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Preparation and determination of zinc(II) chlorophylls by reversed-phase high-performance liquid chromatography

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ABSTRACT

A preparative method for zinc(II) chlorophyll *a* and *b* [Zn(II)-chl-a and -b] was developed on the basis of their purification by semi-preparative high-performance liquid chromatography. Zn(II)-chl-a and -b and the corresponding epimers were separated and determined with an ODS (C_{18} chemically bonded silica gel) column using methanol–acetone (75:25, v/v) as mobile phase. Linear calibration graphs were obtained over the concentration range 0–50 $\mu g\ cm^{-3}$ of each zinc(II) chlorophyll with photometric detection at 425 nm. The present HPLC determination provides an accurate and conventional method with a detection limit of 3.5 ng cm^{-3} for Zn(II)-chl-a, 2.5 ng cm^{-3} for Zn(II)-chl-a′ and 3.0 ng cm^{-3} for Zn(II)-chl-b with a relative standard deviation of less than 2.3% ($n = 10$). The analytical values obtained for synthetic samples by a spectrophotometric method were confirmed to be high compared with those determined by the proposed HPLC method.

INTRODUCTION

A variety of metal-substituted chlorophylls, *i.e.*, metallochlorophylls in which the central magnesium ion of chlorophylls is replaced with other metal ions such as iron(III) and copper(II), are used for colouring materials of foods and some kinds of medicines [1,2]. In recent years a diverse group of metallochlorophylls, including iron(III) and copper(II) chlorophylls, has also received a great deal of attention because of their potential use for electrode materials for photoelectron conversion [3]. This continuing interest in metallochlorophylls requires a conventional method for their preparation and determination. The development of methods suitable for the determination of chlorophylls and metallochlorophylls has been the subject of intense research for many years. In fact, there have been many reports on the chromatographic determination of chlorophylls themselves, *i.e.*, the parent compounds of metallochlorophylls [4–6]. The determination of iron(III), nickel(II) and copper(II) chlorophylls by high-performance liquid chromatography (HPLC) has been reported in previous papers [7–9]. In this paper we propose a semi-preparative HPLC method for purifying zinc(II) chlorophyll *a* and *b* [Zn(II)-chl-a and -b] and also a rapid and accurate HPLC method for their determination. In addition, the liquid chromato-

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graphic behaviour of zinc(II) chlorophylls is described in comparison with that of chlorophylls because Zn(II)-chl-a may serve as an internal standard in the HPLC determination of native chlorophylls.

EXPERIMENTAL

Preparation of zinc(II) chlorophylls

Chlorophylls were extracted from fresh spinach as described [10–12] and purified by reprecipitation from a water–dioxane solution of crude chlorphylls [13]. The chlorophylls thus obtained were pheophytinized by adding 10 cm^3 of dilute hydrochloric acid (0.1 *M*) to 150 cm^3 of an acetone solution of chlorophylls (200 mg) and keeping the mixed solution at 40°C for 30 min. Pheophytins were extracted from this acetone–water solution with hexane and evaporated to dryness on a rotary evaporator. A 20-cm^3 volume of a chloroform solution of 20 mg of pheophytins was added to 40 cm^3 of a methanol solution of 21 mg of zinc(II) acetate dihydrate and the mixture was stirred at 40°C in an argon atmosphere for 1 h. After evaporation of the chloroform–methanol solution, the residue was extracted with hexane and the extract was washed twice with distilled water. The hexane solution was rotary evaporated at 40°C to obtain zinc(II) chlorophylls. Zn(II)-chl-a and -b were separated with a semi-preparative Develosil ODS column (10-μm spherical octadecyl silica, 25 cm × 3 cm I.D.) (Nomura Chemical, Aichi, Japan) equipped with a Kusano–Kagaku loop injector (0.59 cm^3). The flow-rate of the mobile phase of methanol–acetone (75:25, v/v) was 7.4 cm^3 min^{-1}. Each fraction from semi-preparative HPLC was evaporated to dryness on a rotary evaporator at 40°C to obtain Zn(II)-chl-a and -b in pure form.

