Original Research Article

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

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\textbf{ABSTRACT}

Achillea millefolium and Achillea ptarmica are both plants belonging to the Asteraceae 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 millefolium, 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 spasmodytic, anti-inflammatory, analgesic, haemostatic, antidiabetic, chologogue, antitumor, antioxidant, antifungal, antisepptic 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
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 Asteraceae 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 isobutyramides and phenethyl amides, and especially saturated and unsaturated 5- and 6-ring alkylamines (piperidides, pyrrolidides, and pyrrolidides). C10-, C11- and C14-olefinic and acetylenic alkylamines 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 piperidide function. 2E,4E,6Z-decatrienoic acid piperidide 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 piperidide and 2,6-decatrienoic acid piperidide are NAAs identified using gas chromatography–mass spectrometry (GC–MS) in the roots of A. distans Wildl. subsp. distans; however, the correct stereoisomer could not be determined. A. distans Wildl. subsp. distans belongs to the A. millefolium aggregate [2]. Greger and Hofer [23] identified 17 NAAs in A. millefolium, while Greger and Wernert [24] identified two additional NAAs, namely undeca-2E,4E-diene-8,10-diynoic acid piperidide and tetradeca-2E,4E,12Z,14Z-diynoic acid isobutylamide. Besides NAAs, other compounds present in A. millefolium L. are volatile oils, sesquiterpene lactones, vitamins, alkanes, alkaloids and bases, saponins, sterols, sugars, 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 polyenic carboxylic acids with various amines) [18], Kuroيكا 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 denatured ethanol (95% ethanol denaturated with 5% diethylther) 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_{2}O (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_{2}O (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 C18 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_{2}O 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 detector 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,
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 quadrupole temperature were 150 °C and 230 °C, respectively. Helium (Air Products and Chemicals, 
v). The MS2 spectra are shown in Fig. 2, while
the total ion chromatogram (TIC) of the root extract of
3. Results and discussion
A. ptarmica

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].

1. A. ptarmica

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 (Rt), 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_{12}H_{23}N, compounds P2, P9, and P13), 1 NAA with a piperide function (unsaturated 6-ring C_{12}H_{23}N, compound P6), 2 NAAs with a 2-methylbutylamide function (compounds P3 and P8), 1 NAA with a phenylbutyamide 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-dynoic 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-dynoic 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),
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-diyynoic acid isobutyramid). NAAs having a phenylethylamide alkyl group showed similarly formed fragments as the isobutyramid NAAs, i.e., the typical losses, which are presented in Table 1. The typical m/z values of these losses

Fig. 2. MS² fragmentation spectra (CID) of N-alkylamides in the A. ptarmica extract.
| Compound | \( R_t \) (HPLC) (min) \[\text{a} \] | \( R_t \) (GC) (min) | Structure | Chemical name | MW (g/mol) | Classification |
|----------|---------------------------------|-----------------|-----------|--------------|-------------|----------------|
| P1 (=M1) | 53.4 [17.7%]                    | 23.3            | [Diagram] | Undeca-2E,4E-diene-8,10-diynoic acid isobutylamide | 229.32 | F3M1          |
| P2       | 59.7 [14.3%]                    | –               | [Diagram] | Undeca-2E,4E-diene-8,10-diynoic acid piperidide  | 241.33 | F3M5          |
| P3       | 62.4 [3.4%]                     | –               | [Diagram] | Undeca-2E,4E-diene-8,10-diynoic acid 2-methylbutylamide | 243.35 | F3M1          |
| P4       | 62.6 [0.1%]                     | –               | [Diagram] | Undeca-2E,4E-diene-8,10-diynoic acid phenylethylamide | 277.37 | F3M11         |
| P5 (=M3) | 63.4 [73.6%]                    | 15.9            | [Diagram] | Deca-2E,4E,8Z-trienoic acid isobutylamide (8,9-dehydropellitorine) | 221.34 | F3M1          |
| P6       | 65.0 [1.5%]                     | –               | [Diagram] | Undeca-2E,4E-diene-8,10-diynoic acid piperidide  | 239.32 | F3M5          |
| P7       | 65.4 [7.0%]                     | 25.6            | [Diagram] | Deca-2E-ene-4,6,8-trienoic acid isobutylamide | 213.28 | F3M1          |
| P8 (=M4) | 72.5 [3.4%]                     | –               | [Diagram] | Deca-2E,6Z,8E-trienoic acid 2-methylbutylamide (homospilanthol) | 235.37 | F3M1          |
| P9 (=M5) | 73.6 [1.3%]                     | 23.4            | [Diagram] | Deca-2E,4E,8Z-trienoic acid piperidide  | 233.35 | F3M5          |
for compound P4 (undeca-2E,4E-diene-8,10-diyanoic 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 shown 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 MS² 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-diyanoic acid piperidide), P9 (deca-2E,4E,8Z-trienoic acid piperidide) and P13 (tetra-deca-2E,4E,12Z-triene-8,10-diyanoic acid piperidide), 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 2 (continued)

