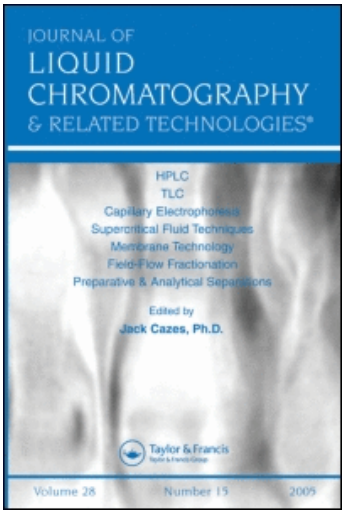

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High Performance Liquid Chromatography of Zinc and Copper Pheophytins

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ZINC AND COPPER PHEOPHYTINS

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ABSTRACT

The zinc or copper chelates of pheophytins a and b were formed and separated on a reversed-phase C-18 column. Allomerized products were produced readily during the chelation reaction. Resolution of the allomerized compounds from the non-allomerized chelates was achieved using a gradient elution technique. Compound identification was facilitated by monitoring the column eluate at both 436 and 658 nm. The method allowed for isolation of individual pigments for further study.

INTRODUCTION

A number of porphyrin metal complexes are readily formed from chlorophyll derivatives(1). Chromatographic methods developed for the analysis of chlorophylls are numerous and have involved liquid chromatography. Review articles summarizing these techniques have been published (2,3,4).

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The formation of zinc and copper pheophytin chelates has been previously reported in canned vegetables (5,6). Recently, the formation of metal chlorophyll complexes has been used to enhance the appearance of heat processed vegetables (7). Copper pheophytin chelates are manufactured commercially as food colorants and are permitted for use in some European countries (8). Jones et al. (9) described a reversed-phase thin layer method for the detection of the zinc and copper complexes in processed foods.

High performance liquid chromatography (HPLC) has recently been used to monitor chlorophylls and their derivatives during the processing of foods (10). However, no HPLC methods are available for the determination of zinc and/or copper metal porphyrin compounds. This report investigates the use of HPLC for the separation and detection of zinc or copper pheophytins.

MATERIALS AND METHODS

Extraction of chlorophylls a and b

Chlorophylls a and b were extracted from forty grams of surface cucumber tissue by blending with 160 g of acetone for two minutes. The extract was filtered through Whatman #1 and #42 filter paper. All pigment extracts were stored at 4,C under nitrogen.

Preparation of pheophytins a and b

Pheophytins a and b were prepared from the chlorophyll extract after acidification with 250 ul of concentrated hydrochloric acid. The pigments were transferred to diethyl ether (100 ml) and the excess acid removed by three successive washings with 100 ml of water. The course of pheophytin formation was monitored by HPLC. After complete conversion (10 min), the diethyl ether layer was dried over anhydrous sodium sulfate. The diethyl ether was removed under nitrogen and the pigments dissolved in dry acetone until further use.

Preparation of copper and zinc pheophytin complexes

The copper and zinc pheophytin complexes were prepared following a procedure similar to that reported by Jones et al. (11). Copper pheophytins a and b were formed by adding 1.0 ml of 2.5 M copper (II) chloride to 4.0 ml of the acetone pheophytin extract (total pheophytin conc. ≃ 0.1 mg/ml). Zinc pheophytins a and b were prepared by adding crystalline zinc chloride (0.3 g) to 4.0 ml of the pheophytin acetone mixture. The samples were periodically mixed and the progress of chelation was monitored by HPLC. Following completion of the reaction (30 - 90 min.), the chelates were transferred to diethyl ether and the organic layer washed with water and dried over anhydrous sodium sulfate. Prior to analysis by

HPLC, the diethyl ether was evaporated under nitrogen and the pigments dissolved in acetone.

Pheophytins a and b were also separated by HPLC and isolated. Each metal chelate was then formed individually and compared to those prepared in pigment mixtures.

