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Integration of non-targeted metabolomics and automated determination of elemental compositions for comprehensive alkaloid profiling in plants

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ABSTRACT

Plants produce a large array of specialized metabolites to protect themselves. Among these allelochemicals, alkaloids display highly diverse and complex structures that are directly related to their biological activities. Plant alkaloid profiling traditionally requires extensive and time-consuming sample preparation and analysis. Herein, we developed a rapid and efficient approach for the comprehensive profiling of alkaloids in plants using ultrahigh performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS)-based metabolomics. Using automated compound extraction and elemental composition assignment, our method achieved > 83% correct alkaloid identification and even > 90% for medium to high intensity peaks. This represented a significant improvement in identification rate compared to generic methods used for EC determination with no *a priori*, such as in untargeted metabolomics studies. The developed approach was then applied to identify specific alkaloids of *Aconitum lycoctonum* L. and *A. napellus* L. (Ranunculaceae) using different parts of the plant (leaf, perianth and pollen). Significant differences in alkaloid profiles between the two species were highlighted and discussed under taxonomic and evolutionary perspectives. Taken together, the presented approach constitutes a valuable chemotaxonomic tool in the search for known and unknown alkaloids from plants.

1. Introduction

Alkaloids are naturally occurring organic compounds that constitute the largest class among the nitrogen containing specialized (often referred to as secondary) metabolites with more than 31,000 compounds already identified (Wink, 1993; Roberts and Wink, 1998; Dictionary of Natural Products database). They are widely distributed in the plant kingdom, especially among angiosperms (more than 20% of all species produce alkaloids) but are also found to a lesser extent in microorganisms and animals (Blum, 1981; Rosenthal and Berenbaum, 1991, 1992; Harborne, 1993; Wink, 1993; Roberts and Wink, 1998). The extraordinary variety and complexity of alkaloid structures as well as their biological properties have long intrigued scientists in several research fields, including ecology, chemistry, toxicology and pharmacology. While humans have long recognized their potential as medicines (e.g. quinine, colchicine) or drugs (e.g. nicotine, cocaine), it is now largely assumed that plants produce alkaloids to protect themselves from various predators including herbivores and pathogens (see e.g. Baldwin, 1988; Bennett and Wallsgrove, 1994; Wink, 1993; Roberts and Wink, 1998; Yang and Stöckigt, 2010). Some alkaloids are also used by plants as herbicides against competing plants (Harborne, 1993; Wink, 1988, 1993). Such chemical defence shapes biological interactions at different trophic levels and then ecological networks (Adler et al., 2001).

Alkaloids are traditionally profiled in plants after extensive sample preparation, which yields extracts that are almost free of other metabolites. Yet, such procedures are relatively time-consuming and difficult to automate as they involve several steps of liquid-liquid partitioning and acid-base extraction. An attractive alternative is to profile alkaloids directly from crude (e.g. methanolic) extracts using non-targeted liquid chromatography-mass spectrometry (LC-MS)-based approaches (Gosselin et al., 2013; Leuthardt et al., 2013; Lucchetti et al., 2016). However, this creates an issue of how to rapidly determine which peak is an alkaloid and which is not, a process sometimes referred to as dereplication (Hubert et al., 2017). The nitrogen rule has often been perceived as a possible tool for alkaloid detection but it is not an infallible method since it fails to detect alkaloids containing an even number of nitrogen atoms and it is unreliable for masses higher than 500 Da (Kind and Fiehn, 2007). Another more powerful option is to use high-resolution mass spectrometry (HRMS), which provides accurate measurements of mass-to-charge (m/z) ratios and of relative isotope abundances for the determination of alkaloid-like elemental

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compositions (ECs). Yet, whilst well-established metabolomics workflows exist for LC-MS analysis and data pre-processing such as peak detection (Smith et al., 2006; Xia et al., 2015; Pluskal et al., 2010), metabolite annotation and/or identification are still regarded as major bottlenecks in metabolomics research (Dias et al., 2016; Weber et al., 2017). In recent years, various approaches for automated structure elucidation have been developed (Dührkop et al., 2015; Tsugawa et al., 2016; Allen et al., 2015; Ridder et al., 2013), but information on their performances for alkaloid detection in complex plant extracts is still limited. In this context, we postulated that having a tool that would enable us to specifically and automatically retrieve alkaloids from metabolomics peak lists based on EC determination would greatly enhance our capacity to profile alkaloids in complex plant matrices.

Here we present an innovative approach for the comprehensive profiling of alkaloids in plant extracts based on the following steps: (i) analysis of crude extracts by non-targeted UHPLC-HRMS metabolomics, (ii) extraction of all markers from the metabolic profiles including alkaloids and non-alkaloids using both commercial and open-access metabolomics softwares, and (iii) rapid and efficient detection of putative alkaloids based on optimized criteria for automated determination of ECs containing C, H, N, and O atoms. To test and validate our method, we selected the alkaloid-containing plant *Aconitum lycoctonum* L. (Ranunculaceae) since the *Aconitum* genus (monkshood) has been extensively studied and recognized as a rich source of structurally diverse and complex C_{18}, C_{19} and C_{20} type diterpenoid alkaloids (Puschner et al., 2004; Xiao et al., 2006), with at least 421 diterpenoid alkaloids isolated from 84 species (Xiao et al., 2006). Finally, as the alkaloid mixture is known to vary among *Aconitum* species (Ralphs et al., 1997) and plant parts (Gosselin et al., 2013; Rawat et al., 2014), the developed approach was applied as a chemotaxonomic tool to identify specific alkaloids of *Aconitum lycoctonum* L. (Ranunculaceae) and *Aconitum napellus* L. (Ranunculaceae) using different parts of the plant (leaf, perianth and pollen).

2. Results and discussion

2.1. UHPLC-HRMS profiling

The main aim of this study was to evaluate the feasibility of using non-targeted metabolomics for the profiling and identification of alkaloids in plants. First, a generic analytical method was developed to analyse crude leaf extracts from *A. lycoctonum* by UHPLC-HRMS (Fig. 1). An HRMS system that has been shown to provide high accuracy of mass measurements (< 2 ppm in routine) and of relative isotope abundances (< 3%) (Glauser et al., 2013) was selected and operated in positive electrospray ionization. For separation, a reversed phase C18 column and mobile phases consisting of water and acetonitrile acidified with formic acid 0.05% to increase ionization in positive mode were employed. The UHPLC-HRMS profile was first processed using the commercial software MarkerLynx XS. Feature (i.e. variable characterized by retention time and *m/z* ratio) detection was performed using generic parameters (Gaillard et al., 2018) but two notable distinctive characteristics, namely retention time range (0.70–4.50 min) and mass range (150–900 Da). These ranges were selected to cover most alkaloids while excluding possibly interfering compounds such as amino acids or phosphatidylcholines/phosphatidylethanolamines. The peak list was then deisotoped and Na^+ and K^+ adducts were automatically removed from the dataset. This provided a list of 619 features in total detected in the whole *Aconitum lycoctonum* leaf extract (Table S1).