| Compound | R<sub>t</sub> (HPLC) | R<sub>t</sub> (GC) | Structure | Chemical name | MW (g/mol) | Classification |
|----------|------------------|----------------|-----------|---------------|------------|----------------|
| P10 (=M17) | 74.5 [11.0%] | 34.5 | | Tetradeca-2E,4E,12Z-triene-8,10-diyanoic 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-diyanoic acid isobutylamide (anacycline) | 271.40 | F3M1 |
| P13 | 82.4 [1.5%] | – | | Tetradeca-2E,4E,12Z-triene-8,10-diyanoic acid piperidide | 281.39 | F3M5 |
| P14 | 83.3 [4.6%] | – | | Deca-2E,4E-dienoic acid N-methyl isobutylamide | 237.38 | F3M1 |

*: not applicable

* Between brackets: estimated relative quantity to pellitorine from total ion chromatogram.
conclusion, the highest amount of NAAs was found in the roots, which
found in the stem as well (data not shown). No NAAs could be found in
observed in the leaves, while compounds
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Furthermore, compounds
P1
P4
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14

P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14

Table 3
MS1 and MS2 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; 146; 133; 129; 128; 123; 117; 116; 115; 105; 98; 93; 91; 79; 72 |
| P2 242   | 222; 214; 198; 179; 176; 159; 157; 145; 131; 129; 112; 91; 86; 84; 69 |
| P3 244   | 216; 181; 174; 157; 146; 131; 129; 116; 103; 91; 70 |
| P4 278   | 245; 218; 205; 186; 174; 168; 157; 145; 131; 129; 128; 115; 105; 91 |
| P5 222   | 194; 168; 167; 152; 149; 132; 121; 110; 107; 100; 93; 91; 81; 67 |
| P6 240   | 224; 212; 184; 177; 159; 157; 155; 129; 110; 108; 82; 80 |
| P7 214   | 198; 172; 159; 158; 141; 130; 115; 103; 89; 72 |
| P8 236   | 221; 182; 181; 180; 166; 151; 149; 138; 125; 123; 121; 107; 95; 93; 81; 79 |
| P9 234   | 205; 179; 166; 149; 131; 112; 93; 86 |
| P10 270  | 246; 242; 228; 214; 197; 171; 169; 154; 143; 129; 117; 105; 91; 79 |
| P11 224  | 209; 204; 182; 168; 154; 151; 140; 133; 123; 109; 105; 95; 83; 69 |
| P12 272  | 244; 216; 199; 173; 171; 167; 145; 131; 117; 91; 81 |
| P13 282  | 254; 240; 224; 212; 197; 179; 169; 165; 141; 143; 129; 112; 103; 86; 84 |
| P14 238  | 220; 210; 209; 197; 182; 168; 151; 133; 109; 95; 81; 69 |

In bold: characteristic product ions or losses.

Fig. 3. MS2 spectra of deca-2E,6Z,8E-trienoic acid 2-methylbutylamide or homospilanthol (compound P8) with [M+H]+ = m/z 236.