TABLE 1

Apparatus and Conditions for the Separation of Zinc and Copper Pheophytins by HPLC

column	μ Bondapak C_{18} (Waters Associates, Milford, MA)
pump A	Waters Associates, Model 510
pump B	Model 510, equipped with an inlet manifold assembly for gradient elution.
solvent A	75:25 CH_3OH:H_2O (v/v)
solvent B	ethyl acetate
initial condition	55% solvent A - 45% solvent B
final condition	50% solvent A - 50% solvent B
gradient	Linear gradient - curve 6 (solvent programmer, Model 680, Waters Associates) for a duration of 15 min.
flow rate	2.0 ml/min.
detector	Waters Associates Model 440, dual channel
injector	Waters Associates Model U6K
sample size	20 ul in acetone
detection wavelength	658 and/or 436 nm

Apparatus and conditions for the HPLC analysis

The HPLC apparatus and conditions used for the separation of the pheophytin metal chelates are outlined in Table 1.

Identification of zinc and copper pheophytins

The metal chelates were identified by their retention times on reversed-phase columns, their visible light absorption characteristics compared to reported literature values, and from observations made of their relative responses at the two monitored wavelengths. Visible absorption spectra were obtained after collecting selected peaks following repeated sample injections. All samples were transferred to diethyl ether for the determination of the visible spectra. Spectra were recorded using a Gilford (Oberlin, Ohio) Model 2600 spectrophotometer and plotted with a Hewlett-Packard (San Diego, CA) Model 7225B graphic plotter.

RESULTS AND DISCUSSION

HPLC chromatograms of fresh cucumber extracts showed only the presence of chlorophylls *a* and *b* when monitored at 658nm. Pheophytins *a* and *b* were formed by the addition of HCl to the chlorophyll *a* and *b* extract. Monitoring the column eluate at 658 nm allowed for selective detection of the chlorophyll compounds and derivatives without interferences from other pigments

present in the extracts. Simultaneously screening the eluate at 436 nm and 658 nm permitted more sensitive detection of the zinc and copper pheophytin b chelates since these compounds have a visible light absorption maxima near the monitored 436 nm wavelength. Measurements of the ratio of the two detector responses also aided in the identification of the separated compounds.

Table 2 summarizes and compares the spectral data for the separated pigments to reported literature values. Since the literature values for the pigment complexes were reported with diethyl ether as the solvent, all experimental pigments were transferred into ether before determining their light absorption characteristics. Use of the eluate mixture as solvent rather than diethyl ether resulted in a slight shift (approx. 2 nm) of the wavelength maxima toward the red region.

Figure 1 shows a representative chromatogram of the zinc pheophytin compounds formed from a mixture of pheophytins a and b. Peaks B (retention time = 8.1 min) and D (retention time = 10.9 min.) were identified as zinc pheophytins b and a, respectively (Table 2). Their order of elution on the reversed-phase column was expected. A similar elution order was observed for the chlorophyll a and b and pheophytin a and b compounds.

TABLE 2

Visible Absorption Spectra Identification Data of Zinc and Copper Chelates[a].

Peak	Peophytin Complex	Retention Time (min)[b]	Absorption max (nm) found	Absorption max (nm) reported[c]
B	Zn b	8.1	448;637	446;634
D	Zn a	10.9	425;654	423;653
B'	Cu b	11.8	441;629	438;627
D'	Cu a	16.2	422;650	421;648

[a] data reported were recorded in diethyl ether.
[b] refer to Figures 1 and 2.
[c] from Jones et al. (11).

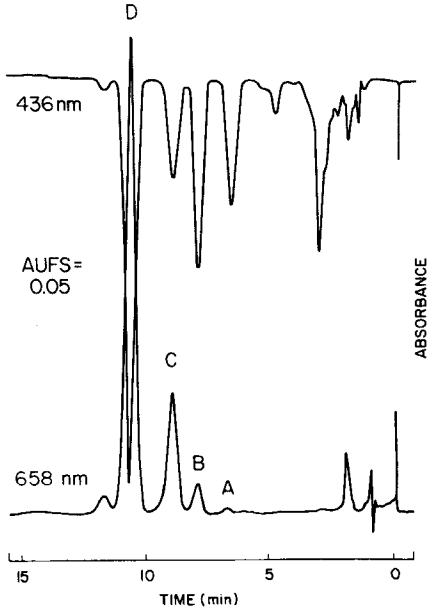

Figure 1. Chromatogram of zinc pheophytins. Peak A = allomerized zinc pheophytin b, peak B = zinc pheophytin b, peak C = allomerized zinc pheophytin a, peak D = zinc pheophytin a.

