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Metabolic discrimination of pine resins using multiple analytical platforms

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ABSTRACT

Resins are one of the first sites of interaction between plants and biotic and abiotic factors. Despite their evident morphological and chemical differentiation from other plant organs, the detailed correlation between resins and biological or environmental factors is not yet clear. In this study, ^{1}H nuclear magnetic resonance (NMR), gas chromatography coupled with mass spectrometry (GC-MS) and high-performance thin-layer chromatography (HPTLC)-based profiling techniques were applied to the metabolic characterisation of plant resins of different species and season of collection, using samples from five different species that were collected during early and late spring. The ^{1}H NMR analysis confirmed the main metabolic groups in the resins to be terpenoids and further GC-MS analysis revealed a notable chemical variation between the species and collection periods. *Abies grandis* displayed a significant differentiation from the other species, showing a higher number of monoterpenes. The HPTLC-based profiling method hyphenated with multivariate data analysis (MVDA) also showed a clear separation confirming the GC-MS terpenoidal profiling results. Additionally, the unknown compounds were obtained by preparative TLC for identification. Based on the results of the three analytical platforms, it was concluded that the major difference in chemical composition of pine species was between species rather than the collection period. Nonetheless, the chemical profiles of resins from different species and collection periods can be well discriminated and correlated to mono- and sesquiterpenes in the case of species and diterpenes for the collection periods.

1. Introduction

Plant resins have been used in traditional medicine for many years and have commercial value (Dell and McComb, 1979). Though there is some information on their herbivore insect repelling efficiency, their full physiological and biological functions in plants require deeper investigation. These activities are probably related to their toxicity or their role as signal molecules that attract specific predators or parasitoids (Price et al., 1980; Raffa and Berryman, 1983; Mumm et al., 2003).

Plant resins contain a diverse array of specialised metabolites, including terpenes and phenolic compounds. Amongst these terpenes, there are roughly equal amounts of monoterpenes and diterpenes and a smaller fraction of sesquiterpenes and triterpenes (Phillips and Croteau, 1999; Martin et al., 2002). The characterisation of resins has progressed greatly over the years thanks to the development of sophisticated molecular and chemical technologies that are now accessible for their analysis.

Recently, diverse metabolomics tools have been applied to the research of resin chemistry and physiology. Amongst these, a study that combined gas chromatography coupled with mass spectrometry (GC-MS) followed by statistical analysis of the data showed that monoterpenes could affect the spread of the invasive mountain pine beetle, *Dendroctonus ponderosae* (Taft et al., 2015). This finding coincided with the results of a study on the variation in the chemical composition of pines with different herbivory levels, using a similar approach (Keefover-Ring and Linhart, 2010). Liquid chromatography coupled with mass spectrometry (LC-MS)-based metabolomics has been used to establish whether the responses to folivores in closely related plant species could be associated with macroevolutionary traits and plant-folivore coevolutionary processes (Rivas-Ubach et al., 2016).

Due to the complexity of the metabolome, one single analytical method is generally insufficient to cover all types of metabolites. It is for this reason that the use of multiple protocols and analytical

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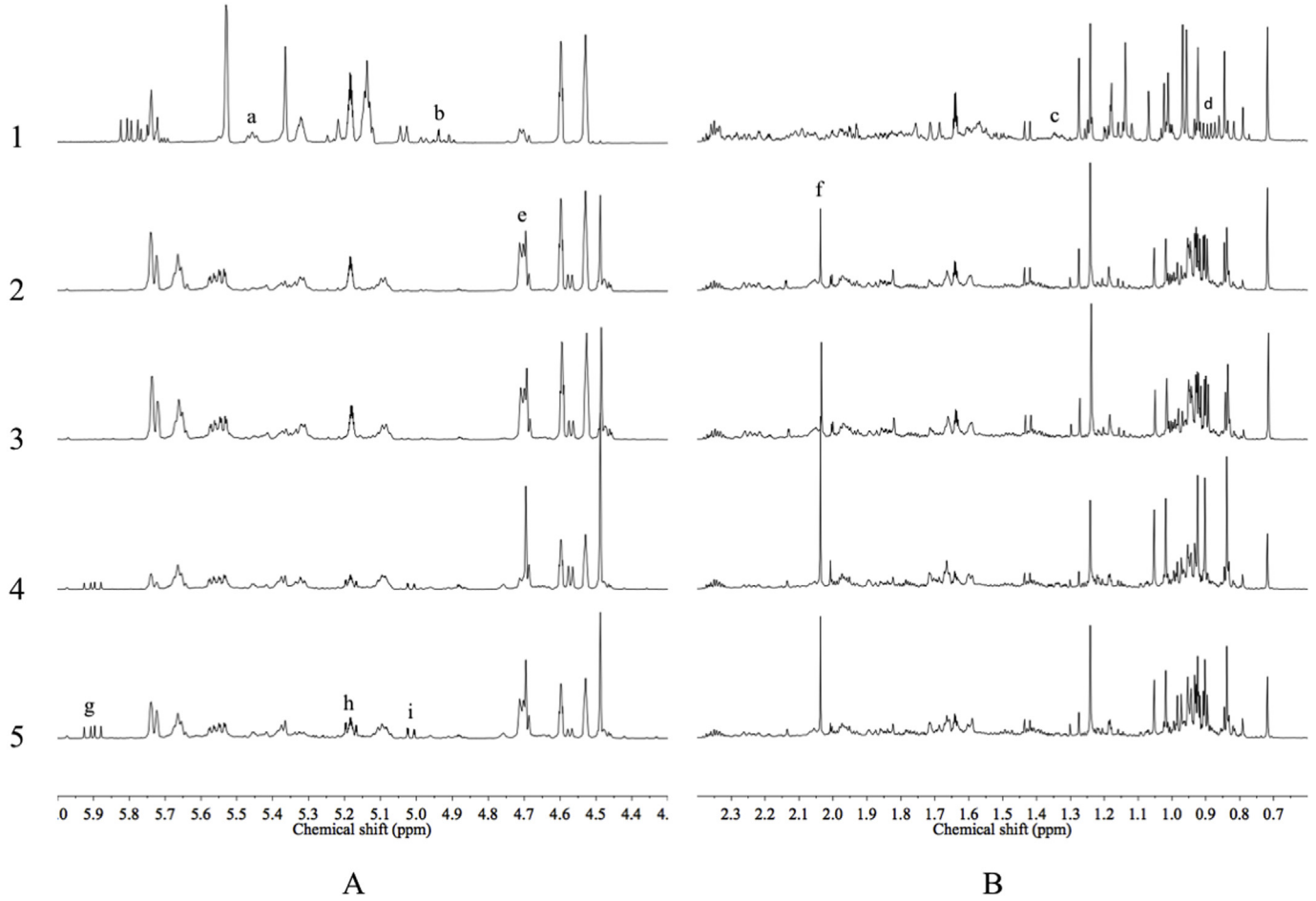

