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Note

Efficient clean up of non-aqueous plant extracts using reversed-phase cartridges

Applications to the determination of phytoalexins from *Brassica* spp. by high-performance liquid chromatography

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Phytoalexins are low-molecular-weight antimicrobial compounds that are synthesized *de novo* by plants in response to microorganism challenge[1]. These metabolites are a chemically heterogeneous group of compounds but certain chemical types tend to be associated with particular plant families[2].

A phytoalexin, brassilexin (Fig. 1), has been recently isolated from *Brassica juncea* and *B. napus*[3,4]. Its accumulation was related to resistance to *Leptosphaeria maculans*, a fungus which causes the blackleg disease of crucifers[4,5]. Brassilexin has been related to sulphur-containing indol phytoalexins, which were isolated from Chinese cabbage (*Brassica campestris* spp. *pekinensis*), Japanese radish (*Raphanus sativus*) and oilseed rape (*B. napus*)[4,6–9] (Fig. 2).

Extraction of phytoalexins from leaves and further steps of purification and determination are usually complicated by the presence of pigments, waxes, sterols and other interfering compounds. All these compounds are also highly damaging to high-performance liquid chromatography (HPLC) equipment and columns. Many authors bypass this problem using non-chlorophyllous or etiolated plant tissues, *e.g.* refs. 9–14, or diffusates from elicited plant tissues, *e.g.* refs. 15 and 16. Extraction with light petroleum or hexane and preparative chromatography have also been used to remove pigments, *e.g.* refs. 17–20. These methods were usually inefficient or their yields were too low and insufficiently consistent for quantitative analysis of brassilexin from small plant samples (fresh weight 1–2 g)[4].

In this paper, we present a new and unusual use of reversed-phase cartridges to eliminate these interfering compounds from non-aqueous plant extracts, prior to HPLC quantitative analysis of sulphur-containing indol phytoalexins.

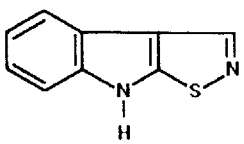

Fig. 1. Chemical structure of brassilexin[3].

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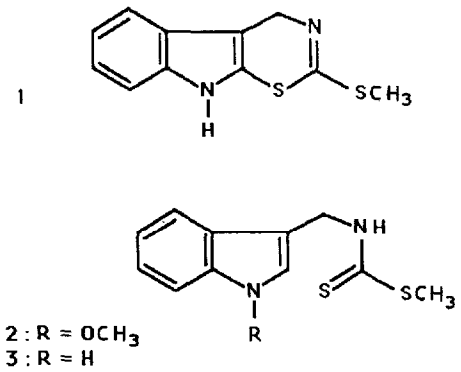

Fig. 2. Chemical structures of cyclobrassinin (**1**), methoxybrassinin (**2**) and brassinin (**3**)[8].

MATERIALS AND METHODS

Plant material and elicitation

B. juncea cv. Aurea and *B. napus* (cvs. Brutor and Primor) plants were grown in the glasshouse, under the conditions previously described[5].

To elicit phytoalexin accumulation, plants in the greenhouse or leaf disks were contaminated with pycniospore suspensions of *L. maculans* or sprayed with a 10 m*M* aqueous solution of $CuCl_2$ containing Tween 80 as described previously[5].

Instrumentation and chemicals

Extra-Sep reversed-phase cartridges (sorbent mass/column volume : 100 mg/1.0 ml) were obtained from Lida Manufacturing Corp. and Bond Elut endcapped reversed-phase cartridges from Analytichem International. The sorbents tested were C_{18}, C_8, C_2, CH, PH and CN.

A Waters 600 multisolvent delivery system equipped with a Waters U6K universal injector was used for analytical HPLC. Chromatographic and spectral data from the eluate were acquired with a Waters 990 photodiode array detector. Data were then stored in the memory and treated by a NEC APC III computer loaded with the Waters 990 software. Reversed-phase HPLC took place through a Brownlee C_{18} column (220 mm × 4.6 mm, particle size 5 μm) which was directly coupled with a 30-mm Brownlee guard column packed with the same stationary phase.

