Chapter 7.5

PHENOLIC COMPOUNDS: EXTRACTION AND ANALYSIS

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Phenolic compounds make up one of the major families of secondary metabolites in plants, and they represent a diverse group of compounds. Phenolics can be broadly divided into non-soluble compounds such as condensed tannins, lignins, and cell-wall bound hydroxycinammic acids, and soluble phenolics such as phenolic acids, phenylpropanoids, flavonoids and quinones. In this article, a method for extracting and analysing the soluble fraction of these phenolics, using HPLC coupled to photodiode array (PDA) detector, is described along with its uses to profile the flavonoid content of Lotus japonicus.

INTRODUCTION

Phenolic compounds are a large and diverse group of molecules, which includes many different families of aromatic secondary metabolites in plants. These phenolics are the most abundant secondary metabolites in plants and can be classified into non-soluble compounds such as condensed tannins, lignins, cell-wall bound hydroxycinammic acids, and soluble compounds such as phenolic acids, phenylpropanoids, flavonoids and quinones. All these groups are involved in many processes in plants and animals. One family, the flavonoids, is of particular interest because of its multiple roles in plants and its impact on human health (Harborne and Williams, 2000).

In plants, flavonoids play a role in flower and seed pigmentation, in plant fertility and reproduction, and in various defence reactions to protect against abiotic stresses like UV light or biotic stresses such as predator and pathogen attacks (Weisshaar and Jenkins, 1998; Winkel-Shirley, 2001; Forkmann and Martens, 2001). Evidence

also suggests their involvement in plant growth and development since they have been shown to regulate polar auxin transport (Murphy et al., 2000; Brown et al., 2001).

In legumes, they play a significant role in the nitrogen-fixing symbiosis at two different levels. First, they regulate the synthesis of Nod factors in *Rhizobium*, which is responsible for the inception of the nodule (Peters et al., 1986; Zaat et al., 1988; Recourt et al., 1992). Second, they act as molecule signals in the transduction pathways of the Nod factor leading to the inhibition of auxin transport within the root cortical cells and therefore to the formation of nodule primordia (Mathesius et al., 1998). Moreover, there is some evidence indicating that flavonoids may also be involved in arbuscular mycorrhiza symbiosis (Harrison and Dixon, 1993; Harrison and Dixon, 1994; Akiyama et al., 2002) although in a non essential manner (Becard et al., 1995).

The flavonoid family is subdivided into different sub-families such as flavones, isoflavones, flavanones, flavonols, anthocyanins, chalcones and condensed tannins. All of these types of compounds comprise three aromatic rings harbouring different substitutions such as methylation and hydroxylation (Figure 1; Aoki et al., 2000). Most of the flavonoid aglycones are found in a glycosylated form in plant cells, this is assumed to protect them from degradation, to reduce their toxic effects and to aid their transport across membranes by increasing their water solubility (Jones and Vogt, 2001). These compounds, in their aglycone or glycosylated forms, are located in the vacuoles within plant cells and are in the polar soluble fraction. Therefore, flavonoids can be easily extracted with polar solvents such as methanol, which is not the case for insoluble lignins and tannins that bind to proteins on cell disruption during the extraction. Then, flavonoids and other phenolics present in the polar soluble fraction can be separated by reverse phase HPLC equipped with a photo diode array detector (HPLC-PDA) which establishes the light absorbance spectrum from visible and UV wavelengths of each detected compound, as phenolic compounds are aromatic and show intense absorption in the UV region of the spectrum.

In this article, we describe a method that was developed to profile soluble phenolics, which are mainly flavonoid glycosides, and its application in profiling the flavonoid content of our model species, *Lotus japonicus*.

PROCEDURE

Materials

The procedure described below is set up for the analysis of the major phenolic compounds on a Water Ass HPLC system. This system is equipped with µ-Nova-Pak reverse phase C_{18} 8x10 RCM column (Water Ass), a photodiode array detector (Water 996 PDA detector, Water Ass) with Millenium 32® software, for the separation, detection and data analysis respectively. All glassware, consumables, and solutions used are described in details in the protocol.

