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Chapter 7.4

SECONDARY METABOLITE PROFILING

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Secondary metabolites include a very wide variety of compounds with different structures and chemical properties. In order to obtain an overview of the secondary metabolite content of a plant species such as Lotus japonicus, a profiling technique coupling sequential extraction with different chromatographic methods (GC-MS, HPLA-DAD/MS) was established. This method allows the qualitative analysis of ionic (charged) compounds such as alkaloids and non-ionic (neutral) compounds such as terpenoids and phenolic compounds. This technique can also be used for the isolation of new compounds

INTRODUCTION

Secondary metabolites are a wide range of compounds from different metabolite families that can be highly inducible in response to stresses. These compounds are not essential for cell structure and maintenance of life but are often involved in plant protection against biotic or abiotic stresses (Weisshaar and Jenkins, 1998; Hattenschwiler and Vitousek, 2000). Some secondary metabolite families such as carotenoids and flavonoids are also involved in cell pigmentation in flower and seed, which attract pollinators and seed dispersers. Therefore, they are also involved in plant reproduction (Winkel-Shirley, 2001).

Plant secondary metabolites also present chemical and pharmaceutical properties interesting for human health (Raskin et al., 2002; Reddy et al., 2003). Compounds belonging to the terpenoids, alkaloids and flavonoids are currently used as drugs or as dietary supplements to cure or prevent various diseases (Raskin et al., 2002) and in particular some of these compounds seems to be efficient in preventing and inhibiting various types of cancer (Watson et al., 2001; Reddy et al., 2003).

Each secondary metabolite family has some specific chemical characteristics, which implies that specific extraction and analysis methods should be developed to study each family in detail. On the other hand, using their polarity properties, which range from polar for polyhydroxylated alkaloids or flavonoid glycosides to non-polar for terpenoids or lipids, and improved database systems, it is possible to profile an increasing number of compounds by using different analytical systems such as GC-MS, LC-MS and HPLC-DAD coupled to a sequential extraction.

The technique described below is aimed at profiling a wide range of compounds from different classes of secondary metabolites. These compounds can be easily extracted from freeze-dried materials using aqueous ethanol, methanol and dichloromethane allowing the extraction of a large range of compounds from water-soluble to non-polar compounds. Then, it is possible to separate them by using different chromatographic methods (reversed phase and ion exchange) and to analyse the fractions obtained by GC-MS and HPLC-DAD/MS (Figure 1). This secondary metabolite profiling is established after removing the major flavonoid glucosides, which are the predominant secondary metabolites along with insoluble tannins and lignin in Lotus plants, as they would mask all the others secondary metabolites present. These flavonoids are the subject of more detailed studies and are therefore analysed separately using another extraction technique (for details see: Chapter 7.5 - Phenolic compounds: Extraction and Analysis Method).
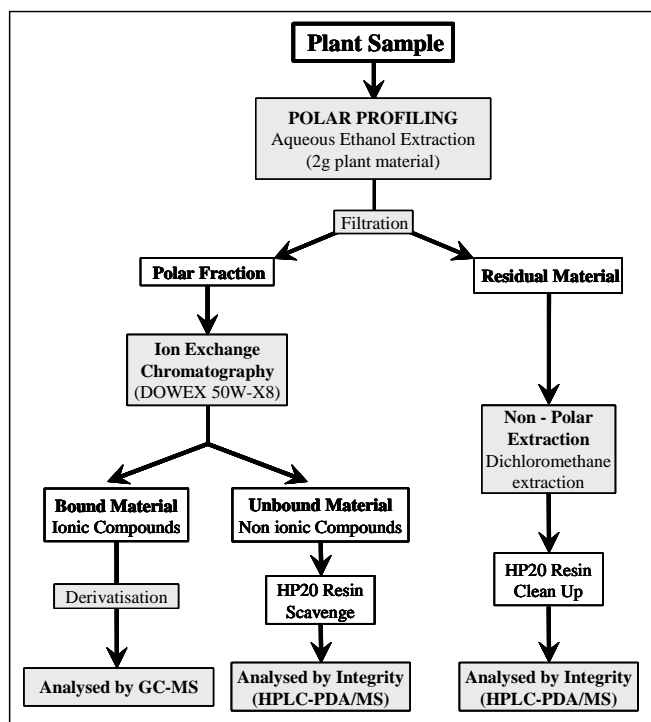

Figure 1. Schematic diagram of secondary metabolite profiling method.

The profiling method described here was originally designed by Molecular Nature Ltd. (International Patent Application Numbers, PCT/GB2003/000905, PCT/GB2003/000880, PCT/GB2003/000892, PCT/GB2003/000906) in order to identify and isolate new compounds which can present a pharmaceutical interest (Watson et al., 2001; Reddy et al., 2003). Since it allows the detection of a large number of compounds from different families of secondary metabolites, it is the method of choice to establish a general profile of secondary metabolites to obtain an overview of the secondary metabolite content of species such as *Lotus japonicus*.