Fig. 4. MS2 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 piperidine 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 flowers 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].
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 MS² spectra, the major ions are the protonated forms of the NAAs. MS² spectra are presented in Fig. 7. All the NAAs assigned in A. millefolium are summarised in Table 5 with their corresponding retention time (R₂), structure, chemical name, molecular weight (MW, average mass) and classification. Structures of ten NAAs were 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 piperideide 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-methoxyphenylethylamide 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-dionoyl isobutylamide), M3 (deca-2E,4E-diene-8,10-dionoyl isobutylamide), compound M5 (tetradeca-2E,4E-diene-8,10-dionoyl isobutylamide) and M7 (tetradeca-2E,4E-diene-8,10-dionoyl isobutylamide or pomolicyline). 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. MS² 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<sub>t</sub> (HPLC) (min)<sup>a</sup> | R<sub>t</sub> (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 piperidide | 231.34 F3M5 |
| M9        | 80.9 [30.3%]                         | –                       |           | Deca-2E,4E-dienoic acid 4-methoxy phenylethylamide | 301.43 F3M12 |
| Compound | \( R_t \) (HPLC) (min) \(^a\) | \( R_t \) (GC) (min) | Structure | Chemical name | MW (g/mol) | Classification |
|----------|-----------------|-----------------|---------|---------------|-----------|----------------|
| M10      | 87.6 [5.4%]     | –               | [Structure Image] | Deca-2E,4E-dienoic acid piperidide | 235.37    | F3M5           |
| M11      | –               | 23.4            | [Structure Image] | Deca-2E,4E-dienoic acid piperideide | 233.35    | F3M5           |
| M12      | –               | 25.3            | [Structure Image] | Dodeca-2Z,4E-diene >8,10-dynoic acid isobutylamide | 243.35    | F3M1           |
| M13      | –               | 26.3            | [Structure Image] | Deca-2E,4E,6Z-trienoic acid piperidide | 231.34    | F3M5           |
| M14      | –               | 26.3            | [Structure Image] | Deca-2E,4E,6E-trienoic acid piperidide | 231.34    | F3M5           |
| M15      | –               | 30.3            | [Structure Image] | Deca-2E,4E,6Z,8Z-tetraenoic acid piperideide | 229.32    | F3M5           |
| M16      | –               | 30.3            | [Structure Image] | Deca-2E,4E,6E,8Z-tetraenoic acid piperidide | 229.32    | F3M5           |
| M17 (=P10) | –               | 34.5            | [Structure Image] | Tetradeca-2E,4E,12Z-triene-8,10-dynoic acid isobutylamide | 269.39    | F3M1           |

\(^a\) Between brackets: estimated relative quantity to pellitorine from total ion chromatogram.

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":
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,6Z,8Z-tetraenoic acid piperideide). 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-methylbutyamide (homosplianthol) 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 piperideide function, namely compounds M5 (deca-2E,4E,8Z-trienoic acid piperideide) and M10 (deca-2E,4E-dienoic acid piperideide), there was the formation of acyl 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 acyl cation 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 acyl cation was also formed in case of compound M8, with a \( m/z \) value of 149 (loss of \( m/z \) 83).

The previously mentioned NAAs in 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-dinyoic acid isobutylamide), M13 (deca-2E,4E,6Z-trienoic acid piperideide) and M17 (tetradeca-2E,4E,12Z-triene-8,10-dinyoic 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 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, \( \alpha \)-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 \( \alpha \)-rearrangement between C4-C5 in the fatty acid chain, i.e., for compound M3: \( m/z \) 81; for...
compound M6: m/z 83; for compound M17: m/z 129; for compound M8: m/z 81; for compound M11: m/z 83; and for compound M13/14: m/z 81.

Compound M6 in the A. millefolium extract corresponds to compound P11 in the A. ptarmica extract and product ions were already described in the literature for pellitorine [2]. Moreover, the MS spectra corroborate well with the structure of homospilanthol or a related isomeric compound. In the A. millefolium extract, 15 NAAs were assigned: six NAAs having a 4-hydroxyphenylethylamide function, one NAA with a 4-hydroxyphenylpropylamide function, one NAA with a phenylethylamide function, one NAA having a piperidine function and one NAA having a N-methylisobutylamide 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-methylisobutylamide, respectively. In the A. millefolium extract, 15 NAAs were assigned: six NAAs having an isobutylamide function, one NAA with a 4-hydroxyphenylethylamide function, one NAA with a 2-methylbutylamide function, two NAAs having a piperidine function, four NAAs with a piperidine 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 with 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 the 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.

### 4. Conclusion

In this research, the N-alcaloid profiling in two ethanolic plant extracts of the Achillea genus, namely Achillea ptarmica and Achillea millefolium, was performed using 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 piperidine function, two NAAs with a 2-methylbutylamide function, one NAA with a phenylethylamide function, one NAA having a piperidine function and one NAA having a N-methylisobutylamide function. Using both analytical
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