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Chlorophyll-derived Porphyrins Co-chromatograph with Phospholipids in High Performance Liquid Chromatographic Separations of Plant Lipid Classes‡

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Total lipid extracts were prepared from the leaves of potatoes, tomatoes, grapes and spinach, and each was analyzed by normal-phase high performance liquid chromatography (HPLC). When these total lipid extracts were subjected to mild alkaline hydrolysis and the hydrolysates analyzed by normal-phase HPLC, distinct peaks were detected in the region where common phospholipids typically elute (i.e. at 48, 50, and 68 min). The ultra violet-visible spectra of these peak fractions revealed that each exhibited absorption maxima at 400 and 660 nm, suggesting that the peaks were porphyrins, most likely derived from chlorophylls. Mild alkaline hydrolysis apparently cleaved the ester bond of the chlorophylls and released the porphyrin and phytol components. This explanation was verified when commercially prepared chlorophylls *a* and *b* were subjected to the same alkaline hydrolysis conditions and identical peaks at 48 and 68 min were observed. Experiments with buffered (pH 6.0) aqueous homogenates of potato and tomato revealed that similar chlorophyll-derived porphyrins were generated by endogenous enzymes. With the increasing popularity of HPLC as a tool for plant lipid analysis, users of this methodology should be cautioned as to the occurence of these non-phospholipid peaks in the retention time region where phospholipids commonly elute. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

High-performance liquid chromatography with light-scattering detection (HPLC-ELSD) is becoming a valuable tool for the analysis of plant lipids (Conforti *et al.*, 1993; Picchioni *et al.*, 1996). Mild alkaline hydrolyis is a common procedure to hydrolyze fatty acyl lipids and retain other types of lipids intact (e.g. sphingolipids, plasmalogens, and hopanoids). In our recent study of leaf lipids of potatoes, we noted that alkaline hydrolysis appeared to create three new peaks in the polar lipid region. The present work confirms that these peaks occur in the hydrolysates of other green tissues, and it identifies the probable chemical structures of these peaks as being those of chlorophyll-derived porphyrins.

EXPERIMENTAL

Plant material and purified reagents. Potato (c.v. Homestead), tomato (c.v. Pixie), and grape (c.v. Remaily seedless) plants were grown in a greenhouse. The grape plants were derived from tissue culture. Spinach was purchased at a local market. Leaves were harvested and the lipids were extracted immediately as described below. Chlorophylls *a* and *b* (each from spinach) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Lipid extraction. Leaf samples (2 g) were homogenized in a mixture of chloroform:methanol:water (8 mL:16 mL:4.8 mL) with a Polytron homogenizer (Brinkman, Westbury, NY, USA), and lipids were extracted according to the method of Bligh and Dyer (1959).

Analytical normal phase—HPLC. The lipids were separated and quantified using a method similar to one that we have used for lipid analysis in plant tissues (Moreau *et al.*, 1990) and for hopanoid analyses in other species (Moreau *et al.*, 1995). The column was a LiChrosorb 5 Si 60 (100×3 mm i.d.) from Chrompack Inc. (Raritan, NJ, USA), operated at a flow-rate of 0.5 mL/min. The solvents were: **A**—hexane, **B**—isopropanol and **C**—0.04% triethylamine in water (the latter being prepared fresh daily). The linear gradient of **A**:**B**:**C** was: 0 min—100:0:0 (v/v/v), 5 min—19:1:0, 10 min—85:15:0, 15 min—8:12:0, 53 min—40:51:9, 68 min—40:51:9, 73 min—8:12:0, 78 min—100:0:0, and 100 min—100:0:0. The HPLC system consisted of an ISCO (Lincoln, NE, USA) model 2350 pump, an ISCO model 2360 gradient programmer, an ISCO model V4 UV-visible detector, and an Alltech-Varex Mark III evaporative light-scattering detector (Alltech Associates, Inc., Deerfield, IL, USA) operated at 40 °C with 1.60 L/min of nitrogen as the nebulizing gas. When collecting the peaks for determination of the UV-visible spectra, the column effluent was split by placing a Valco "T" between the

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column and the detector such that 10% of the effluent entered the ELSD and 90% was collected.

Mild alkaline hydrolysis. The total lipid extract (10–15 mg) was hydrolyzed in 2 mL 1.5 N KOH in methanol and 200 μL water for 1 h at 70 °C. The hydrolyzates were then cooled, acidified with HCl, and the lipids were re-extracted with chloroform:methanol as described above.