SECONDARY METABOLITE PROFILING PROCEDURE

Materials

The procedure described below is set up for the use of a Perkin Elmer's GC-MS system (model Q-Mass 910; Perkin Elmer, UK) and a Waters' Integrity HPLC-PDA/MS system (Waters, UK) with Millenium 32® software for the data analysis. All glassware, consumables, and solutions used are described in detail in the protocols.

Secondary metabolite extraction

Secondary metabolite profiling entails a sequential extraction using 50% ethanol (v/v) to collect the polar compounds then a dichloromethane extraction for the non-polar compounds.

Polar extraction

Two grams of freeze-dried material is ground and extracted overnight with 50% ethanol (v/v) at room temperature in a 250 ml conical flask. Then, the extracted mixture is filtered on a Buchner funnel and the flask is washed once with 10 ml of 50% ethanol (v/v). The washing solution is filtered and added to the extraction solution. The residual material on the filter is then store at -20ºC and freeze-dried before the dichloromethane extraction.

The extracted solution is fractionated by ion-exchange chromatography. The sample is applied to a 10x1 cm glass column containing 3 cm of DOWEX 50W-X8 (HCl form, Sigma, UK) resin previously regenerated by adding to it excess 2N HCl, soaking for 15 min, then washing it few times with deionised water until it reaches pH 7 and finally equilibrated with 25 ml of 50% ethanol (v/v). The unbound fraction of the sample, containing the non-ionic compounds, is collected on a 500 ml round bottom flask (RBF). The column is washed once with 75 ml of 50% ethanol (v/v) and with 75 ml of deionised water. The effluents are collected, added to the unbound fraction and stored at -20ºC before subsequent fractionation and analysis.

After the washing steps, the column is eluted with 100 ml of 2M ammonium hydroxide (NH_4OH) and the effluent containing the ionic compounds is collected in a new 500 ml RBF. This fraction is then carefully evaporated at 35 ºC using a rotary – evaporator (Büchi, Switzerland). The evaporation step is stopped when around 5

ml are left in the RBF. At this stage, the extraction liquid is transferred into 10 ml glass vial and, if a deposit is observed on the RBF, the flask is washed with few drops of 70% methanol (v/v) using a Pasteur pipette and this washing solution is added to the 10 ml vial. The vial is then stored at -20 ºC and freeze-dried before GC-MS analysis.

Non–ionic fractionation

The unbound fraction from the ion-exchange chromatography previously collected is further scavenged by reversed-phase chromatography using an HP 20 column on a BioFlash chromatography system (Biotage).

Before the separation, the HP20 column is washed with 200 ml of 100% acetone and equilibrated with 25% methanol (v/v). Then, the unbound fraction is applied onto the column and the HP20 column is washed with 25% methanol (v/v). The washed solution along with the unbound fraction containing mainly sugars and most of the hydrophilic flavonoid glycosides, is discarded.

The column is then eluted with 400 ml of 25% acetone in methanol (1:4, v/v). The effluent, collected in a 1 l RBF, is then carefully evaporated at 35ºC with a rotary-evaporator (Büchi, Switzerland). When around 5 ml are left in the RBF, the evaporation step is stopped and the solution is transferred into a 10 ml glass vial. The RBF is rinsed with few drops of 25% acetone in methanol (1:4, v/v) that are added to the 10 ml vial. Then the vial is stored at -20ºC and freeze-dried before HPLC-DAD/MS analysis.

Non-polar extraction

The residual material from the polar extraction, previously freeze-dried, is placed in a filter paper thimble inside a glass Soxhlet. In parallel, a 500 ml RBF containing 200 ml of dichloromethane and two or three glass beads is placed on a heating mantle. The Soxhlet is attached on top of the RBF and 150 ml of dichloromethane is slowly added to the sample. Then, a refrigeration column is fixed to the Soxhlet apparatus and the cooling water is turned on. The heating mantle is then switched on ensuring a steady refluxing rate and left overnight to extract.

At the end of the extraction, the heating mantle is switched off and the Soxhlet apparatus is allowed to cool to room temperature before being dismantled. The dichloromethane extract is added to 100 ml of HP20 resin in a 1 l RBF and the resin is completely dried with a rotary-evaporator (Büchi, Switzerland) at 35ºC. It is then transferred in a 250 ml conical flask where it is eluted three times with 100 ml of 10% acetone in methanol (1:10, v/v). The solution is filtered through tissue paper into a 500 ml RBF and evaporated up to 5 ml at 35ºC. The solution left is then transferred in a 10 ml glass vial and the RBF is rinsed with few drops of 10% acetone in methanol (1:10, v/v) which are added to the 10 ml vial. Then the vial is stored at -20ºC and freeze-dried before HPLC-DAD/MS analysis.