Solvents were obtained from Carlo Erba [ethanol 95% (v/v) RPE-ACS, methanol RS HPLC] or Prolabo (hexane R. P. Normapur, dichloromethane HPLC). Twice distilled water was produced in the laboratory. HPLC solvents were filtered through 0.45 μm Millipore filters and degassed by flushing through with helium.

Extraction of phytoalexins

To extract brassilexin from *B. juncea*, elicited leaves or leaf disks (fresh weight 1–2 g) were macerated in 95% (v/v) ethanol at 80°C for 15 min as previously described[5]. To extract methoxybrassinin and cyclobrassinin from *B. napus*, elicited leaves or leaf disks (1–2 g) were homogenized in 95% (v/v) ethanol at room temperature (Waring blender)[5]. The ethanolic extracts were evaporated to dryness under reduced pressure at 40°C and the residues taken up in hexane (2 × 2 ml).

Sample clean up (Fig. 3)

The cartridges were dried in an oven (40°C, 4 h) before use. Hexane samples were passed through the *unsolvated* cartridges and the sorbent washed with hexane until elution of a colourless solution. Cartridges were then eluted with 3 × 1 ml methanol–water (50:50, v.v). The colourless eluates containing the phytoalexins were dried under reduced pressure (40°C) and recovered in dichloromethane (3 × 3 ml). Dichloromethane was evaporated under a stream of nitrogen (50°C, Reacti-therm and Reacti-vap, Pierce) to prevent the strong oxidation of these compounds in the air. Finally the residue was taken up in 100 μl 95% (v/v) ethanol.

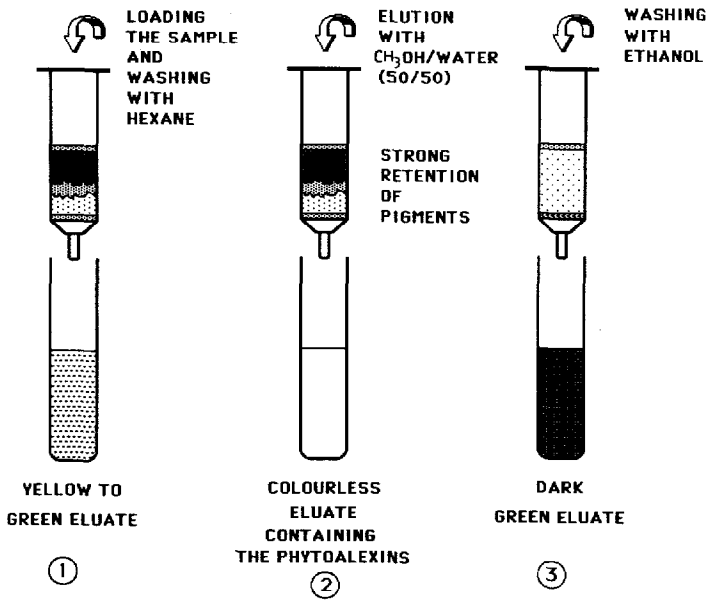

Fig. 3. Sample clean up protocol using reserved-phase cartridges.

Quantitative analysis of phytoalexins

Samples (20 μl) were chromatographed on the analytical C_{18} column eluted with a methanol–water gradient as previously described[5]. Known quantities of brassilexin (purified in the laboratory), brassinin, cyclobrassinin and methoxybrassinin (calculated from their absorption coefficients recorded with a Pye Unicam SP8-200 spectrophotometer) were chromatographed under the same conditions as standards.

Protocol efficiency

The efficiency of the protocol was estimated by adding 5–10 μg of purified phytoalexins to the ethanol during the extraction of unelicited plant samples. The quantities of phytoalexins recovered after sample clean up and HPLC analysis were compared to the quantities added to the ethanol.