Autolytic generation of chlorophyll fragments. Leaves (2 g) of potato and tomato were homogenized with a mortar and pestle in 20 mL of buffer containing 0.3 M sucrose, 0.1 M MES, 2 mM EDTA, 5 mM dithiothreitol and 5 mM β-mercaptoethanol adjusted to pH 6.0 with KOH (as previously described by Moreau, 1987). The filtered homogenate was incubated for 4 h at 30 °C, with shaking. The lipids were then extracted with chloroform:methanol and analyzed by HPLC as described above.

Solid phase extraction. Lipid extracts were dissolved in 5 mL chloroform and applied to a dry Mega-Bond-Elute silica gel (10 g) solid phase extraction cartridge (Varian, Harbor City, CA, USA). Non-polar lipids were eluted with 50 mL of chloroform. An intermediate polarity lipid fraction (generally considered glycolipids) was eluted with 50 mL acetone, and polar lipids were eluted with 50 mL methanol.

UV-visible spectra. Sample peaks, which eluted from the HPLC column in the effluent stream composed of hexane:isopropanol:water approximately 8:11:1 (v/v/v), were collected. A 1 mL aliquot of each sample was placed in a quartz cuvette and its UV-visible spectrum (190–900 nm) was obtained with a Shimadzu UV 1601PC UV-visible spectrophotometer (Shimadzu Scientific Instrument Co., Columbia, MD, USA) equipped with UVPC kinetics software.

RESULTS AND DISCUSSION

The total lipid extract from potato leaves was analyzed via normal-phase HPLC-ELSD (Fig. 1A). Typical polar lipid peaks consisted of galactolipids (monogalactosyldiglyceride (MGDG) and digalactosyldiglyceride (DGDG)) and common phospholipids (phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC)). When this total lipid extract was subjected to mild alkaline hydrolysis and the hydrolysate analyzed using the same HPLC system, the peaks of PE, PI, and PC were absent, and two new major peaks were observed in the polar lipid region, wtih retention times of about 50 and 66 min (Fig. 1B). Solid phase extraction (SPE) with a silica cartridge column was used to fractionate the alkaline hydrolysate into three fractions. The first of these fractions, the chloroform fraction, which typically contains non-polar lipids did not exhibit any peaks in the polar lipid region (25–75 min) when analyzed by HPLC-ELSD (data not shown). The second fraction, eluted with acetone, which typically includes plant galactolipids and sulpholipids, contained one major peak with a retention time of about 48 min (Fig. 1C). The third fraction, eluted with methanol, which typically includes phospholipids, contained major peaks with retention times of 50 and 66 min (Fig. 1D). It appears that the broad 50 min peak in the total leaf hydrolysate (Fig. 1B) was apparently fractionated into two sharper peaks by SPE with silica, a 48 min peak in the acetone fraction (Fig. 1C) and a 50 min peak in the methanol fraction (Fig. 1D).

Based on their retention times it was suspected that these hydrolysate peaks at 48, 50, and 66 min might be phospho-sphingolipids (although the occurrence of sphingomyelin and other phospho-sphingolipids has yet to be unequivocally demonstrated in plants) or plasmalogens (Felde and Spiteller, 1994). However, when each was collected and analyzed by UV-visible spectroscopy (Fig. 2), each was found to have absorption maxima at 400 and 660 nm. These absorption maxima are almost identical to those of chlorophylls *a* and *b* (Devlin, 1975), suggesting that mild alkaline hydrolysis cleaved the phytol tail from the chlorophylls, and that the resulting porphyrins (chlorophyllide *a* and *b*) exhibit HPLC and solid phase extraction retention times that are almost identical to those of common phospholipids. In order to test this hypothesis, samples of purified chlorophylls *a* and *b* were purchased and hydrolyzed under the same alkaline conditions as used with the leaf lipid extracts. When these chlorophyll hydrolysates were then analyzed by HPLC-ELSD, the chlorophyll *a* hydrolysate had two major peaks—one at 13.6 min which was previously reported to be the retention time for phytol in this HPLC system (Moreau *et al.*, 1990) and one at 48 min which apparently was the retention time for the porphyrin, chlorophyllide *a*. Furthermore, the chlorophyll *b* hydrolysate had two major peaks, the phytol peak at 13.6 min and one at 66 min which

