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SHORT COMMUNICATION

ISOLATION OF CHLOROPHYLL *A* FROM SPINACH LEAVES

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ABSTRACT. An efficient method for separating chlorophyll *a* from spinach leaves by column chromatography and solvent extraction techniques has been developed. The purity and identity of the chlorophyll *a* have been confirmed by UV-Vis, IR and mass spectrometry. Yields from 100 g of freeze-dried spinach were 23 – 24 mg of chlorophyll *a*.

KEY WORDS: Chlorophyll *a*, Spinach leaves, Separation, Column chromatography, Solvent extraction, Pheophytin *a*

INTRODUCTION

The chlorophylls are one of a number of pigments usually contained in green organelles of higher plants [1] where they play a central role in the primary stage of photosynthesis. The entire structure of chlorophyll *a* and *b* was put forward by Fischer and Wenderoth [2] while the first chemical synthesis was reported by Woodward and co-workers [3]. The first successful separation of chlorophyll was reported by Tswett [4] giving birth to modern chromatographic techniques. A variety of chromatographic procedures, including paper [5], thin-layer [6], conventional column [7], and high-performance liquid chromatography [8] have been developed and used for analytical and preparative separation of chlorophylls and their derivatives. Low yields have been reported due to susceptibility of chlorophyll *a* to a number of chemical transformations which might occur during the extraction. Recently [9], the counter-current chromatography (CCC) technique has been applied to the separation of chlorophyll *a* from spinach leaves.

In view of the high cost of high performance liquid chromatography (HPLC), and counter current chromatography (CCC) techniques, we describe in the present work an improved column chromatographic method coupled with solvent extraction for the extraction and purification of chlorophyll *a*. This is the first example where rapidity and simplicity of solvent extraction and column chromatography have been successfully combined for medium scale extraction of chlorophyll derivatives. The purity of chlorophyll *a* was close to that of commercial chlorophyll *a* (Sigma-Aldrich).

EXPERIMENTAL

Equipment

IR spectra were recorded on a Nicolet 410 FTIR spectrophotometer (4000-400 cm^{-1}) using Nujol mulls or liquid films between potassium bromide cells. The UV-Vis spectra were recorded on a Pye-Unicam 8700 spectrophotometer (200-900 nm) in 80% aqueous acetone solution for chlorophyll *a*, and in petroleum ether (60-80 °C) for pheophytin *a*.

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A Finnigan LCQ Mass Spectrometer with the ESI source was employed for mass spectrum determination. The determination conditions were as follows: sheath gas flow rate, 0.65 L min^{-1}; auxiliary gas flow rate, 0.06 L min^{-1}; ion spray voltage, 3.5 kV; capillary temperature, 200 ºC; capillary voltage, 27 V; tube lens offset, 55 V. Samples were injected by a Finnigan syringe pump connected to the ESI source by a 100 μL – m.i.d. fused silica capillary, flow rate was 3 μL min^{-1}.

Column chromatography was carried out with silica gel (Merck Kieselgel 60; 230-400 mesh) or alumina powder (Wako alumina, activated 300 mesh).

Reagents

All the chemicals used were obtained from commercial suppliers and used without further purification.

Extraction procedure

All the work was performed in dim light. Fresh spinach leaves were first separated from the mid-ribs and washed with cold water. They were then frozen for storage. The freeze-dried spinach leaves (100 g) were crushed and extracted with four 50 mL portions of 80% aqueous acetone. The combined extracts (200 mL) were concentrated to about 50 mL and washed with petroleum ether (60-80 °C), (3 x 20 mL).

The aqueous acetone layer, which contained mostly carotenes and other acetone-soluble plant constituents, was discarded. The petroleum ether layer was washed with aqueous methanol (60:40 v/v). The petroleum ether layer was analyzed by UV-Vis and IR spectroscopy. The petroleum ether layer containing the chlorophyll *a* was chromatographed on a silica gel column. The column was eluted with petroleum ether and with 0.5% n-propanol in petroleum ether to give the chlorophyll *a*. Acidification with dilute acetic acid gave pheophytin *a*.

RESULTS AND DISCUSSION

Extraction and purification

The yields from 100 g of freeze-dried spinach were 23-24 mg of chlorophyll *a* (obtained in triplicate).