RESULTS

Extraction of phytoalexins

Under the analysis conditions, the retention times of brassilexin, brassinin, methoxybrassinin and cyclobrassinin were 7.4 (± 0.5), 8.2 (± 0.3), 9.2 (± 0.2) and 9.7 (± 0.2) min respectively.

The extraction of elicited *B. napus* tissues with hot ethanol allowed the elimination of a compound with a retention time close to that of brassilexin. As this phytoalexin was usually present in small amounts in these plants[4,5], this extraction facilitated the determination of brassilexin. The extraction led to important losses of brassinin, methoxybrassinin and cyclobrassinin and was inadequate for quantitative analysis of these phytoalexins (Table I). Extraction by homogenization of leaves in ethanol at room temperature permitted an excellent recovery of all phytoalexins (Table I) but quantitative analyses of brassilexin was less precise in elicited *B. napus* tissues.

TABLE I

RECOVERY (%) OF PHYTOALEXINS AFTER EXTRACTION, CLEAN UP AND HPLC ANALYSIS

Amounts of 5–10 μg of phytoalexins were added to ethanol at 80°C or ethanol at room temperature during the extraction of unelicited plant samples. Plant extracts were then cleaned up using various reversed-phase cartridges. The recovery is expressed as percent of the quantity of phytoalexin added to the ethanol. Each value is the mean of 12–15 measurements (three experiments) and the standard deviation is noted.

Cartridge	*Brassilexin*	*Brassinin*	*Methoxy-brassinin*	*Cyclo-brassinin*
C_{18} (extraction with ethanol at 80°C)	77.8 ± 5.7	47.0 ± 3.4	27.6 ± 7.8	51.8 ± 9.4
C_{18}	83.9 ± 5.7	82.9 ± 5.9	82.5 ± 5.6	67.2 ± 6.0
C_8	82.0 ± 4.9	82.4 ± 7.2	80.7 ± 9.9	66.5 ± 4.8
C_2	75.6 ± 3.0	82.8 ± 3.4	68.2 ± 12.4	65.8 ± 5.2
Ph	67.3 ± 8.9	87.5 ± 9.3	84.2 ± 7.2	71.3 ± 11.7

Efficiency of various reserved-phase cartridges in sample clean up

As shown in Fig. 3, washing by hexane, whatever the volume introduced, gave a yellow to green eluate containing no traces of brassilexin, brassinin, methoxybrassinin and cyclobrassinin. A nearly colourless eluate containing these phytoalexins was obtained upon elution with methanol–water. Pigments and other interfering compounds were strongly retained on the sorbent from which they were eluted by 95% (v/v) ethanol. In this case, the cartridges could be used again with the same efficiency if carefully dried (with the exception of endcapped reversed-phase, see below). This sample clean up (Fig. 4) permitted the elimination of polar compounds, which were not dissolved with hexane and dichloromethane, and of non-polar compounds (retention time > 11 min) showing a strong absorbance at 420 nm.

All types of bonded silicas tested, whether endcapped or not, cleaned up the *Brassica* extracts equally efficiently (Fig. 5) without interfering with brassilexin. The

