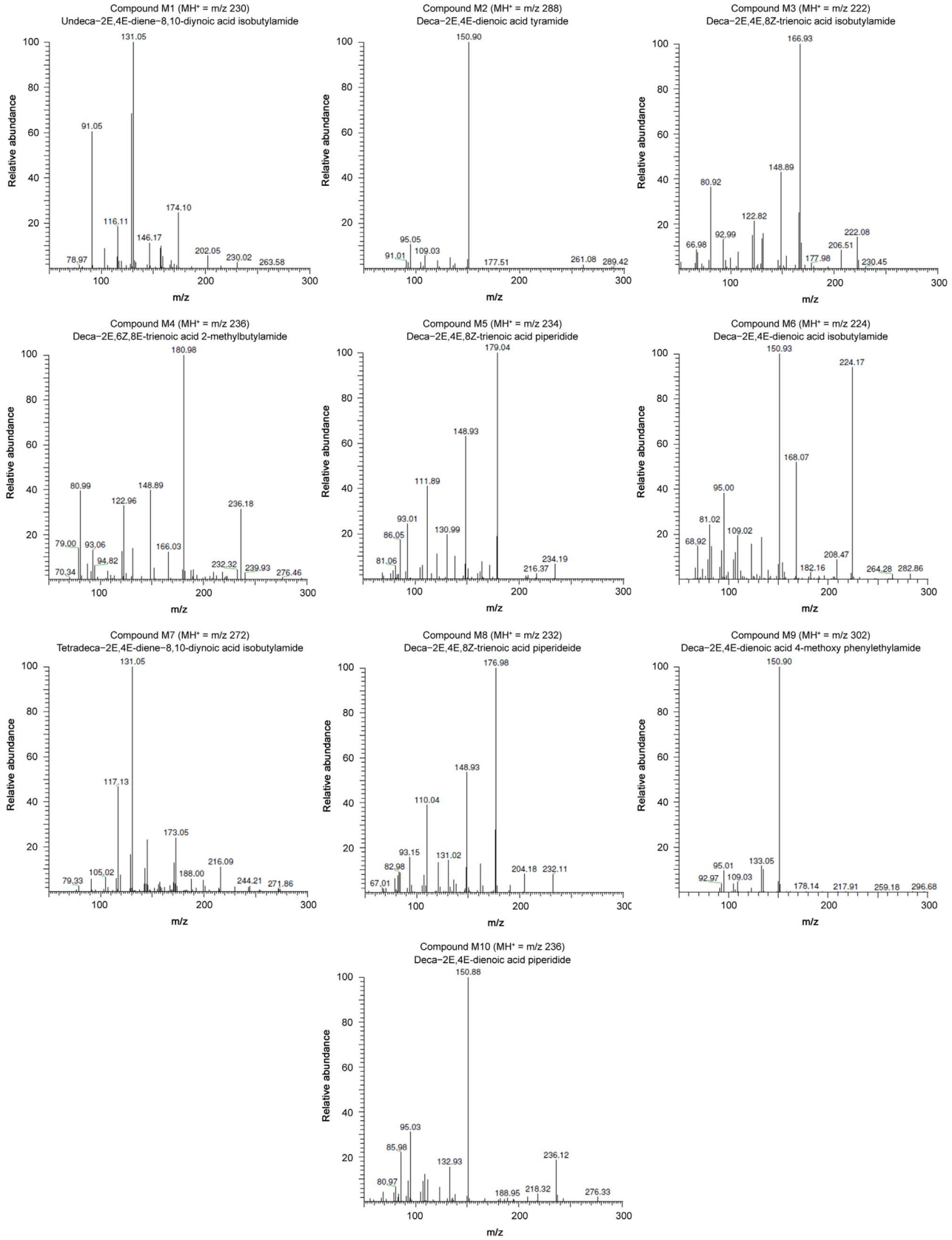


Fig. 7. MS^2 fragmentation spectra (CID) of *N*-alkylamides in the *A. millefolium* extract.