Identification and purity

The identification and purity of the chlorophyll *a* extract, was determined by UV-Vis, IR and mass spectrometry. The spectrophotometric monitoring was carried out wherever possible at every stage.

UV-Visible spectra

The spectrum of the whole chlorophyll extract showed several absorption bands between 200 - 400 nm indicating a mixture of several pigments. That of the yellow acetone layer indicated that it contained mainly β-carotene [10], while the petroleum ether layer contained xanthophylls [11] and chlorophyll. The UV-Vis absorption characteristics where found to be close to literature values [12,13] as shown in Table 1.

Table 1. Electronic spectral data (λ_{max}) of chlorophyll *a*.

Observed bands λ_{max} (nm)	328	430	451	535	579	615	662
Literature values (nm) [12, 13]	327	431	450	534	580	617	662

The spectrum has the soret bands at 430 and 451 nm (B_y and B_x) and two Q bands at 615 and 662 nm Q_x and Q_y.

The spectra can be understood on the basis of Gouterman's four-orbital model for porphyrins and their derivatives [14]. For D_{4h} metalloporphyrins, the two degenerate lowest unoccupied molecular orbitals (LUMO, e_g^*) and two nearly degenerate highest occupied molecular orbitals (HOMO, a_{1u} and a_{2u}) give rise to a strong soret band in the near-UV region and weak Q bands in the visible region. However in the D_{2h} symmetry the degenerate e_g^* (D_{4h}) LUMOs split into x and y components (b_{3g} and b_{2g}). The b_{3g} and a_u orbitals are more destabilized relative to b_{2g} and b_{1u} because of their orbital nodal pattern.

IR spectra

The interpretation of chlorophyll infrared spectra is based mainly on the work of Goodwin [15] and Katz [16]. The region of greatest significance is the carbonyl region where most of the ligand absorption bands are obtained. These ligand absorption positions are more or less independent of the metal ion in the centre of the tetrapyrrole ring.

In petroleum ether, the νC-H vibrations are observed at 2970, 2927 and 2876 cm^{-1}. This is in agreement with band assignment in literature of these C-H stretching modes at 2960, 2925 and 2875 cm^{-1} [16, 17]. The C-9 ketone ν(C=O) band which is reported to appear as two bands at ~1700 cm^{-1} (normal ketone absorption) and at ~1650 cm^{-1} (ketone oxygen coordination to magnesium) [16] is observed at 1702 cm^{-1} and 1642 cm^{-1}.

Bands assignable to νC-O, νC-C and νC-N vibrations are also observed. νC-N vibrations of the tetrapyrrole ring assigned [17, 18] at 1350 cm^{-1} are observed at 1370 cm^{-1} while νC-C skeletal vibration is observed at 1089 cm^{-1}. νC-O vibration gives a strong sharp band at 1242 cm^{-1}. The alkyl C-H anti-symmetric and symmetric deformation is observed at 1429 cm^{-1}.

Mass spectrometry

Electron spray ion mass spectral analysis of chlorophyll *a* produced several fragmentation peaks. The most important peaks in this analysis are the peaks at m/z 591.3_{obs} (590.9_{calc}) and 613.2_{obs} (613.9_{calc}). The peak at m/z 613 is the base peak. This peak is due to the fragmentation of chlorophyll with the loss of the phytyl chain. This peak's relative abundance of 100% shows the extract to be chlorophyll *a*.

These observations are strengthened when compared to the mass spectrum of pheophytin *a*. The peak at m/z ratio of 591.3_{obs} results from the fragmentation of pheophytin-*a* formed in solution as a degradation product. The relative abundance of this peak at m/z 591.3_{obs} of about 10% could be reasonably interpreted as showing that the percentage of chlorophyll *a* that has converted into pheophytin *a* is low.

In the mass spectra of pheophytin *a*, the peak due to the loss of the phytyl chain during fragmentation is observed at m/z 593.2_{obs}. In the pheophytin *a*, this peak has a relative abundance of 100% also indicating the loss of the phytyl chain during fragmentation.

CONCLUSION

This work presents a successful application of column chromatography coupled with solvent extraction to the isolation and purification of chlorophyll *a*. The advantages of the procedure described in this study include: use of common solvents, simplicity of apparatus, high yield and purity of the product and a relatively time-saving process.

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