Apparatus and measurement method

The analytical HPLC system consisted of a JASCO BIP-1 pump, a Unisilpak $5C_{18}$ column (10-μm spherical ODS, 250 mm × 4.6 I.D.) or an Inertsil ODS-2 column (5-μm spherical ODS, 250 mm × 4.6 mm I.D.), a Rheodyne Model 7125 injector with a 100-mm^3 injection loop and a Shimadzu C-R3A Chromatopac integrator. The detector used for this system was a Uvidec-1000 variable-wavelength UV–Vis detector or a Shimadzu SPD-MIA photodiode-array detector. Each HPLC solvent or solvent mixture was filtered through a fresh 0.45-μm hydrophilic Millipore filter and degassed every time before use by ultrasonically vibrating the solvent container. The mobile phase was methanol–acetone (75:25, v/v), the flow-rate was 0.5–1.0 cm^3 min^{-1}, the inlet pressure was 4.9–9.8 MPa and the analytes were usually detected at 425 nm. The ^1H NMR spectra at 270 MHz of samples in C^2HCl_3 solution containing tetramethylsilane were recorded with a JEOL JNM-GX 270 FT-NMR spectrometer. The chemical shifts are reported in ppm relative to $CHCl_3$ ($\delta = 7.23$). The electronic absorption spectra were recorded with a Hitachi U-2000 spectrophotometer using 1-cm quartz cells. Every product container was wrapped with aluminium foil and stored under an argon atmosphere to prevent photo-decomposition from exposure to light.

Analytical procedure

A 100 μg cm^{-3} stock solution of Zn(II)-chl-a and -b was prepared in glass-stoppered volumetric flasks, which were then sealed and stored at 5°C in the dark. Standard solutions for calibration graphs were prepared with a solvent composition as near to the sample as possible, by diluting the appropriate volume of the stock solution with a mixture of methanol and acetone (75:25, v/v). All sample solutions were filtered (0.2 μm) prior to injection into the chromatograph. The mobile phase was an isocratic mixture of HPLC-grade methanol and acetone (75:25, v/v). The analytical columns used were operated at ambient temperature with a flow-rate of 1.0 cm^3 min^{-1}.

RESULTS AND DISCUSSION

Identification of zinc(II) chlorophylls

Zinc(II) chlorophylls consist of a relatively hydrophilic zinc(II)–chlorin complex and a lipophilic phytyl group. Zn(II)-chl-a has a methyl group on the chlorin ring, whereas Zn(II)-chl-b

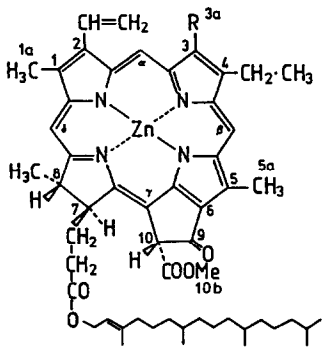

Fig. 1. Structure of zinc(II) chlorophylls. R = CH_3, Zn(II)-chl-a; R = CHO, Zn(II)-chl-b.

has an aldehyde group (Fig. 1). This difference in the substituent attached to the chlorin ring causes a dramatic change in their spectroscopic properties and chromatographic behaviour.

The three-dimensional chromatogram of a mixture of zinc(II) chlorophylls is shown in Fig. 2. The absorption spectra of Zn(II)-chl-a and -b are very similar to those of the corresponding epimers, *i.e.*, Zn(II)-chl-a′ and Zn(II)-chl-b′. Further detailed observation revealed that the absorption spectra of the epimers are identical with those of their parent compounds. The absorption spectra give rise to only small red shifts of the Soret band on going from pheophytins to the corresponding zinc(II) chlorophylls. Typical absorption spectra of Zn(II)-chl-a and -b are presented in Fig. 3. The absorption maxima and molar absorptivities of these compounds have been given in a previous paper [14]. These spectroscopic characteristics were useful in the present HPLC and absorption spectrophotometric determination.

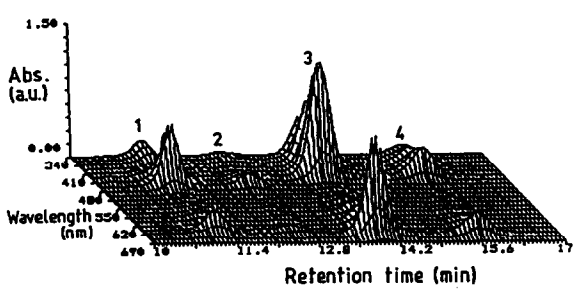

Fig. 2. Three-dimensional chromatogram of zinc(II) chlorophylls. 1 = Zn(II)-chl-b; 2 = Zn(II)-chl-b′; 3 = Zn(II)-chl-a; 4 = Zn(II)-chl-a′.