Fig. 1. Typical ^{1}H NMR (600 MHz in CH_3OH-d_4) spectra of pine resins in the range of δ 4.3 – δ 6.0 (A) and d 0.6–2.4 (B). 1: *Abies grandis*, 2: *Pseudotsuga menziesii*, 3: *Picea abies*, 4: *Pinus sylvestris*, 5: *Pinus strobus*. a: H-3 of *α*-pinene, b: H-10a and H-10b of *β*-pinene, c: H-9 of *α*-pinene, d: H-8 of *α*-pinene, e: H-2 from bornyl acetate, f: CH_3CO- of bornyl acetate, g: H-15b from sclareol, h: H-14 from sclareol, i: H-15a from sclareol.

instruments is necessary to obtain truly comprehensive information (Gromski et al., 2015).

For untargeted metabolic analysis, there are various analytical platforms that can be used, including both stand-alone or hyphenated systems. Among the existing analytical technology, nuclear magnetic resonance spectroscopy (NMR)- and mass spectrometry (MS)-based methods are currently the most popular analytical tools for metabolomics, since the data they can provide, fulfils the essential requirements for these studies, i.e., the detection of a broad chemical range of metabolites with adequate resolution and sensitivity. Though both methods have advanced greatly in recent years their individual strengths and limitations in terms of metabolite coverage, resolution and sensitivity prevail and each method has a specific target range of metabolites for which its response is optimum. Hence, hyphenated platforms are essential to obtain the most comprehensive information on the metabolome. Amongst the recently introduced systems, thin-layer chromatography (TLC) has shown an interesting potential as a complementary tool for metabolomics.

Thin-layer chromatography, a type of planar chromatography, has several advantages as an analytical tool, such as a short overall measuring time considering that it allows simultaneous analysis of multiple samples, a high signal robustness and requires a relatively low initial investment in equipment and running costs compared to other chromatographic methods (Morlock et al., 2014). Amongst all of the advantages of TLC, the broadness of the range of detectable metabolites is regarded to be its strongest feature as a tool for metabolomics. Many groups of metabolites can be selectively visualised using numerous chemical derivatisation methods (Fichou et al., 2016). Moreover, when used preparatively, TLC bands can be easily separated for further analyses or bioactivity tests. In view of the evident potential of TLC, it is worth reopening the debate as to whether it may be a realistic option that can compensate for the limitations of current NMR- or MS-based metabolomics methods.

When considering TLC as a metabolomics tool, it is important to be aware of inherent features such as its low resolution and reproducibility. However, throughout the last decade, great efforts have been made to improve these limitations, and the development of highly efficient sorbents has resulted in a greatly increased resolution. Additionally, other technological advances focussed on a full automation of all steps required for a TLC analysis, such as sample application, development and chemical derivatisation have increased the robustness of TLC as a profiling method. This advanced version of TLC, known as high-performance thin-layer chromatography (HPTLC), has been successfully used as a fingerprinting tool in metabolomics (Nicoletti et al. 2012; Nicoletti, 2012; Nicoletti et al. 2013a).

Added to these technological improvements in TLC hardware and resolution, the development of better methods for the processing of TLC-generated data has also been achieved, converting this method in a robust tool for metabolic profiling. This is the case of an efficient data processing tool that has recently been developed and applied to MVDA by Fichou et al. (2016).

The aim of this study was to investigate the profiles of pine resins collected from five species of the Pinaceae family, *Abies grandis* Douglas ex D.Don Lindl. (Pinaceae), *Pseudotsuga menziesii* (Mirb.) Franco