2.2. Automated determination of elemental compositions

For optimisation of the automated determination of ECs in MarkerLynx, several parameters were optimized in an empirical manner (i.e. trial-and-error process), such as the nature and number of atoms, the mass tolerance, the electron state and the number of isotopic peaks to be used for spectral accuracy matching (i-FIT™). The number of non-specific atoms (i.e. C, H, and O atoms) was set to cover most natural products (Iijima et al., 2008). In contrast, the range of N (0–3) atoms was found to be critical to minimize wrong assignment. Indeed, forcing the number of N to at least one would generate numerous false positive hits within non-alkaloids, whereas increasing the number of N to more than 3 would increase wrong assignments among alkaloids. Altogether, the selected range of 0–3 N atoms encompasses well the diversity of alkaloids encountered in nature since more than 87% of all reported alkaloids and more than 99% of diterpenoid alkaloids contain less than 4 nitrogen atoms, according to the Dictionary of Natural Products (DNP). Furthermore, it was also advantageous to include 1 Na atom in the list of elements to reduce the probability of false positive assignment, although it slightly increased false negative assignment. Another important parameter was the mass tolerance; we chose a quite conservative window of 4 ppm to prevent any risk of overlooking alkaloids. Furthermore, only even ions were accepted while odd ions were discarded in order to detect mostly ions of the molecular species but no fragments. Finally, the number of peaks for isotopic pattern determination was set to 3 (i.e. M, M + 1 and M + 2) as the best compromise between statistics (the more peaks the better) and peak abundance (M + 3, M + 4 habitually display too low intensities in small molecules).

After setting the optimal parameters in MarkerLynx, a threshold of 800 counts was applied, since peaks of lower intensity could not be reliably assessed due to too weak ion statistics. The obtained list of elemental compositions (i.e. 354 markers remaining) was further processed by removing ECs that displayed i-FIT™ values > 0.3. Actually,

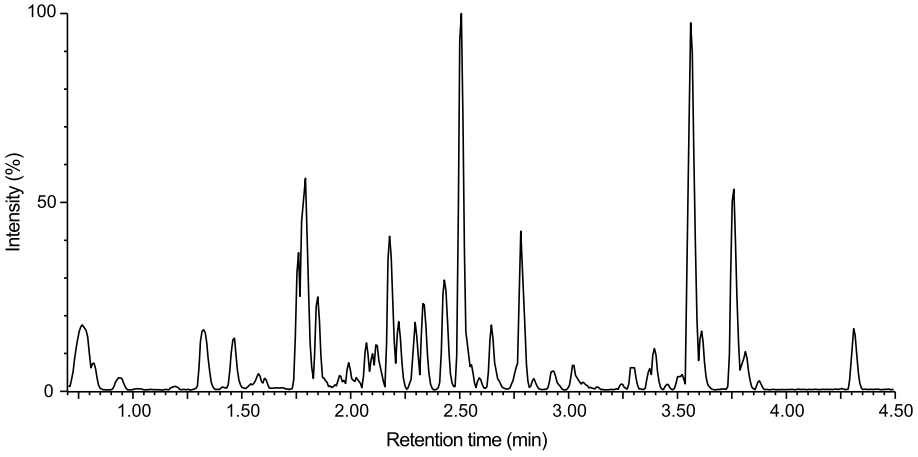

Fig. 1. UPLC-HRMS profile of *Aconitum lycoctonum* leaf extract.

Table 1
Validation of the automated assignment of elemental compositions of all the alkaloid candidates (n = 74) obtained with the optimized MarkerLynx method. In bold, correct assignment; in bold italic, wrong assignment but correct EC (i.e. false-positives); in regular, wrong assignment and incorrect EC (i.e. false-positives). Molecular formulae generated by the generic method are also listed.