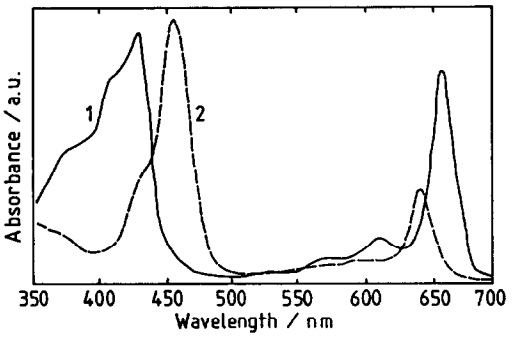

Fig. 3. Absorption spectra of zinc(II) chlorophylls. 1 = Zn(II)-chl-a; 2 = Zn(II)-chl-b.

^{1}H NMR spectroscopy provides a good opportunity to identify each derivative of zinc(II) chlorophylls because their central zinc(II) ion is diamagnetic. The ^{1}H NMR spectra of zinc(II) chlorophylls and their epimers fractionated by semi-preparative HPLC were measured to confirm the identification of each peak component on the chromatogram. The proton resonance signals observed were assigned in analogy with those for pheophytins and chlorophylls [15] and the partial results of the assignments are summarized in Table I. It is noteworthy that the diamagnetic ring-current shifts of the meso-protons are accompanied by the formation of zinc(II) chlorophylls from pheophytins.

Chromatographic separation

Taking into account previous studies of chlorophylls [16,17] and metallochlorophylls [14], a reversed-phase ODS column in combination with a methanol–acetone mobile phase was chosen for zinc(II) chlorophylls. A good separation of Zn(II)-chl-a and -b and of their corresponding epimers was achieved on an ODS column with methanol–acetone (75:25, v/v) as the mobile phase (Fig. 2). The chromatogram of zinc(II) chlorophylls prepared under the present experimental conditions illustrates the appearance of Zn(II)-chl-a′ and −b′, *i.e.*, the C-10 epimers of Zn(II)-chl-a and -b, respectively. As expected, Zn(II)-chl-b and -b′ are eluted earlier than Zn(II)-chl-a and -a′ because there is less interaction of the former with the non-polar ODS stationary phase. This elution order agrees with the general tendency that the aldehyde

TABLE I

^{1}H NMR CHEMICAL SHIFTS OF ZINC(II) CHLOROPHYLL *a* AND *b*

Zn(II)-chl-a			Zn(II)-chl-b		
δ (ppm)	Multiplicity	Proton	δ (ppm)	Multiplicity	Proton
9.52	s	β-H	9.16	s	β-H
9.29	s	α-H	9.41	s	α-H
8.45	s	δ-H	8.24	s	δ-H
6.11	s	10-H	6.04	s	10-H
3.88	s	10b-CH_3	3.99	s	10b-CH_3
3.64	s	5a-CH_3	3.57	s	5a-CH_3
3.33	s	1a-CH_3	3.42	s	1a-CH_3
3.21	s	3a-CH_3	10.20	s	3a-CHO

group attached to the chlorin ring is more polar than the methyl group. Hence the retention behaviour of zinc(II) chlorophylls is interpreted on the basis of both hydrophobic interactions between the phytyl group and the octadecyl chain of the ODS stationary phase and hydrophilic interactions between the chlorin ring including the aldehyde group and the mobile phase.

It is of interest to compare the retention behaviour of the Zn(II)-chls with that of normal chlorophylls [Mg(II)-chls] because the skeletal structures are very similar except for the central metal ion. A typical chromatogram obtained for a mixture of Zn(II)-chls and Mg(II)-chls with photometric detection at 425 nm, using an Inertsil ODS-2 column and a flow-rate of 1.4 cm^3 min^{-1}, is shown in Fig. 4. The peak of Zn(II)-chl-b overlaps partially with that of Mg(II)-chl-a,

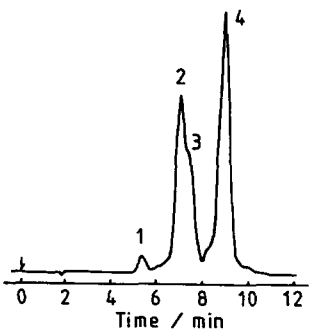

Fig. 4. Typical chromatogram of a mixture of Zn(II)-chls and Mg(II)-chls. 1 = Mg(II)-chl-b; 2 = Mg(II)-chl-a; 3 = Zn(II)-chl-b; 4 = Zn(II)-chl-a.

but the peak of Zn(II)-chl-a is well resolved from those of Mg(II)-chl-a and -b. Therefore, Zn(II)-chl-a in pure form can be used as an internal standard in the HPLC determination of native chlorophylls. Advantages are that Zn(II)-chl-a is relatively stable and can be easily prepared by the proposed method.