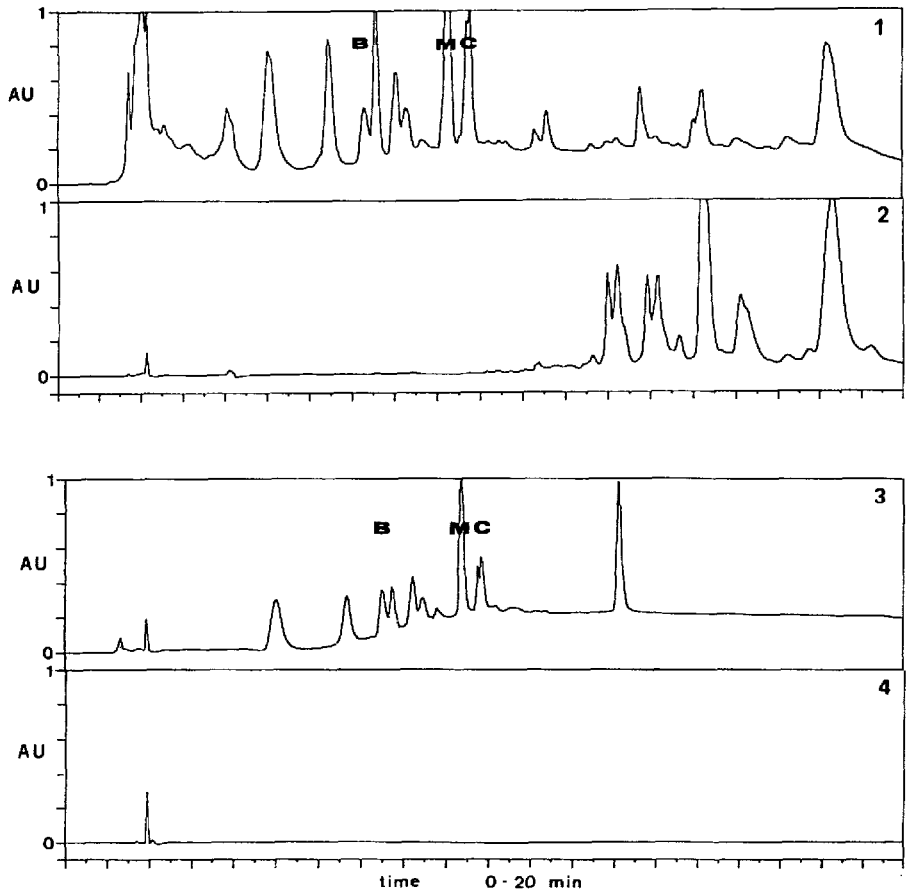

Fig. 4. Chromatograms at 218.2 (1 and 3) and 420 nm (2 and 4) of an ethanolic extract of elicited *B. napus* before (1 and 2) and after (3 and 4) sample clean up. The leaves were macerated in ethanol 80°C and the extract was cleaned up using C_{18} cartridges (see Fig. 3). The cleaned up samples (20 μl) were injected on a C_{18} analytical column (5 μm particle size). The solvent delivery consisted of a linear gradient from 50% (v/v) methanol in water to 100% methanol in 5 min, then maintained for 10 min. The flow-rate was constant at 1.5 ml min^{-1}. B = brassilexin; M = methoxybrassinin; C = cyclobrassinin.

total yield of the method was very high for brassilexin, brassinin and methoxybrassinin, using C_{18} or C_8 cartridges to clean up samples (Table I). It was lower for cyclobrassinin, whatever the sorbent used.

DISCUSSION

Dahiya and Rimmer recently described procedures for determining methoxybrassinin and cyclobrassinin from elicited *B. napus* or *B. juncea* tissues. Since these authors used callus tissues[14] and leaves or stem segments incubated in the dark[9], their analyses were less complicated by interfering compounds, *e.g.*, pigments than ours (see Fig. 4), and the use of a guard column was sufficient to protect the column.

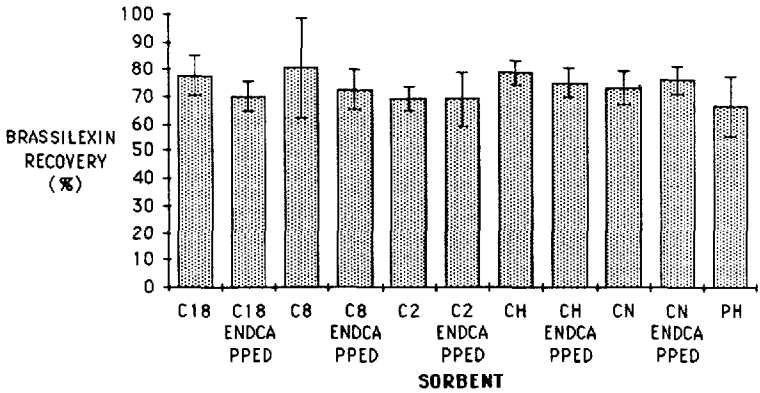

Fig. 5. Brassilexin recovery after sample clean up and HPLC analysis. Amount of 5–10 μg of the phytoalexin were added to ethanol during the extraction of unelicited plant samples. These samples were then cleaned up using various endcapped or non-endcapped reversed-phase cartridges. The recovery is expressed as percent of the phytoalexin added to the ethanol. Each value is the mean of 12–24 measurements (3–6 experiments) and the standard deviation is noted.