Marker ID	*m/z*	Rt (min)	Intensity (counts)	MarkerLynx molecular formula Generic method	MarkerLynx molecular formula Optimized method	Additional Comments
ALK-1	669.3379	3.58	184,816	**$C_{37}H_{47}N_3O_7Na$**	**$C_{36}H_{48}N_2O_{10}$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-2	468.2944	2.50	180,170	**$C_{25}H_{41}NO_7$**	**$C_{25}H_{41}NO_7$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-3	697.3323	3.77	107,209	**$C_{37}H_{48}N_2O_{11}$**	**$C_{37}H_{48}N_2O_{11}$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-4	388.2124	1.75	61,663	**$C_{22}H_{29}NO_5$**	**$C_{22}H_{29}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-5	496.2914	2.78	52,469	**$C_{26}H_{41}NO_8$**	**$C_{26}H_{41}NO_8$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-6	454.2815	1.85	44,320	$C_{25}H_{38}N_2O_4Na$	**$C_{24}H_{39}NO_7$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-7	342.1706	2.22	33,167	**$C_{20}H_{23}NO_4$**	**$C_{20}H_{23}NO_4$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-8	524.2851	2.64	30,485	$C_{25}H_{39}N_4O_8$	**$C_{27}H_{41}NO_9$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-9	697.3315	3.62	30,237	$C_{35}H_{61}N_2P_5S$	**$C_{37}H_{48}N_2O_{11}$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-10	683.3523	4.32	25,920	**$C_{37}H_{50}N_2O_{10}$**	**$C_{37}H_{50}N_2O_{10}$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-11	472.2703	3.39	20,826	**$C_{27}H_{37}NO_6$**	**$C_{27}H_{37}NO_6$**	Reported as alkaloid in other plant genera (DNP)
ALK-12	334.1507	1.32	19,374	***$C_{14}H_{23}NO_8$***	***$C_{14}H_{23}NO_8$***	Correct EC but alternative ID: Ethyl 3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside
ALK-13	669.3373	3.81	17,939	$C_{39}H_{50}O_8Na$	**$C_{36}H_{48}N_2O_{10}$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-14	655.3218	3.02	12,036	**$C_{35}H_{46}N_2O_{10}$**	**$C_{35}H_{46}N_2O_{10}$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-15	328.1916	3.38	11,182	**$C_{20}H_{25}NO_3$**	**$C_{20}H_{25}NO_3$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-16	496.2901	2.55	11,062	**$C_{26}H_{41}NO_8$**	**$C_{26}H_{41}NO_8$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-17	458.2546	2.93	10,170	$C_{24}H_{33}N_4O_5$	**$C_{26}H_{35}NO_6$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-18	346.2015	0.95	9541	**$C_{20}H_{27}NO_4$**	**$C_{20}H_{27}NO_4$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-19	482.2752	2.10	7667	**$C_{25}H_{39}NO_8$**	**$C_{25}H_{39}NO_8$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-20	388.2134	1.95	7473	$C_{23}H_{29}N_2O_2Na$	**$C_{22}H_{29}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-21	456.276	3.52	7435	$C_{26}H_{42}O_3NaP$	**$C_{27}H_{37}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-22	472.2708	3.52	7084	$C_{23}H_{40}N_2O_6P$	**$C_{27}H_{37}NO_6$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-23	543.2417	2.10	6509	$C_{21}H_{38}N_2O_{14}$	$C_{21}H_{38}N_2O_{14}$	Incorrect EC (alternative EC: $C_{24}H_{40}O_{12}Na$)
ALK-24	430.2598	2.84	5876	$C_{26}H_{35}N_2O_2Na$	**$C_{25}H_{35}NO_5$**	Reported as alkaloid in other plant genera (DNP)
ALK-25	492.2388	3.50	5575	$C_{25}H_{36}N_2O_6P$	**$C_{29}H_{33}NO_6$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-26	476.2437	3.61	4913	$C_{27}H_{31}N_4O_4$	**$C_{29}H_{33}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-27	372.2174	1.75	4906	$C_{16}H_{35}O_7S$	**$C_{22}H_{29}NO_4$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-28	501.1873	1.96	4903	$C_{27}H_{35}O_3P_3$	**$C_{25}H_{28}N_2O_9$**	Reported as alkaloid in other plant genera (DNP)
ALK-29	501.1877	1.89	4700	$C_{24}H_{33}NO_7NaP$	**$C_{25}H_{28}N_2O_9$**	Reported as alkaloid in other plant genera (DNP)
ALK-30	458.2552	3.07	4440	$C_{22}H_{38}N_2O_6P$	**$C_{26}H_{35}NO_6$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-31	492.2377	3.60	4225	$C_{25}H_{36}N_2O_6P$	**$C_{29}H_{33}NO_6$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-32	482.2764	1.95	3896	$C_{26}H_{39}N_2O_5Na$	**$C_{25}H_{39}NO_8$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-33	454.2808	2.03	3860	**$C_{24}H_{39}NO_7$**	**$C_{24}H_{39}NO_7$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-34	450.2271	2.95	3812	$C_{28}H_{31}N_2O_2Na$	**$C_{27}H_{31}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-35	330.2069	1.89	3728	$C_{17}H_{30}N_3NaP$	**$C_{20}H_{27}NO_3$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-36	685.3326	3.06	3474	**$C_{36}H_{48}N_2O_{11}$**	**$C_{36}H_{48}N_2O_{11}$**	Reported as alkaloid in other plant genera (DNP)
ALK-37	314.1757	2.49	3269	$C_{17}H_{21}N_4O_2$	**$C_{19}H_{23}NO_3$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-38	342.118	1.18	2724	$C_{13}H_{17}N_4O_7$	***$C_{15}H_{19}NO_8$***	Correct EC but alternative ID: N-caffeoylglucosamine
ALK-39	442.2597	3.00	2690	$C_{23}H_{38}N_3O_2NaP$	**$C_{26}H_{35}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-40	541.1535	1.54	2689	$C_{18}H_{33}NO_{14}NaP$	$C_{19}H_{28}N_2O_{16}$	Incorrect EC (alternative EC: $C_{22}H_{30}O_{14}Na$)
ALK-41	314.1761	2.91	2602	$C_{20}H_{23}N_2Na$	**$C_{19}H_{23}NO_3$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-42	372.1287	1.80	2425	$C_{16}H_{24}N_2O_4PS$	***$C_{16}H_{21}NO_9$***	Correct EC but alternative ID: ammonium adduct of *m/z* 355.1032
ALK-43	366.0666	0.76	2412	$C_{13}H_{15}N_2O_9Na$	***$C_{12}H_{15}NO_{12}$***	Correct EC but alternative ID: Ammonium adduct of *m/z* 349.0418
ALK-44	480.2946	2.75	2065	$C_{30}H_{43}NP_2$	**$C_{26}H_{41}NO_7$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-45	563.2098	2.40	2004	$C_{21}H_{39}O_{15}P$	$C_{23}H_{34}N_2O_{14}$	Incorrect EC (alternative EC: $C_{26}H_{36}NO_{12}Na$
ALK-46	427.1714	2.07	1626	$C_{17}H_{34}NO_5P_2S$	$C_{19}H_{26}N_2O_9$	Incorrect EC (alternative EC: $C_{22}H_{28}O_7Na$)
ALK-47	416.2441	2.35	1624	**$C_{24}H_{33}NO_5$**	**$C_{24}H_{33}NO_5$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-48	344.1872	3.57	1590	$C_{19}H_{30}O_2NaP$	**$C_{20}H_{25}NO_4$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-49	731.1766	1.46	1566	$C_{28}H_{47}N_3O_6NaP_5S$	$C_{29}H_{34}N_2O_{20}$	Incorrect EC (alternative EC: $C_{32}H_{36}O_{18}Na$)
ALK-50	414.2636	2.82	1536	$C_{26}H_{35}N_2ONa$	**$C_{25}H_{35}NO_4$**	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-51	540.0828	0.75	1509	$C_{18}H_{21}NO_{18}$	$C_{18}H_{21}NO_{18}$	Incorrect EC

(*continued on next page*)

Table 1 (*continued*)