Determination of zinc(II) chlorophylls

Four kinds of zinc(II) chlorophylls, *i.e.*, Zn(II)-chl-a, -a′, -b and -b′, were fractionated on a semi-preparative ODS column with methanol–acetone (75:25, v/v) as the eluent. The calibration graphs for zinc(II) chlorophylls fractionated in pure form were constructed by analytical HPLC with a Uvidec-1000 variable-wavelength UV–Vis detector. The amounts of Zn(II)-chl-a, -a′ and -b fractionated were sufficient to construct their calibration graphs, but the amount of Zn(II)-chl-b′ was not. The linear range of the calibration graphs was 0–50 μg cm^{-3} for the three zinc(II) chlorophylls.

The detection limits of the HPLC method with visible detection at 425 nm were calculated from the amount of each zinc(II) chlorophyll which yielded a signal-to-noise ratio of 2. The minimum detectable concentrations were 3.5, 2.5 and 3.0 ng cm^{-3} for Zn(II)-chl-a, -a′ and -b, respectively, with relative standard deviations ($n = 10$) of 1.92%, 2.19% and 1.49%, respectively.

For comparison purposes, calibration graphs for spectrophotometry with a Hitachi U-2000 spectrophotometer were constructed for Zn(II)-

chl-a, -a′ and -b at 427, 427 and 455 nm, respectively. The detection limits calculated from the standard deviation at the lowest concentration level of the standard solution were 15, 14 and 16 ng cm^{-3} for Zn(II)-chl-a, -a′ and -b, respectively. Hence the spectrophotometric method seems to be less sensitive to the determination of Zn(II)-chls than the proposed HPLC method, probably because of the differences in the analytical procedure and/or handling of the standard solutions. However, the detection limits of these two methods cannot be directly compared because the detector used and the definition of detection limit are different in each method.

Evaluation of analytical results

The RP-HPLC method proposed here was evaluated in comparison with the spectrophotometric method because the latter has been used as a conventional method for determination of chlorophylls [18] and metallochlorophylls [1]. The analytical HPLC method was applied to artificial samples prepared by mixing Zn(II)-chl-a and -b obtained by the semi-preparative HPLC method. These artificial samples prepared using pure Zn(II)-chls were also analysed spectrophotometrically for comparison purposes. The analytical values obtained by the spectrophotometric method are plotted against those determined by the HPLC method in Fig. 5, where the concentrations of the artificial samples, *i.e.*, the mixtures of Zn(II)-chl-a and -b, are employed as analytical values instead of the content of each component. The slope of the regression line calculated for Zn(II)-chl-a was greater than unity: $y = 1.07x + 0.045$, with a correlation coefficient $r = 0.997$. The slope calculated for Zn(II)-chl-b was clearly larger than unity: $y = 1.32x + 0.085$ with $r = 0.999$. These results suggest that the spectrophotometric method overestimates the contents of Zn(II)-chl-a and -b at least under the present analytical conditions. This overestimation is ascribed to the partial overlapping of the absorption bands of Zn(II)-chl-a and -b (*cf.*, Fig. 3). In other words, the spectrophotometric determination of Zn(II)-chl-a could be subject to interference to some extent in the presence of Zn(II)-chl-b, although

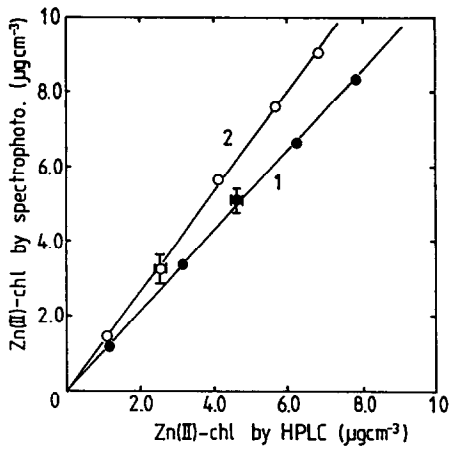

Fig. 5. Comparison of analytical results obtained by the HPLC method with those by the spectrophotometric method. The errors are expressed as bars on the plot of the averages for at least three independent determinations. 1 = Zn(II)-chl-a; 2 = Zn(II)-chl-b.

the exact composition of the mixture of Zn(II)-chl-a and -b can be calculated from the absorbances at the λ_{max} of each Zn(II)-chl using multiple-component software.

A typical plot of the analytical results obtained by the spectrophotometric method against those by the HPLC method is shown only for Zn(II)-chl-a and -b in Fig. 5, but a similar plot with a slope larger than unity was also obtained for Zn(II)-chl-a′. The comparison of the HPLC and spectrophotometric methods demonstrated that the RP-HPLC method developed here is an accurate and conventional method for the determination of zinc(II) chlorophylls.

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