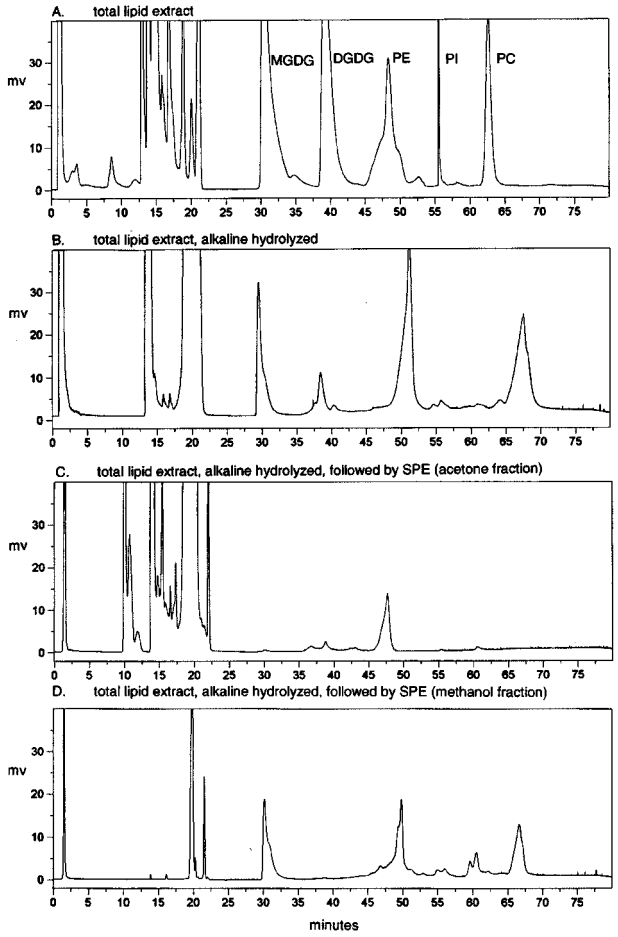

Figure 1. High performance liquid chromatograms with light scattering detection of A—the total lipid extract from potato leaves; B—mild alkaline-hydrolyzed total lipid extract from potato leaves; C—the acetone fraction from SPE with silica of mild alkaline-hydrolyzed total lipid extract from potato leaves; and D—the methanol fraction from SPE with silica of mild alkaline-hydrolyzed total lipid extract from potato leaves.

apparently was the retention time of the porphyrin, chlorophyllide *b*. The absorption spectra of these chlorophyll *a* and *b* hydrolysates were nearly identical (data not shown) to those obtained with the hydrolyzed total leaf extacts (Fig. 2), with sharp peaks at 400 and 660 nm.

In order to investigate whether these types of artefacts could be generated from the leaves of species other than potatoes, leaves of tomatoes, grapes and spinach were also extracted and the total lipid extacts were subjected to mild alkaline hydrolysis. When analyzed by HPLC-ELSD, the hydrolysates of each of these three species also contained artefact peaks at 48, 50, and 66 min. We conclude that these types of artefacts can be generated from leaf extracts of each of the four species of plants examined in this study and are probably quite widespread.

Also, to see whether these artefacts could be generated by endogenous plant enzymes, autolytic experiments (similar to those described previously; Moreau, 1987), were performed (Fig. 3). When buffered (pH 6.0) aqueous homogenates of potato leaves were incubated at 30 °C for 4 h, most (but not all) of the abundant phospholipids were degraded as previously described (Fig. 3A showing ELSD detection) and new peaks of several coloured compounds appeared (Fig. 3B showing visible detection at 400 nm), suggesting that porphyrin fragments were generated by endogenous enzymes. The identity of four peaks (labelled) in the phospholipid region were noted with ELSD detection (Fig. 3A), and their identity as porphyrins was clearly implicated by their strong absorbance with detection at