GC-MS analysis

Gas-liquid chromatography is designed to separate volatile compounds from a complex mixture. This technique uses the temperature of vaporisation specific to each compound to separate them from a solution by passing the sample through a heated column where it is partitioned between an inert gas under pressure and a thin layer of non-volatile liquid coated on an inert support inside the column.

Many compounds are difficult to vaporise, like the polyhydroxylated alkaloids. Their capacities to vaporise can be improved by replacing the hydroxyl groups by other chemical groups like trimethylsilyl groups before the injection onto the GC-MS. Therefore, before the injection onto the GC-MS, the ionic fraction obtained after the polar extraction is derivatised by silylation in order to ensure a good vaporisation of the sample. This step is realised by adding 100 μl of Sigma Sil A, a mixture of trimethylchlorosilane, hexamethyldisilazane and pyridine (1:3:9, v/v/v; Sigma Chemical Company, UK), to each mg of freeze-dried sample and heating for 15 min at 60°C.

This fraction is then injected onto the GC-MS system (model Q-Mass 910, Perkin Elmer) and separated by a temperature gradient (180°C for 5 min, then from 180°C to 300°C at 10°C/min and a further 5 min at 300 °C) on a capillary column coated with a non-polar liquid phase (25 m x 0.22 mm id x 0.25 μm BPX5 stationary phase, SGE Ltd.). Then, the effluent, containing the separated and vaporised compounds, pass through the mass spectrometer (TMD detector) where it is ionised and fragmented before been picked up by the analyser, which collect the mass spectral data between 71 and 600 m/z.

HPLC-PDA/MS

High-pressure liquid chromatography is designed to separate compounds from a mixture of compounds in solution. This technique uses the solubility and size properties of each compound to separate them with a solvent mixture that is pumped under pressure through the column. Compounds are detected by a photodiode array detector (Water 996 PDA detector, Waters Ass), which establish the light absorbance spectra from visible and UV wavelengths of each detected compound, and a spectrometer (TMD detector), collecting their corresponding mass spectra.

This technique is used to analyse the non-ionic fraction obtain after the polar extraction and the non-polar fraction from the dichloromethane extraction. Before injection, the samples previously freeze-dried are dissolved in 100% methanol and adjusted to the concentration of 10 mg.ml^{-1}. Then, three μl is injected onto an HPLC-PDA/MS (Waters, UK) and the separation is realised onto a C_8 HPLC column (50 mm x 2.1 mm id x 3.5 μm, Waters) with a linear gradient of two solvent, 100% deionised water and 100% acetonitrile (with 0.01% trifluoroacetic acid), at a flow rate of 0.35 ml.min^{-1}, starting at 90% deionised water, 10% acetonitrile (with 0.01% trifluoroacetic acid), rising to 100% acetonitrile over 6 min, which was held for a further 6.5 min. Then, data of the eluted peak are collected from 200 to 600 nm

and between 71 and 600 m/z by the photo-diode array detector and the mass spectrometer respectively. The data analysis is processed using an adapted processing method of Millennium 32® software and the spectra of the eluted peaks are matched with those contained in our own libraries.

COMMENTS

In this article, we describe a method for secondary metabolite profiling. Other profiling methods are also available for profiling of secondary metabolites and will give different profiles. Thus, the method used must be carefully chosen and adapted according to the facilities available and the aim of the research undertaken.

The method, described here, was originally established for the isolation of unknown secondary metabolites. Therefore, it may not be the most suitable for secondary metabolite profiling as it necessitates a large quantity of dried material (at least two g). However, this technique was successfully used to gain an overview of the secondary metabolite content of *Lotus japonicus* and *Lotus corniculatus*, a related species with a better-documented secondary metabolite content.