Brassilexin appears to be less apolar than methoxybrassinin and cyclobrassinin. We thus need a more polar elution system than that used by Dahiya and Rimmer[14] to get a good separation of indol phytoalexins. With this elution system, chlorophyllous pigments and other interfering compounds precipitate in aqueous solvents, and with repeated injections, are damaging to HPLC apparatus and columns (see Fig. 4). The filters and guard column used for analysis were inappropriate to protect the column and pipes.

We previously proposed a protocol for brassilexin analysis, in which ethanolic extracts were fractionated between water and diethyl ether prior to HPLC analysis[5]. This permitted us to discard polar compounds but most of the pigments were still present in the samples and interfered with the analysis. We therefore tested classic uses of silica or bonded silica cartridges to clean up samples. Neither silica cartridges with non-polar solvents (hexane or dichloromethane) nor bonded silica cartridges with polar solvents (methanol–water) allowed us to eliminate undesirable compounds.

Finally, we considered a less classic separation. First hexane was used to dissolve samples, thus discarding most polar compounds. The separation obtained with the novel strategy, described here using bonded silicas and appropriate solvents, may first be due to polar interactions between some compounds of the sample and non-bonded silanol groups in the non-polar solvent (Fig. 3, No. 1). In this case, the most non-polar metabolites, *e.g*, lipids or carotenoids are not retained on the sorbent and are directly eluted with hexane. Other compounds are retained by polar interaction with silanol groups. Elution with a polar solvent (methanol–water) solvates the sorbent and suppresses polar interactions between the metabolites and silanol groups (Fig. 3 No. 2). The most non-polar compounds still present, *e.g*, chlorophyllous pigments will then interact with the functional groups of bonded silicas (non-polar interaction in a polar solvent) and be retained. Moderately non-polar compounds, like the phytoalexins, are eluted with the solvent. Finally, the retained compounds are eluted with ethanol (Fig. 3, No. 3).

To support this hypothesis it is important to note that all bonded silicas tested were equally efficient for the separation of phytoalexins from unwanted compounds (Table I, Fig. 5). On the other hand, the use of endcapped bonded silicas (mostly C_{18}) showed that chlorophyllous pigments, which were previously retained on the sorbent, are profusely eluted with hexane. As for brassilexin, it was identically retained on endcapped and non-endcapped sorbents (Fig. 5). This suggests, if our hypothesis is sound, that all silanol groups are not methylated during the endcapping process. In this respect we note that non-endcapped cartridges can be used many times with equal efficiency. On the contrary, endcapped cartridges can never be used more than twice to clean up samples. On the third use of the same cartridge, phytoalexins and pigments are no longer retained on the sorbent. This may mean that irreversible bonding to silanol groups occurred during the cleaning up the samples. The fewer free silanol groups present in endcapped bonded silicas than in non-endcapped ones would then explain why the latter can be reused many times, which is not the case with the former. The same phenomenon was observed with insufficiently dried sorbent. In this case, unactivated silanol groups led to important losses of phytoalexins and pigments.

The protocol presented here for quantitative analysis of *Brassica* phytoalexins is easy to use, rapid and accurate. Its yield is excellent. It would then be interesting to see whether it can be used to separate other metabolites from various kind of matrices. In our case, this protocol is particularly efficient for comparison of the accumulation of phytoalexins between single plantlets or even single leaves, and to screen for plants possessing the genes conditioning the hypersensitive resistance to *L. maculans* in the population of interspecific crosses between *B. juncea* and *B. napus*[4,21].

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