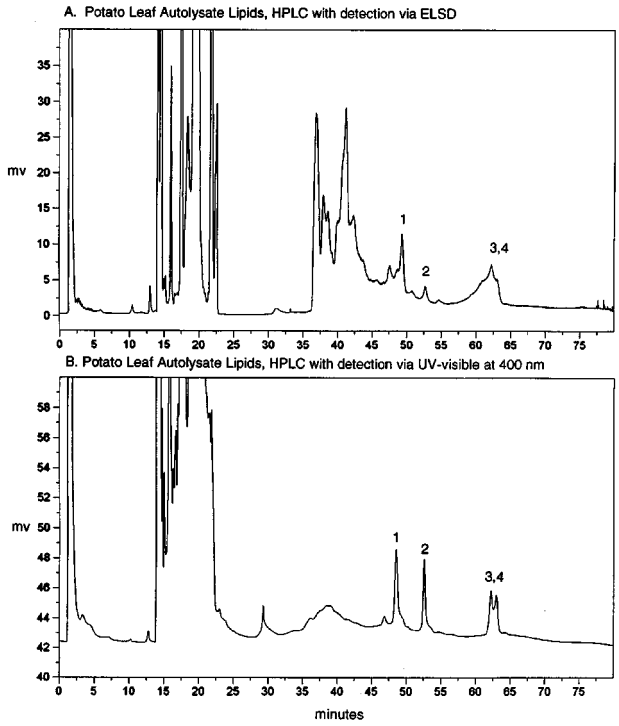

Figure 3. High performance liquid chromatograms, A—with light scattering detection; and B—with UV-visible detection at 400 nm, of the lipids observed in buffered potato leaf homogenates after 4 h incubation at pH 6.0 and 30 °C.

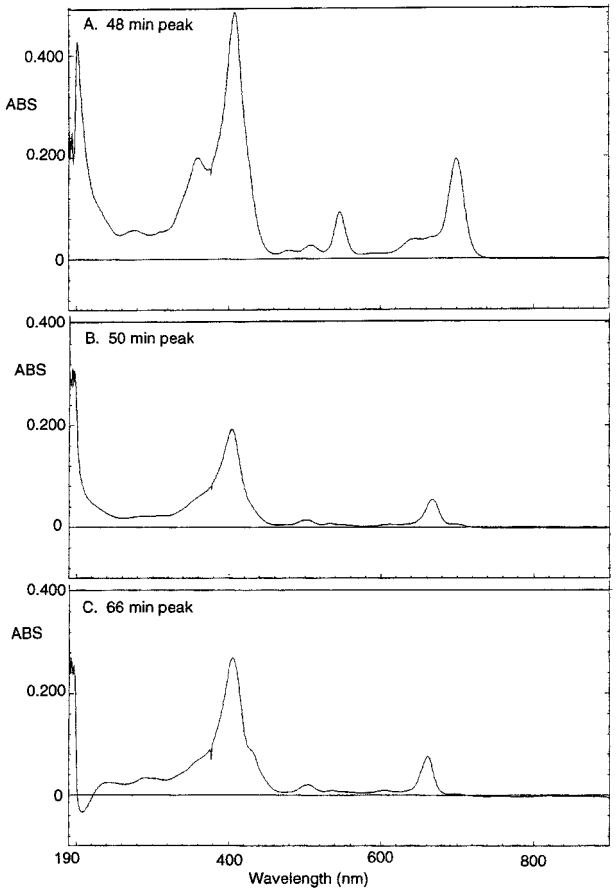

Figure 2. UV-visible spectra of the three major "polar lipid" HPLC peaks in potato leaf lipid extracts at retention times of A—48 min; B—50 min; and C—66 min after mild alkaline hydrolysis of potato leaf lipid extracts.

400 nm (Fig. 3B). Similar peaks were observed when tomato leaf homogenates were similarly prepared, incubated, extracted and analyzed (data not shown). Whereas the retention times of the porphyrins generated autolytically were at 48, 52, 62, and 63 min (Fig. 3), the retention times of the major peaks generated by alkaline hydrolysis were 48, 50, and 66 min, with numerous minor peaks (Fig. 1). These differences in retention times may be due to the fact that alkaline conditions could potentially cause structural changes other than just ester bond hydrolysis. The fact that some of the same peaks were generated by both processes, and that all of the peaks are in the region where common phosphlipids elute, is noteworthy.

The autolytic experiments indicated that some of the plethora of esterases and lipolytic enzymes found in many plant tissues (Moareau, 1987) are capable of cleaving the ester bond in chlorophylls and thereby generating porphyrins (chlorophyllides) under physiological conditions. During processes such as senescence, where there is a rapid breakdown of chlorophyll, the involvement of these endogenous esterases to cleave the porphyrin and phytol parts of the chlorophyll molecule is a biochemical possibility.

The formulation of porphyrin fragments by mild alkaline hydrolysis is not surprising since phytol is bound to the porphyrin ring by an ester bond. However, the findings that (a) these porphyrin fragments have HPLC retention times nearly identical to common phospholipids, and (b) they can be generated by endogenous plant enzymes, are noteworthy. With the increasing popularity of HPLC-ELSD for plant lipid analysis, those employing this valuable tool need to be aware of this information.

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