Although these two species are very similar at the metabolic level, we observed some differences in their profile, allowing us to discriminate between these two species (Figure 2).
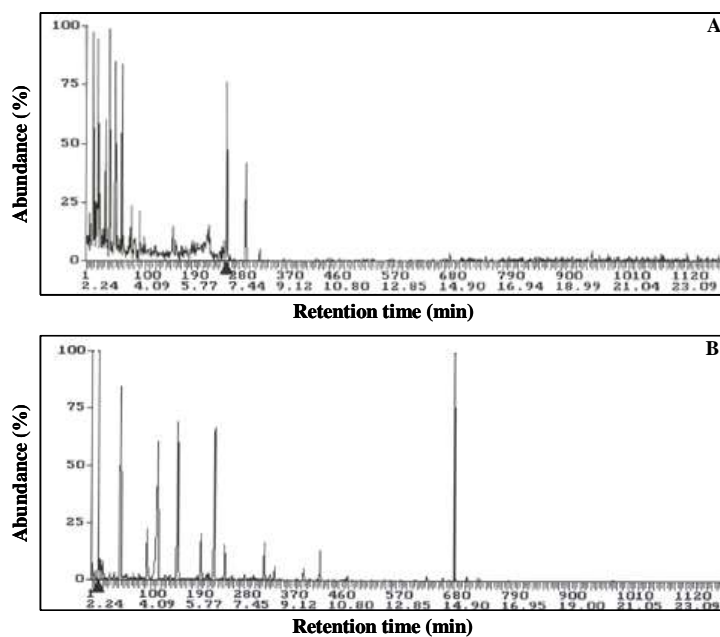

Figure 2. Comparison of GC-MS chromatograms of (A) L. japonicus and (B) L. corniculatus roots. Chromatograms were obtained by separation on a capillary column coated with a non-polar liquid phase using a temperature gradient of 180 ℃ for 5 min, then 180 ℃ to 300 ℃ at 10 ℃/min and further 5 min at 300 ℃.

Moreover, profiling allowed us to detect a novel alkaloid in stems of both species and at a smaller quantity in roots and leaves of *L. japonicus*. This metabolite seems to be a new polyhydroxylated pyrrolidine alkaloid related to DMDP (2R, 5R-dihydroxymethyl- 3R, 4R- dihydroxypyrrolidine) and HomoDMDP (Figure 3) and is likely to be a glycosidase enzyme inhibitor, which may play a role in the plant defence reaction against insect or nematode attacks. Thus, further studies of this compound are currently in process in collaboration with Dr Nash and Molecular Nature Ltd.
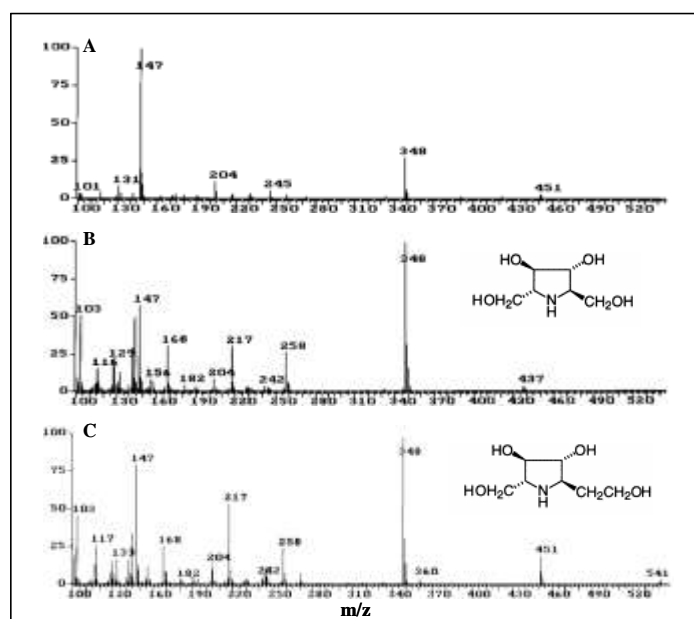

Figure 3. Mass spectra of the novel compound (A) and comparison with the mass spectra of DMDP (B) and HomoDMDP (C). Each spectra was collected with a mass spectrometer (TMD detector) between 71 and 600 m/z after GC-MS separation on a capillary column coated with a non-polar liquid phase using a temperature gradient of 180 °C for 5 min, then 180 °C to 300 °C at 10 °C/min and further 5 min at 300 °C.

REFERENCES

Hattenschwiler S, and Vitousek P. (2000) **The role of polyphenols in terrestrial ecosystem nutrient cycling**. *Trends in Ecology and Evolution* 15, 238-243.

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, and Yakoby N. (2002) **Plants and human health in the twenty-first century**. *Trends in Biotechnology* 20, 522-531.

Reddy L, Odhav B, and Bhoola KD. (2003) **Natural products for cancer prevention: a global perspective**. *Pharmacology & Therapeutics* 99, 1-13.

Watson AA, Fleet GWJ, Asano N, Molyneux RJ, and Nash RJ. (2001) **Polyhydroxylated alkaloids -- natural occurrence and therapeutic applications**. *Phytochemistry* 56, 265-295.

Weisshaar B, and Jenkins G. (1998) **Phenylpropanoid biosynthesis and its regulation**. *Current Opinion in Plant Biology* 1, 251-257.

Winkel-Shirley B. (2001) **Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology**. *Plant Physiology* 126, 485-493.