Prior to the extraction of the major phenolic compounds, the plant samples to be analysed are freeze-dried and ground to a fine powder.
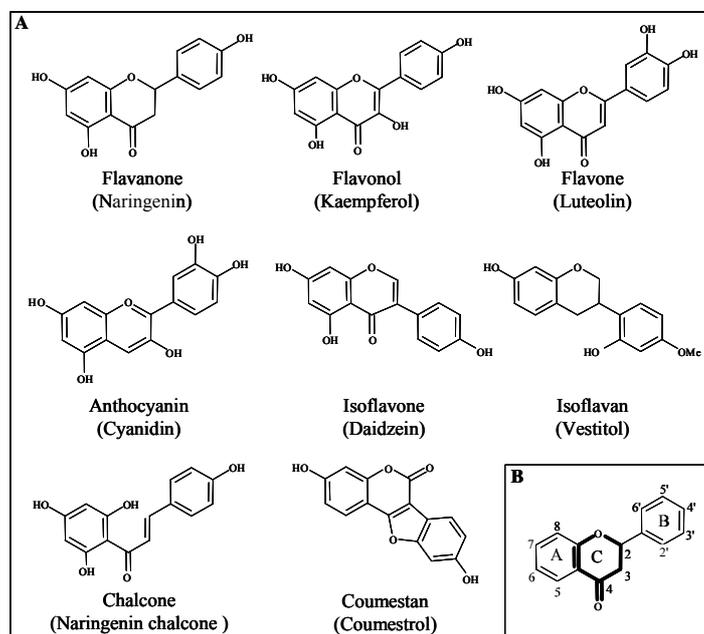
Figure 1. Flavonoid structures (Adapted from Aoki et al*., 2000). **A:** Chemical structure of selected subclasses and biologically active flavonoids. **B:** General structure of flavonoid compounds. The different flavonoid classes are defined according to the structure of the C-ring and the functions carried by C_3 and C_4 (in bold).*

Extraction of Phenolic Compounds

Flavonoid compounds are extracted from 50 or 100 mg of freeze-dried sample in 5 ml of 70% methanol HPLC grade (Fisher Chemical, UK), and shaken for 15 min at room temperature. After centrifugation (3,000 rpm, 5 min), the supernatant is filtered on Fisherbrand QL125 90 mm filter paper (Fisher Science Corporation, UK). The pellet is then re-extracted with 5 ml of 70% methanol and finally rinsed with 5 ml of 100% methanol. All three supernatants are pooled together before removal of the methanol under vacuum with a rota-evaporator at 70°C (Büchi, Switzerland). When the methanol has evaporated, the extract is concentrated and purified using an activated Sep Pack C_{18} column (Sep-Pak®RC Cartridge, Water) and eluted with 4 ml of 100% HPLC grade methanol before being stored at 4°C prior to analysis.

Analysis of Phenolic compounds

Between 0.4 to 4 ml of extracted sample is dried using a SC-3 sample concentrator (Techne) and resuspended in 30 μl of 70% methanol.

Samples are analysed by high performance liquid chromatography on a Waters Millennium 32® system. Flavonoids are separated by gradient HPLC on a μ-Nova-Pak reverse phase C_{18} (8x10) RCM column (Water Ass) with a linear methanol/acetic acid (5%) gradient from 0 to 100% methanol in 50 min at a flow rate of 2 ml/min. Eluting peaks are monitored with a photodiode array detector (Water 996 PDA detector, Water Ass) at 280, 340, and 380 nm and spectra are recorded between 240 to 400 nm. Detection is realised using these three wavelengths in order to observe a wide range of phenolic classes: phenolic acids and isoflavonoids at 280nm, flavonoids and coumestans at 340nm and chalcones at 380nm. Data analysis is processed using an adapted processing method using Millennium 32® software and the UV/visible spectra of the eluted peaks were matched with those contained in our own libraries containing previously analysed standards and unknown compounds.