However, a shift to shorter retention times occurred upon chelation of the pheophytins with zinc.

The rate of chelation of pheophytin a with zinc was much more rapid than that of pheophytin b. Within ten minutes after the addition of zinc chlorides, the chromatogram showed a peak for the zinc pheophytin chelate and no peak for pheophytin a. Approximately one hour was required before a peak for zinc pheophytin b could be detected.

Peaks A and C are believed to be the allomerized (oxidized) zinc pheophytins b and a, respectively. These peaks form in greater concentrations after the mixture was exposed to the atmosphere and continued to increase during the chelation reaction. These findings are in agreement with those reported by Jones et al. (11). These authors found that pigment changes attributed to allomerization were readily induced during the formation of the metal complexes. The allomerized products were found to be more strongly bound (more polar) to a sugar column. The shorter retention times of peaks A and C on the reversed-phase C-18 column suggests this relationship. The visible absorption spectra of certain allomerized complexes lack a secondary peak or a plateau at a wavelength shorter than that of the major absorption peak in the blue region (11). The spectra of the allomerized compounds found in this study agree with these characteristics.

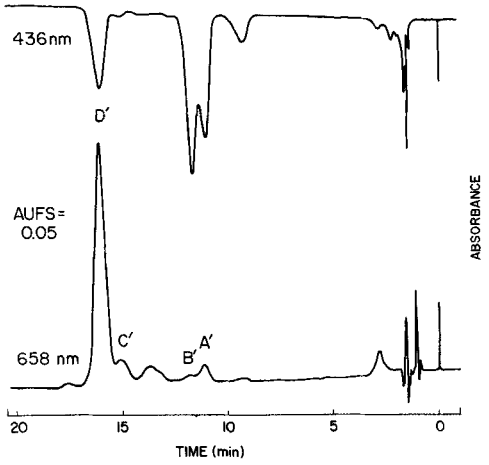

Figure 2. Chromatogram of copper pheophytins. Peak A' = allomerized copper pheophytin b, peak B' = copper pheophytin b, peak C' = allomerized copper pheophytin a, peak D' = cooper pheophytin a.

Figure 2 is a representative chromatogram of the copper pheophytin compounds formed from the reaction of pheophytin a and b with copper chloride. Peaks B' (retention time = 11.8 min) and D' (retention time = 16.2 min) were identified as copper pheophytins b and a, respectively (Table 2). In contrast to the zinc chelates, the copper a and b derivatives have longer retention times than their parent pheophytins. Allomerization compounds (peaks A' and C') were also apparent during the course of the chelation reaction. If a solution of pheophytins a and b were simultaneously injected with the above pheophytin copper ion mixture, the oxidation products were found to co-elute with

pheophytin b (Peak A') and a (Peak C'). Therefore, in order to obtain the copper allomerization compounds, it is necessary to allow sufficient time to complete the chelation reaction. As noted in the formation of the zinc complexes, the pheophytin a copper chelate formed much more rapidly than the b complex and allomerized products were noted particularly in the presence of oxygen. If the chelation reactions were performed under N_2, allomerization products were detected, but to a much lesser extent.

The HPLC method described in this study allows for the separation of either zinc or copper pheophytin complexes. Other pigments present in the sample mixture did not hinder the metal chelation reaction or interfere with the separation. An isocratic solvent system could be used to achieve a similar separation. However, the time required to complete the analysis was shortened considerably by using a gradient elution technique. The developed method also allowed for isolation of individual pigments for further study.

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