Marker ID	*m/z*	Rt (min)	Intensity (counts)	MarkerLynx molecular formula Generic method	MarkerLynx molecular formula Optimized method	Additional Comments
ALK-52	324.1613	3.60	1442	$C_{19}H_{26}ONaP$	$\mathbf{C_{20}H_{21}NO_3}$	Reported as alkaloid in other plant genera (DNP)
ALK-53	264.0881	1.47	1340	$\mathbf{C_{13}H_{13}NO_5}$	$\mathbf{C_{13}H_{13}NO_5}$	Reported as alkaloid in other plant genera (DNP)
ALK-54	310.0934	1.62	1318	$C_{12}H_{25}OP_4$	$\mathbf{C_{14}H_{15}NO_7}$	Reported as alkaloid in other plant genera (DNP)
ALK-55	372.2184	1.58	1252	$C_{16}H_{37}O_5P_2$	$\mathbf{C_{22}H_{29}NO_4}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-56	430.2586	3.33	1159	$\mathbf{C_{25}H_{35}NO_5}$	$\mathbf{C_{25}H_{35}NO_5}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-57	434.2324	3.72	1157	$C_{28}H_{31}N_2ONa$	$\mathbf{C_{27}H_{31}NO_4}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-58	404.2089	1.06	1139	$C_{17}H_{31}N_4O_5S$	$\mathbf{C_{22}H_{29}NO_6}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-59	440.2659	1.23	1115	$C_{26}H_{35}N_2O_4$	$\mathbf{C_{23}H_{37}NO_7}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-60	538.3023	3.11	1048	$C_{24}H_{46}N_2O_9P$	$\mathbf{C_{28}H_{43}NO_9}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-61	318.061	2.26	1041	$C_9H_{17}O_{10}S$	$C_{15}H_{11}NO_7$	Incorrect EC
ALK-62	414.2655	3.62	1033	$C_{18}H_{41}N_4ONaP_2$	$\mathbf{C_{25}H_{35}NO_4}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-63	530.3104	3.52	988	$C_{34}H_{45}NP_2$	$\mathbf{C_{30}H_{43}NO_7}$	Reported as alkaloid in other plant genera (DNP)
ALK-64	472.2705	4.20	978	$C_{21}H_{48}NO_2P_3S$	$\mathbf{C_{27}H_{37}NO_6}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-65	330.2081	1.17	939	$C_{23}H_{25}N_2$	$\mathbf{C_{20}H_{27}NO_3}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-66	274.092	1.60	932	$C_{10}H_{23}NNaP_2S$	$\mathbf{C_{11}H_{15}NO_7}$	Reported as alkaloid in other plant genera (DNP)
ALK-67	388.2128	1.24	931	$C_{23}H_{29}N_2O_2Na$	$\mathbf{C_{22}H_{29}NO_5}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-68	671.2305	2.14	922	$C_{29}H_{43}N_3O_9P_3$	$C_{29}H_{38}N_2O_{16}$	Incorrect EC
ALK-69	434.2332	2.95	911	$C_{20}H_{37}N_4ONaP_2$	$\mathbf{C_{27}H_{31}NO_4}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-70	482.2758	2.17	895	$C_{29}H_{41}NOP_2$	$\mathbf{C_{25}H_{39}NO_8}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)
ALK-71	406.2444	2.07	892	$C_{16}H_{38}N_3O_5NaP$	$\mathbf{C_{19}H_{35}NO_8}$	Unambigous EC assignment, potentially a previously undescribed alkaloid (not reported in DNP)
ALK-72	294.1349	1.96	877	$C_{16}H_{19}N_2O_2Na$	$\mathbf{C_{15}H_{19}NO_5}$	Reported as alkaloid in other plant genera (DNP)
ALK-73	294.1348	1.88	846	$C_{14}H_{24}O_3NaP$	$\mathbf{C_{15}H_{19}NO_5}$	Reported as alkaloid in other plant genera (DNP)
ALK-74	328.1906	2.26	843	$C_{21}H_{25}N_2Na$	$\mathbf{C_{20}H_{25}NO_3}$	Reported as alkaloid in *Aconitum, Delphinium* and/or *Consolida* genus (DNP)

the i-FIT™ provides a measure of the likelihood that the theoretical isotope pattern of an elemental composition matches peaks in a measured spectrum and is a more reliable value for the determination of ECs than mass accuracy alone (Ibáñez et al., 2009). The smaller the i-FIT™, the better the match between experimental and theoretical data. Only ECs whose i-FIT value ranked first among all possible formulae were considered for data evaluation. The filtered list (i.e. 214 markers) was split into two: (i) a list of ECs including one or two nitrogen atoms but no sodium atom (i.e. 78 alkaloid candidates), and (ii) a list containing all other ECs (i.e. 136 non-alkaloid candidates). The alkaloid list was further filtered by keeping ECs displaying H/C ratios between 0.65 and 2.25, while H/C ratios comprised between 0.2 and 3.1 were considered for the non-alkaloid list (Kind and Fiehn, 2007). According to the DNP, > 98% of all known alkaloids have H/C ratios within 0.65 and 2.25. The final, refined alkaloid list consisted of 74 ECs (Table 1) and the final, refined non-alkaloid list consisted of 136 ECs (Table S2).

We then evaluated the identification performance of this automated optimized method by manually computing the automatic determination of ECs against the DNP database. Among the 74 selected alkaloid-like ECs, 49 were already reported as alkaloids in the genus *Aconitum* and/or its two closely related genera *Delphinium* and *Consolida*, and 12 in other plant species. Moreover, one alkaloid-like EC not found in the DNP database could correspond to a previously undescribed alkaloid (ALK-71, Table 1). Among the 12 remaining alkaloid-like ECs, 8 were possibly incorrectly assigned whereas 4 were correctly assigned but did not correspond to alkaloids (i.e. NH_4^+ adducts, amino sugars, etc.). Altogether, the method reached an identification rate of 83.78% and even 90.24% above the 2500 counts limit, which corresponds to the average background noise of a base peak intensity chromatogram under the conditions employed. In comparison, we also computed molecular formulae with a generic method based on parameters set with no *a priori* such as those used in untargeted metabolomics (Iijima et al., 2008). Strikingly, the generic method was able to find only 17 alkaloids within this restrained list (identification rate 27.42% relative to the optimized method, Table 1). In other words, generic methods such as those usually employed in untargeted metabolomics are prone to a large proportion of false-negatives and present a great risk of not correctly annotating important biomarkers in the case of alkaloids. Regarding the list of non-alkaloid candidates, we also performed a manual verification, which revealed only five potential false-negatives (Table S2). This suggests that our method generates a minimal number of both false-positive and negative assignments.

