Phytochemistry 154 (2018) 1–9

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

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

Maryse Vanderplanck[a], Gaétan Glauser[b,*]

[a] Analytical Chemistry, AgroBioChem Department, University of Liège - Gembloux Agro-Bio-Tech, Passage des Déportés 2, B-5030 Gembloux, Belgium
[b] Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Avenue de Bellevaux 51, CH-2000 Neuchâtel, Switzerland

ARTICLE INFO

Keywords:
Aconitum lycoctonum
Aconitum napellus
Ranunculaceae
Alkaloids
Metabolite profiling
UHPLC-HRMS
Elemental composition
Chemotaxonomy

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

* Corresponding author.
E-mail address: gaetan.glauser@unine.ch (G. Glauser).

https://doi.org/10.1016/j.phytochem.2018.06.011
Received 16 January 2018; Received in revised form 5 June 2018; Accepted 13 June 2018

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,

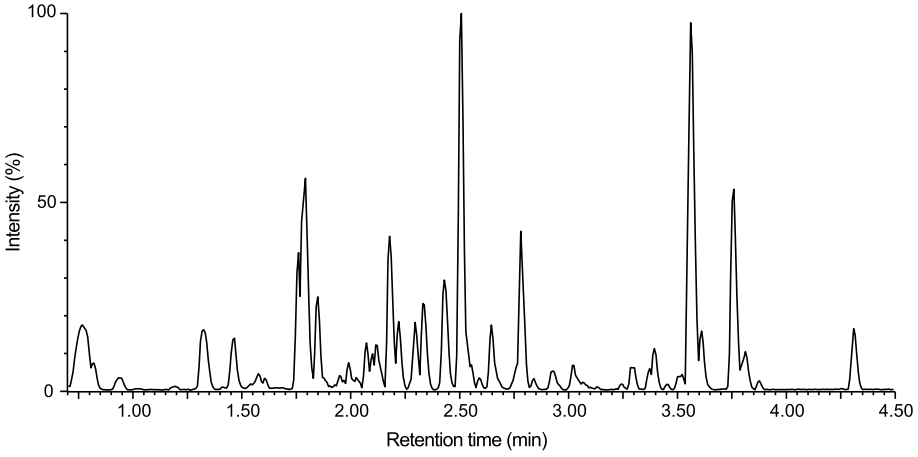

Fig. 1. UPLC-HRMS profile of *Aconitum lycoctonum* leaf extract.