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

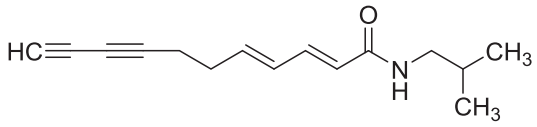
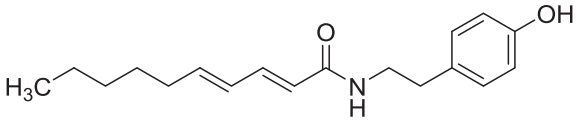
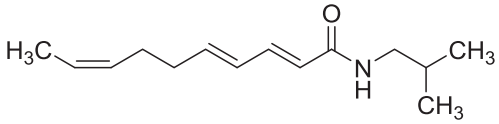
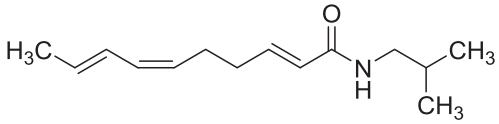
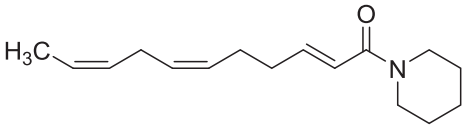
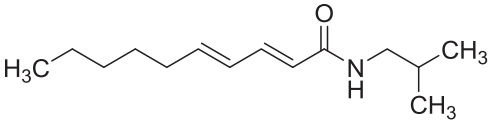
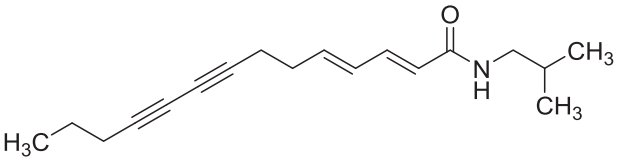
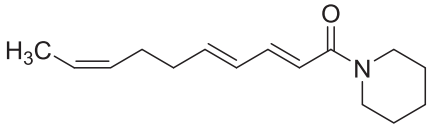
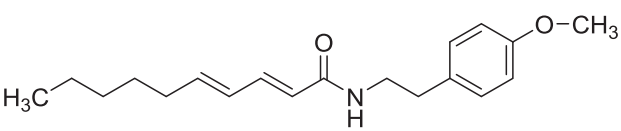
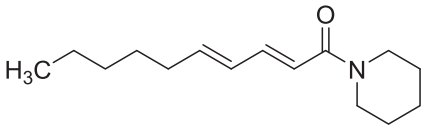
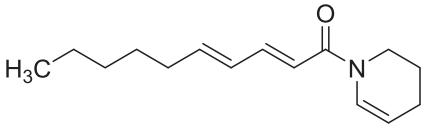
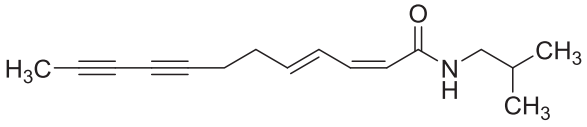
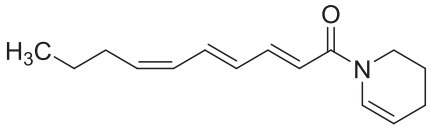
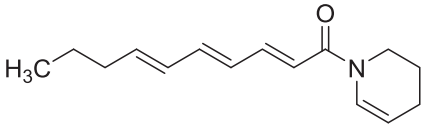
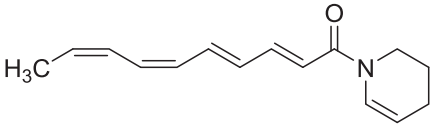
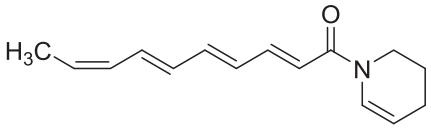
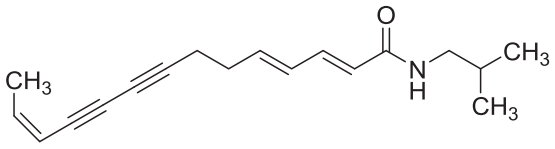
Compound	R_t (HPLC) (min)[a]	R_t (GC) (min)	Structure	Chemical name	MW (g/mol)	Classification
M1 (=P1)	53.1 [13.7%]	23.3		Undeca-2E,4E-diene -8,10-diynoic acid isobutylamide	229.32	F3M1
M2	62.9 [21.0%]	–		Deca-2E,4E-dienoic acid tyramide	287.40	F3M12
M3 (=P1)	63.1 [86.6%]	15.8		Deca-2E,4E,8Z-trienoic 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

Table 5 *(continued)*

Compound	R_t (HPLC) (min)[a]	R_t (GC) (min)	Structure	Chemical name	MW (g/mol)	Classification
M10	87.6 [5.4%]	–		Deca-2E,4E-dienoic acid piperidide	235.37	F3M5
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.

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.

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 [28].

Characteristic fragment ions for NAAs with a 4-methoxy phenylethylamide function are described in Table 1 and 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).

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 Fig. 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) [34]. 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 was also the formation of a cationic species (C5-C6+ loss of H, *m/z* 162) [34]. An acylium ion was also formed in case of compound **M8**, with a *m/z* value of 149 (loss of *m/z* 83).

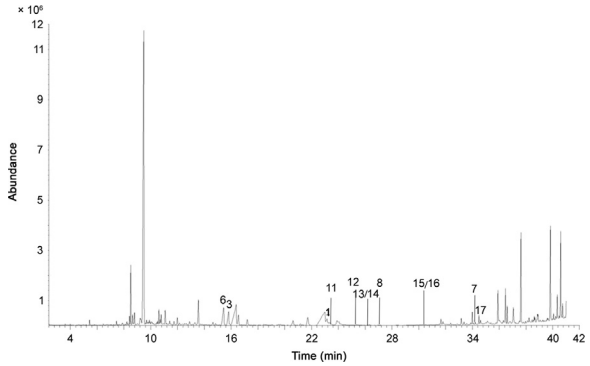


Fig. 8. TIC of *Achillea millefolium* obtained using GC–EI–MS.

The previously mentioned NAAs in *A. 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.

3.2.2. *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 Fig. 8, with the same numbers as indicated in HPLC-MS. Additional compounds assigned using GC–MS were indicated starting numbering from 11.

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 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** and **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, i.e., for compound **M3**: *m/z* 81; for

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%)
M13/14	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
M15/16	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: no % between brackets means intensity < 7% of base peak.
Product ions indicated in bold: characteristic product ions for the amide functional group of the NAA.

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 the literature for pellitorine [2]. 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.

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 nuclear magnetic resonance (NMR) and synthetic standards. In the *A. ptarmica* extract, a total of 14 NAAs 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 reported in *A. 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 *A. 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** and **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 or a related isomeric compound has been assigned in *A. ptarmica* and *A. millefolium*.

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