We then evaluated whether the performance of the method was overall correlated with the peak intensity, the *m/z* ratio and/or the retention time. The identification quality was positively correlated with the ion intensity ($r_p = 0.556$, $p < 0.001$), meaning that most peaks of high intensity were correctly identified while wrong assignments were mainly related to low intensity peaks (Fig. 2). This intensity effect is certainly due to poor ion statistics for peaks of low intensity, which may affect both mass and spectral accuracies of QTOFs, and therefore interfere with the determination of the EC. It would be interesting to determine if such correlation also exists for other types of HRMS systems (e.g. Orbitrap, FT-MS). As expected, no correlation occurred between identification performance and retention time ($p = 0.444$), but the *m/z* value was negatively correlated with the identification accuracy ($r_p = -0.150$, $p = 0.007$), meaning that the higher the *m/z* value, the lower the probability to successfully achieve EC assignment (Fig. 2). As we did not find any influence of the *m/z* value on mass or spectral accuracies, this trend is most likely due to the increasing number of possible elemental formulae caused by an *m/z* increase, which renders an unequivocal determination of EC more challenging.

2.3. Comparison with an open-access software

We assessed the performance of MarkerLynx in comparison with the well-established software MZmine 2 (Pluskal et al., 2012). A method for peak deconvolution and EC prediction was optimized in MZmine using similar parameters as those applied to MarkerLynx, although slight adaptations were necessary due to the different features provided by both softwares. MZmine detected more potential alkaloids (111 ECs *versus* 74 ECs for MarkerLynx), however it also generated much more false-positives (49 for MZmine *versus* 12 for MarkerLynx). This finally led to the same number of true-positives (62 alkaloid candidates) in both MZmine and MarkerLynx alkaloid lists (Table S3 and Table 1, respectively), 53 of which were common to both lists. The high number of false-positives in MZmine may be possibly explained by the following factors: first, the detection of sodium and potassium adducts after generation of the raw peak list was clearly less efficient in MZmine, and this certainly impacted the subsequent determination of ECs. Second, whereas the inclusion of a sodium atom in the EC method was beneficial to MarkerLynx, for some unknown reason the trend was reversed in MZmine and we had to remove the sodium atom. Finally, the fact that *m/z* tolerance (set either in mDa or ppm for MarkerLynx but both in mDa and ppm for MZmine), isotope pattern filters and element count heuristic filters were different may also have affected the results. Taken together, these results show that, in our hands, both softwares were able to detect true alkaloids with similar efficiency but that MZmine produced more false positives during that process. Yet, given that MZmine is a relatively complex software with many features, it remains possible that some parameters may be further optimized to reduce the number of false positives. Moreover, it should be reminded that several other open-access softwares are currently available for automated compound identification through ECs determination (Böcker et al., 2009) and/or MS/MS spectral annotation (Tsugawa et al., 2016; Dührkop et al., 2015; Allen et al., 2015; Ridder et al., 2013).

2.4. Application to Aconitum samples

Using the MarkerLynx metabolomic approach, a total of 359 alkaloid candidates was detected in the leaf, perianth and pollen samples of *A. lycoctonum* and *A. napellus*. 296 molecular formulae matched with known alkaloids from the DNP database, including 282 already found in the *Aconitum* genus. The remaining 63 candidates (17.55%) represent potentially yet unknown alkaloids, thus this dataset may be useful for future phytochemical investigations of *Aconitum* tissues. To reduce data complexity, a principal component analysis (PCA) was performed on the refined dataset consisting of alkaloid candidates and revealed that the samples were clearly clustered in two different groups (Fig. 3A), with the two species being distributed on both sides of the PC1 axis that explained 29.06% of the total variance. This species-clustering was corroborated by a perMANOVA that detected a significant difference between the two *Aconitum* species ($F_{1,16} = 6.107$, $p < 0.001$). Such chemical variation between *A. lycoctonum* and *A. napellus* supports the use of specialized metabolites, especially diterpenoid alkaloids, as chemical markers valuable to plant taxonomy. The separation between the two *Aconitum* species was further investigated by partial least squares discriminant analysis (PLS-DA) (Fig. 3B), a supervised method that explains maximum separation among defined classes of samples. Once again, the ordination map showed a clear discrimination between the two species according to the LV 1 axis that explained 28.9% of the total variance (Table 2). Following PLS-DA, the variable influence in projection (VIP) scores were used to select the most influential alkaloid-like metabolites that were mainly responsible for species separation (i.e. VIP ≥1). The 5 most influential candidates for each species were tentatively identified as known alkaloids by matching the obtained molecular formulae with the DNP database (Table 3). All of them but one have been reported in the *Aconitum* and/or *Delphinium* genera (Ranunculaceae family). Regarding the marker candidates of *A. lycoctonum*: (i) $C_{36}H_{48}N_2O_{10}$ was tentatively assigned as lycaconitine, (ii) $C_{37}H_{48}N_2O_{11}$ as potanidine B, (iii) $C_{24}H_{33}NO_5$ as a hetisane-type diterpenoid alkaloid, probably ternatine or cardionine, and (iv) $C_{25}H_{35}NO_5$ as a yuzurimine-type alkaloid, probably yuzurimine A or E.