COMMENTS

The method described here allows separation and analysis of the soluble fraction of the major phenolic compounds present in plant tissue. It also allows the quantification of the different detected compounds. Therefore, it is the method of choice to profile the phenolic content of plant species like *Lotus japonicus* but also for observing changes in the phenolic profile after challenging the plant with symbionts, pathogens or other stresses.

Although the extraction method used here is one of the easiest and quickest, other methods are available based on differential solvent extraction with increasing polarity, which may be more useful for profiling minor phenolic components. Therefore, the method used must be carefully chosen according to the research aim.

This technique provides us with the UV spectrum of the different compounds detected. In the absence of authentic standards, these UV spectra only allow determination of the compound class. Therefore, this method must be coupled to HPLC-MS and/or NMR analyses in order to identify completely the compounds detected and get some insight into their structure. However, this method was successfully used to obtain the phenolic profile of different tissue of *Lotus japonicus*. For instance, the leaf profile revealed mainly flavonol glycosides derived from kaempferol and quercetin, deduced by comparison with their respective UV spectra (Figure 2).

We also used this technique to evaluate the effect of the arbuscular mycorrhizal fungus colonisation on the phenolic content of *Lotus japonicus* and found some significant qualitative differences (Figure 3).
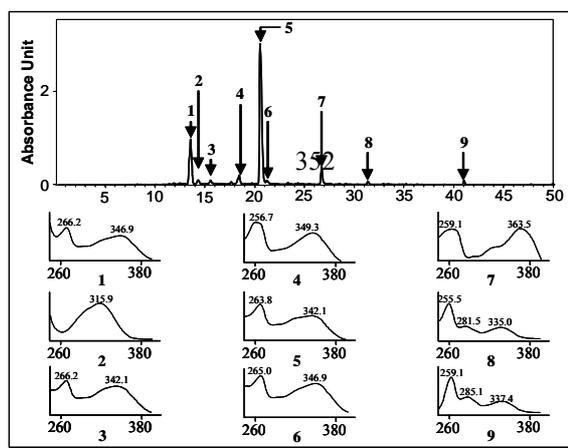
Figure 2. Phenolic compound profile of Lotus japonicus *leaves. Chromatograms were obtained* via *a gradient of 0 to 100 % methanol in 50 minutes using 5 % acetic acid and 100 % methanol after injection onto a μ-Nova-Pak reverse phase C18 column. The UV spectrum of each peak, established by the photo diode array detector, was shown underneath the chromatogram. Compounds 1,3,4,5 and 6: Flavonol Glycoside. 2: Hydroxycinnamic Acid Ester. 7: Kaempferol. Compounds 8 and 9: Unknown.*
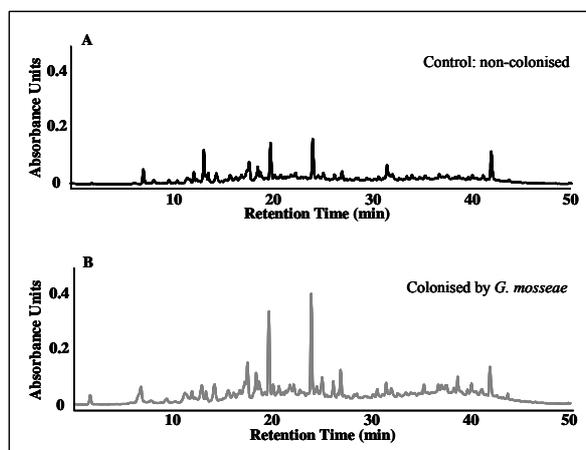

Figure 3. Influence of inoculation with Glomus mosseae *(AM fungus) on flavonoid content of* Lotus japonicus *roots. Comparison of HPLC-PDA chromatograms of* Lotus japonicus *var Gifu non-colonised (A) and colonised by* Glomus mosseae *(B). Chromatograms were obtained* via *a gradient of 0 to 100 % methanol in 50 minutes using 5 % acetic acid and 100 % methanol after injection onto a μ-Nova-Pak reverse phase C_{18} column.*

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