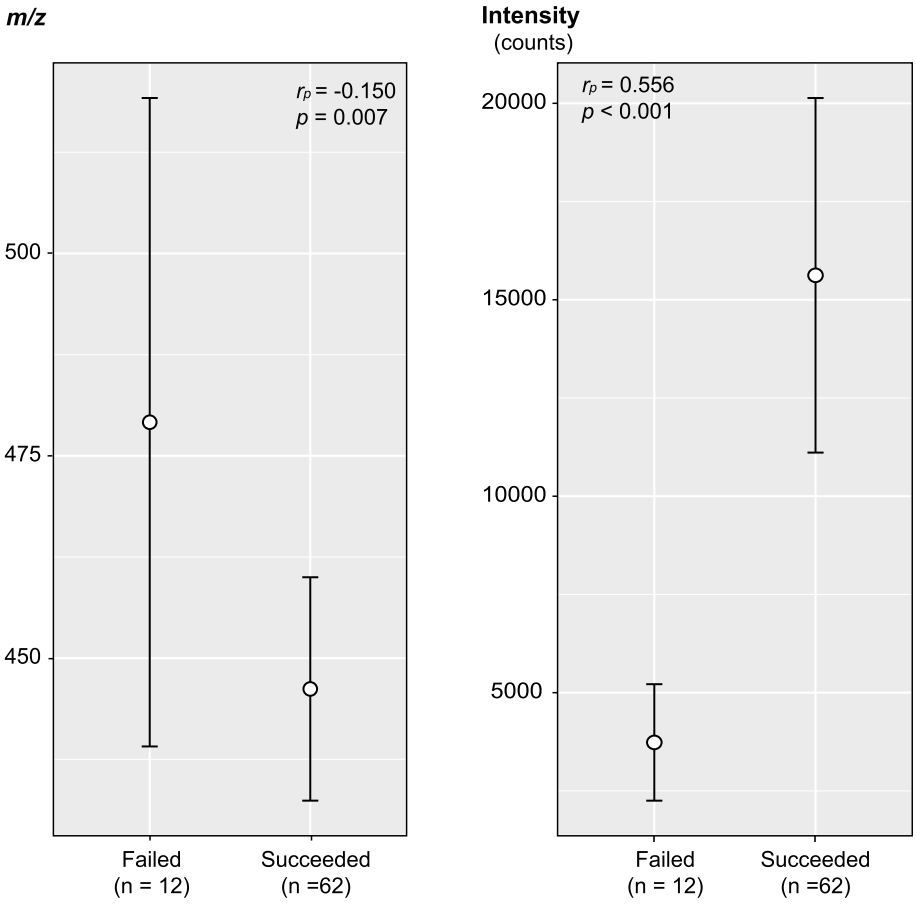

Fig. 2. Correlations of identification quality of the optimized method with *m/z* ratio and intensity. The polyserial correlation coefficients (r_p) and p-values (p) for each correlation are shown.

Four putative markers were tentatively identified in *A. napellus*, namely anthriscifoldine A ($C_{25}H_{37}NO_7$), 14-*O*-acetylegenicunine B ($C_{25}H_{37}NO_7$), bullatine C ($C_{26}H_{41}NO_7$) and napellonine ($C_{22}H_{31}NO_3$). It was not possible to propose unequivocal assignments for $C_{24}H_{33}NO_4$ (alkaloid-1AL) and $C_{24}H_{37}NO_6$ (alkaloid-1AN) because of the relatively high number of isomers reported in *Aconitum* for these molecular formulae.

From the review of the literature, lycaconitine, a C_{19} diterpenoid alkaloid, is known to occur in *A. lycoctonum* since its isolation from the roots of the plant in 1884 (Dragendorff and Spolm, 1884). Lycaconitine has been recently found in corollas of *A. lycoctonum* (Barlow et al., 2017). Potanidine B is a lycoctonine-type C_{19} diterpenoid alkaloid that was isolated from the root of *Delphinium potaninii* Huth

Table 2
PLS-DA cross-validation metrics.

Component	R^2X	R^2X (cum)	R^2Y	R^2Y (cum)	Q^2
LV 1	0.289	0.289	0.965	0.965	0.942
LV 2	0.104	0.393	0.286	0.994	0.556

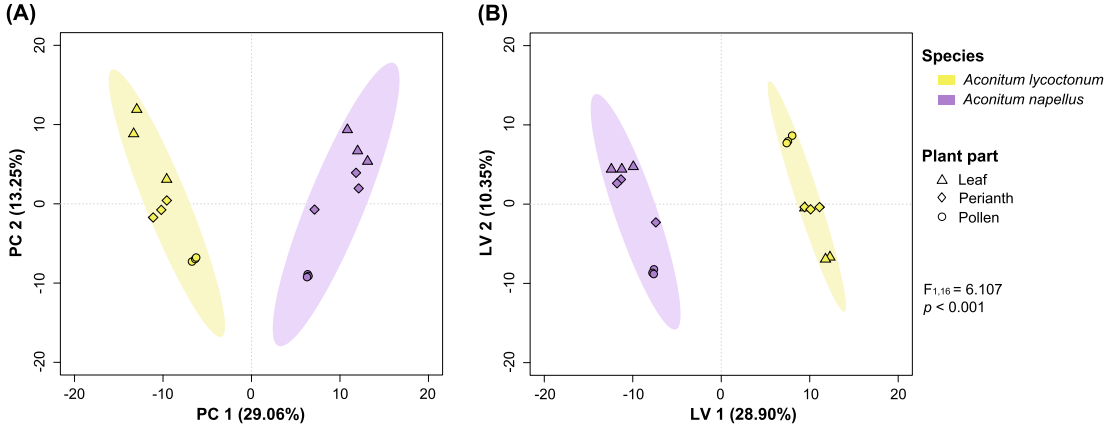

Fig. 3. PCA score plot for first and second components (PC 1 and PC 2) showing a clear separation between *A. lycoctonum* and *A. napellus* alkaloid profiles (A). PLS-DA score plot for first and second latent variables (LV 1 and LV 2) seeking to sharpen the discrimination between *Aconitum* species. Ellipsoids represent 95% confidence ellipses.

Table 3
Alkaloid-like metabolites highlighted by means of PLS-DA (Fig. 3B) and representative of either *A. lycoctonum* or *A. napellus* were tentatively identified using the DNP database. Elemental compositions, mass-to-charge ratios (*m/z*) and retention times (Rt) are provided for the 10 markers.

LV 1 loading (VIP scores)	*Aconitum* species	Measured *m/z*	Rt (min)	MarkerLynx EC	Tentative assignment or number of isomers reported
0.0895 (1.67)	*A. lycoctonum*	669.3373	3.57	$C_{36}H_{48}N_2O_{10}$	Lycaconitine (reported in *Aconitum lycoctonum*)
0.0853 (1.59)	*A. lycoctonum*	697.3319	3.76	$C_{37}H_{48}N_2O_{11}$	Potanidine B (lycoctonine derivative reported in *Delphinium* genus)
0.0840 (1.57)	*A. lycoctonum*	400.2489	2.31	$C_{24}H_{33}NO_4$	Alkaloid-1AL (6 isomers reported in *Aconitum* genus)
0.0823 (1.55)	*A. lycoctonum*	416.2442	2.35	$C_{24}H_{33}NO_5$	Hetisane-type diterpenoid alkaloid (reported in *Delphinium* genus)
0.0800 (1.51)	*A. lycoctonum*	430.2597	2.85	$C_{25}H_{35}NO_5$	Yuzurimine A or E (reported in *Daphniphyllum* genus)
−0.0984 (1.85)	*A. napellus*	464.2653	2.40	$C_{25}H_{37}NO_7$	Anthriscifoldine A (reported in *Delphinium* genus)
−0.0976 (1.83)	*A. napellus*	450.2860	2.17	$C_{25}H_{39}NO_6$	14-*O*-Acetylgenicunine B (reported in *Aconitum variegatum*)
−0.0976 (1.83)	*A. napellus*	480.2959	2.52	$C_{26}H_{41}NO_7$	Bullatine C (reported in *Aconitum napellus)*
−0.0929 (1.73)	*A. napellus*	436.2703	1.72	$C_{24}H_{37}NO_6$	Alkaloid-1AN (5 isomers reported in *Aconitum* genus)
−0.0924 (1.72)	*A. napellus*	358.2390	1.80	$C_{22}H_{31}NO_3$	Napellonine (reported in *Aconitum napellus*)

(Ranunculaceae) for its structural characterization (Pu and Wang, 1994). Its occurrence in other tissues than roots is, to our knowledge, unreported. The hetisane-type diterpenoid alkaloid detected in *A. lycoctonum* might correspond to ternatine, previously isolated from the aerial parts of *Delphinium ternatum* Huth (Ranunculaceae) (Narzullaev et al., 1997), or to cardionine previously isolated from above-ground parts of *Delphinium cardiopetalum* DC (Ranunculaceae) (De la Fuente et al., 1990), both being C_{20} diterpenoid alkaloids. The yuzurimine-type alkaloid might correspond to either yuzurimine A, a minor alkaloid from the bark and the leaves of *Daphniphyllum macropodum* Miq. (Daphniphyllaceae) (Sakurai et al., 1967), or yuzurimine E, an alkaloid from the seed of *Daphniphyllum calycinum* Benth. (Daphniphyllaceae) (El Bitar et al., 2004) and the leaves of *Daphniphyllum glaucescens* Blume (Daphniphyllaceae) (Takatsu et al., 2004). With regards to the markers of *Aconitum napellus*, anthriscifoldine A is a C_{19} diterpenoid alkaloid that was obtained from the whole herbs of *Delphinium anthriscifolium* var. *savatieri* Hance (Ranunulaceae) for its structure elucidation (Song et al., 2009). 14-*O*-acetylegenicunine B is a norditerpene alkaloid isolated from aerial parts of *Aconitum variegatum* L. (Ranunculaceae) (Diaz et al., 2005) that is a closely related species of *A. napellus* (Luo et al., 2005). Bullatine C is a C_{19} diterpenoid alkaloid found in the nectar and galeas of *Aconitum napellus* (Barlow et al., 2017), while napellonine (i.e. dehydronapelline) is known as a C_{20} diterpenoid alkaloid occurring in herb and flowers of *A. napellus* (Chen et al., 1999). Regarding the chemotaxonomic significance of these features, previous studies suggested that C_{18} and C_{19} diterpenoid alkaloids display a taxonomic importance relative to the C_{20} diterpenoid alkloids (Wang and Chen, 2010). Whereas species that exhibit more C_{20} diterpenoid alkaloids may be regarded as more primitive, species with a major chemical composition based on C_{19} diterpenoid alkaloids may be more evolved (Wang and Chen, 2010). Such occurrence of C_{20} diterpenoid alkaloids in ancient species is supported by the fact that C_{20} diterpenoid alkaloids display much simpler backbone structures and are considered as biogenetic precursors of C_{18} and C_{19} diterpenoid alkaloids (Wang and Chen, 2010).

3. Conclusion

The presented approach constitutes an efficient and applicable tool for profiling alkaloids in plant samples. By carefully optimizing the various parameters that are essential to correct EC assignment and by automating the process, numerous alkaloid candidates can be identified within a very short time (less than 30 min for a batch of ca. 500 features). The most promising molecules may then be further confirmed by MS/MS acquisition and comparison with existing databases. Such efficient analytical workflow is the basis of the modern approach of chemotaxonomy and might prove highly useful in the search for known and previously undescribed alkaloids from medicinal plants as well as to identify reliable marker compounds during exposure to toxic species such as *Aconitum* spp.

4. Experimental

4.1. Plant material

Plants were sampled in August 2013 in Switzerland in two localities: Kandersteg (place called Gastereholz, GPS coordinates: 46.45912 N, 7.67072 E; elevation: 1369.5 m) for *Aconitum lycoctonum* L. (Ranunculaceae), and Boltigen (place called Ramsere, GPS coordinates: 46.63934 N, 7.38029 E; elevation: 1338.3 m) for *Aconitum napellus* L. (Ranunculaceae). Leaves and perianths were sampled from single specimens (n = 3 per species). Pollen was collected on several flowers and pooled to obtain sufficient amounts for analyses. Plant samples were stored at −80 °C until extraction and chromatographic analysis.

4.2. Sample preparation

Fresh leaves and perianths (i.e. merged calyx and corolla) were ground to a fine powder under liquid nitrogen and an accurately weighed amount (ca. 50 mg) was placed in a 1.5 mL microcentrifuge tube (Eppendorf, Hamburg, Germany) with 5–10 glass beads (diameter = 2 mm). Alkaloids were extracted using a tissue homogenizer (Retsch Mixer Mill MM300, Düsseldorf, Germany) at 30 Hz during 3 min in presence of 1 mL of aqueous methanol (70%) and 0.5% formic acid. Following centrifugation at 14,000 rpm for 5 min (Centrifuge 5424, Eppendorf, Hamburg, Germany), 600 µL of the clear solution was transferred to an HPLC vial. Pollen samples (ca. 1 mg) were extracted following the same procedure with 100 µL of extraction solvent and 4 glass beads. The pollen samples were diluted 10 times with the extraction solvent prior to injection in the analytical system.

4.3. UHPLC-HRMS acquisition

Alkaloids were profiled by ultrahigh performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS). An Acquity UPLC™ coupled to a Synapt G2 QTOF mass spectrometer from Waters (Milford, MA, USA) was employed. The separation was performed on an Acquity UPLC™ BEH C18 column (50 × 2.1 mm internal diameter, 1.7 µm particle size, Waters) at a flow rate of 0.4 mL/min using a gradient of formic acid 0.05% in water (phase A) and in acetonitrile (phase B). The following program was used: 2–45% B in 5 min, 45–100% B in 1 min, 100% B for 2 min, 2% B for 1.5 min. The column temperature was maintained at 25 °C. The injection volume was of 2 µL. Detection was performed in positive electrospray ionization over a mass range of 85–1200 Da. The scan time was set to 0.4 s. The following MS parameters were applied: capillary voltage +2.8 kV, source temperature 120 °C, sampling cone voltage +40 V, extraction cone voltage +3.0 V, desolvation gas temperature and flow, 350 °C and 800 L/h, respectively. The mass spectrometer was internally calibrated using a 500 ng/mL solution of leucine-enkephalin infused through the Lockspray probe over the entire run. In this configuration, the instrument switched every 15 s on the Lockspray probe to acquire one single

scan of 0.5 s and 5 scans were then summed for mass correction. All aspects of the system were controlled by MassLynx v.4.1 (Waters).

4.4. Feature extraction and automated determination of elemental composition (EC)

MarkerLynx XS – Automated feature extraction performed in MarkerLynx XS (Waters) required a dataset containing at least three samples, therefore a representative sample prepared from *Aconitum lycoctonum* leaves was analysed once by UHPLC-HRMS and then triplicated in order to foul the software while reducing feature variability to a minimum. The following parameters were employed: chromatographic window 0.7–4.5 min, mass range 150–900 Da, marker intensity threshold 500 counts (corresponding in general to a signal to noise ratio of about 10), mass window 0.02 Da, retention time window 0.06 min, peak width at 5% height 6 s, peak-to-peak baseline noise 50, smoothing applied (Savitzky-Golay), noise elimination level automatic, deisotoping function activated. The resulting table comprised samples in columns, features characterized by retention time and mass to charge ratio in lines, and an intensity value for each feature in each sample. Na^+ and K^+ adducts, but not NH_4^+ adducts, were then automatically detected and discarded from the list of features.

The automated determination of ECs was achieved by importing an EC method from MassLynx into the MarkerLynx method. Different parameters were optimized to maximize identification accuracy. Five different atoms were included and the following minimum and maximum numbers of atoms were set at C 0–95, H 0–182, N 0–3, O 0–40, Na 0–1. The mass tolerance was set to 4 ppm and only even electron ions were accepted. A double bond equivalence range (RDBE) of −1.5-50 was used, and the number of peaks for i-FIT™ determination was 3. The EC list obtained was then exported and filtered by applying an intensity threshold of 800 counts and by keeping only ECs with i-fit values inferior or equal to 0.3. The obtained EC list was then separated in potential alkaloid (ECs containing 1 or 2 N atoms but no Na atom) and non-alkaloid (other ECs) lists. The list of alkaloids was further filtered by applying H/C ratios comprised between 0.65 and 2.25. For the non-alkaloid list, H/C ratios between 0.2 and 3.1 were allowed. For the generic method, two additional atoms were selected (P and S) and the number of atoms was set as follows: C 0–95, H 0–182, N 0–10, O 0–45, S 0–5, P 0–6, Na 0–1 (Iijima et al., 2008). The mass tolerance was 5 ppm, both even and odd electron states were afforded, double bond equivalence range was −1.5-50, and the number of peaks for i-FIT™ determination was 3.

MZmine 2 – The raw MassLynx file was converted to CDF (common data format) using the Databridge software provided in the MassLynx package. The CDF file was imported into MZmine 2 and crop filtered using retention time and mass windows of 0.7–4.5 min and 150–900 Da, respectively. The cropped chromatogram was then deconvoluted using the Wavelets (ADAP) algorithm and deisotoped using an *m/z* tolerance of 0.002 Da or 8 ppm and a retention time tolerance of 0.05 min. Na^+ and K^+ adducts were excluded from the obtained peak list. EC prediction was then performed using the following parameters: ionization type $(M+H)^+$, *m/z* tolerance 0.003 Da or 5 ppm, number of C (0–95), H (0–182), N (0–3) and O (0–40), heuristics filters for H/C ratio, NOPS/C ratio and multiple element counts activated, RDBE tolerance −1 to 40, and isotope pattern filter activated (*m/z* tolerance 0.004 Da or 8 ppm, minimum intensity value $1.0e^2$, minimum score 90%). The obtained list of ECs was then exported to Excel and divided into alkaloid candidates (i.e. ECs displaying 1 or 2 N atoms) and non-alkaloid candidates (i.e. other ECs).

4.5. Data analyses

Identification quality – Each EC determined by both MarkerLynx and MZmine 2, and identified as a potential alkaloid candidate was manually imported in the Dictionary of Natural Product (DNP) database (CRC Press, USA, version 6.1 on DVD). Only ECs matching with known alkaloids, especially those isolated from the *Aconitum* genus and/or its two closely related genera *Delphinium* and *Consolida*, were considered as true-positives. The other ECs (i.e. no match with known alkaloids) were considered as false-positives and divided in two categories after manual verification: (i) probably correct EC but not typical of alkaloids (e.g. alternative identity in the DNP, presence of ammonium adduct in the mass spectrum, etc.), and (ii) probably incorrect EC (e.g. peptide structure, alternative EC manually predicted based on adduct and fragment interpretation, too low mass defect to be an alkaloid, etc.). In addition, the list of non-alkaloid candidates was also manually verified to assess potential false-negatives. While this approach is subject to some degree of uncertainty given the absence of reference standards for unambiguous identification, it is conservative in that it accepts as alkaloids only those reported in the DNP (i.e. worst case scenario). Moreover, it provides a way to evaluate EC determination in real plant samples and thus in the presence of potentially interfering molecules from a complex natural extract. Finally, we also evaluated whether the accuracy of compound identification in the alkaloid list was correlated with peak intensity, mass-to-charge ratio (*m/z*) and/or retention time. We computed polyserial correlations using the “polyserial” function from the R-package polycor (Fox, 2016).

Application to *Aconitum* samples – After automatic determination of EC using the optimized MarkerLynx method, we selected compounds displaying an alkaloid-like elemental composition (i.e. including one or two nitrogen atoms but no sodium atom) and applied the aforementioned filters (i.e. i-FIT value ≤ 0.3, intensity ≥800 and H/C ratio between 0.65 and 2.25). Prior to statistical analyses, data were normalized to unit norm (i.e. normalized to the summed total ion intensity per chromatogram) and unit-variance scaled using the “scaling” function from the MetabolAnalyze package in R (Gift et al., 2010). The alkaloid compositions were compared between *A. lycoctonum* and *A. napellus* using permutational multivariate analysis of variance (perMANOVA) based on Euclidean distances and 999 permutations (“adonis” command, R-package vegan; Oksanen et al., 2017). Prior to perMANOVA, the multivariate homogeneity of within-group covariance matrices was verified using the “betadisper” function implementing Marti Anderson's testing methods ($p = 0.673$). A principal component analysis (PCA) was performed to visually assess the interspecific difference (score plot), using the “prcomp” command of the R statistical software. Moreover, a partial least squares discriminant analysis (PLS-DA) including a leave-one-out cross-validation was performed in order to sharpen the separation between species and to understand which variables carry the species separating information (“plsDA” function in R-package DiscriMiner). The markers accountable for interspecific difference were ranked by VIP and tentatively identified using the DNP database, taking into account the consistency of the biological source. All analyses were conducted in R version 3.4.0 (R Core Team, 2017).

Declaration of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2018.06.011.

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