Journal of Chromatography, 154 (1978) 302–305

CHROM. 10,930

Note

Micro-method for the qualitative and quantitative analysis of photosynthetic pigments using high-performance liquid chromatography

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(Received February 3rd, 1978)

During the course of investigations of photosynthetic pigments *in vitro*[1–7] as well as *in vivo*, a simple micro-method was needed for qualitative and quantitative analysis of chlorophyll components and minor components in chlorophyll preparations. Wilson and Nutting[8] developed a spectrophotometric method for the determination of chlorophylls as pheophytins at concentrations of 5 μg/ml. However, an amount of contaminant too small to cause a change in the shape of the visible absorption spectrum might go undetected if masked by a component present in greater concentration. Then, chromatographic separation of test materials made visual, densitometric and spectrophotometric detection of small amount of contaminants more sensitive. A number of investigations of analytical and separative methods for plant pigments, including column[9], paper[9–11], and thin-layer chromatography (TLC)[7,9,12,13], have been reported. It is only possible to analyse pigment amounts up to the order of 10^{-6} g using paper chromatography[1], and to detect qualitatively amounts of contaminant up to the order of 10^{-9} g using TLC[3]. However, it is very difficult to detect small amounts of contaminants with similar R_F values to that of the main pigment using these techniques. Therefore, we have used micro-scale high-performance liquid chromatography (HPLC) to improve separability and sensitivity. In this paper, we report the preliminary results obtained with this method, which enables the micro-scale quantitative and qualitative analysis of photosynthetic pigments down to 10^{-9} g.

MATERIALS AND METHODS

All the experiments were performed at room temperature in total darkness or under dim green light.

Spinach leaves (100 g fresh weight) were homogenized for 3 min in a Warling blender with 500 ml of acetone. The green juice obtained was filtered through a pad of cotton to remove coarse debris, and the filtrate was centrifuged at 10,000 *g* for 5 min to remove insoluble materials. The deep green supernatant solution (acetone extract) was used to prepare chlorophylls *a* and *b*, according to the method of Iriyama *et al.*[5,6]. Chlorophylls *a'* and *b'* were obtained by re-fractionation of chlorophylls *a* and *b* on a sugar column, according to the method of Sievers and Hynninen[14]. Pheophytins *a* and *b* were prepared according to the method of Hynninen[15]. β-

Carotene (E. Merck, Darmstadt, G.F.R.) was used without further purification. All pigment mixtures were dissolved in 10% (v/v) diethyl ether in hexane after evaporating and drying of each pigment solution, and used as sample solutions. Pigments were characterized by comparison of their visible spectra with literature values. In addition, the pigments were thin-layer chromatographed according to the method of Shiraki *et al.*[7].

All reagents and solvents used were analytical-reagent grade and were used without further purification unless otherwise stated. Solvents used for the preparation of chlorophylls were dried and distilled with usual methods.

HPLC was carried out with a Familic-100 instrument (Japan Spectroscopic, Tokyo, Japan) with 65 × 0.5 mm I.D. PTFE tube packed with silica gel powder SS-05 (particle size 0.5 μm; Japan Spectroscopic). Elution patterns were monitored at 380 nm with Uvidec-100 (Japan Spectroscopic). The light path-length was 0.5 mm. Runs were made at room temperature with a solvent flow-rate of 16 μl per min. A 1-μl volume of test solution was charged.

RESULTS AND DISCUSSION

Fig. 1a shows a typical chromatogram for an acetone extract from spinach leaves. Good resolution of chlorophylls *a* and *b*, as well as the major yellow pigments, was obtained with hexane containing isopropyl alcohol by a solvent program of 1, 2, 5, and 10% (v/v) isopropyl alcohol. The column was equilibrated with hexane before injection of sample solution. An unidentified yellow pigment peak appeared between the lutein and violaxanthin peaks.

Fig. 1b shows a typical chromatogram for the pigment mixture (pheophytin *a*, chlorophyll *a'* and chlorophyll *a*) eluted with hexane containing 1% (v/v) isopropyl alcohol. The column was equilibrated with hexane before injection of sample solution. Fig. 1c shows a typical chromatogram for the pigment mixture (pheophytin *b*, chlorophyll *b'* and chlorophyll *b*) eluted with hexane containing 1 and 2% (v/v) isopropyl alcohol. The column was also equilibrated with hexane before injection of sample solution. These mixtures were also well resolved. A programmed elution was necessary because straight elution using a greater concentration of isopropyl alcohol than 2% (v/v) failed to resolve each peak completely, whereas a lower concentration of isopropyl alcohol greatly extended the elution time. It is generally recognized that chlorophyll *a* is converted into chlorophyll *a'* and pheophytin *a*, and chlorophyll *b* into chlorophyll *b'* and pheophytin *b*. Moreover, it has been reported that the absorption spectra of chlorophyll *a'* and chlorophyll *b'* are too similar to those of chlorophyll *a* and chlorophyll *b*, respectively, for spectroscopic detection to be reliable. Therefore, purity of chlorophyll samples should be checked by both spectroscopic and chromatographic methods.

Fig. 2 shows the calibration curves of β-carotene and chlorophyll *a*. Volumes of 1 μl of pigment solutions of various concentrations were eluted with hexane containing 1% (v/v) isopropyl alcohol. The column was equilibrated with hexane containing 1% (v/v) isopropyl alcohol before injection of sample solution. The peaks on the elution curves were integrated by multiplying the peak height by the width at half the height. This method of integration is rapid and satisfactorily accurate for quantitative analysis of pigments. A linear relationship between the integrated

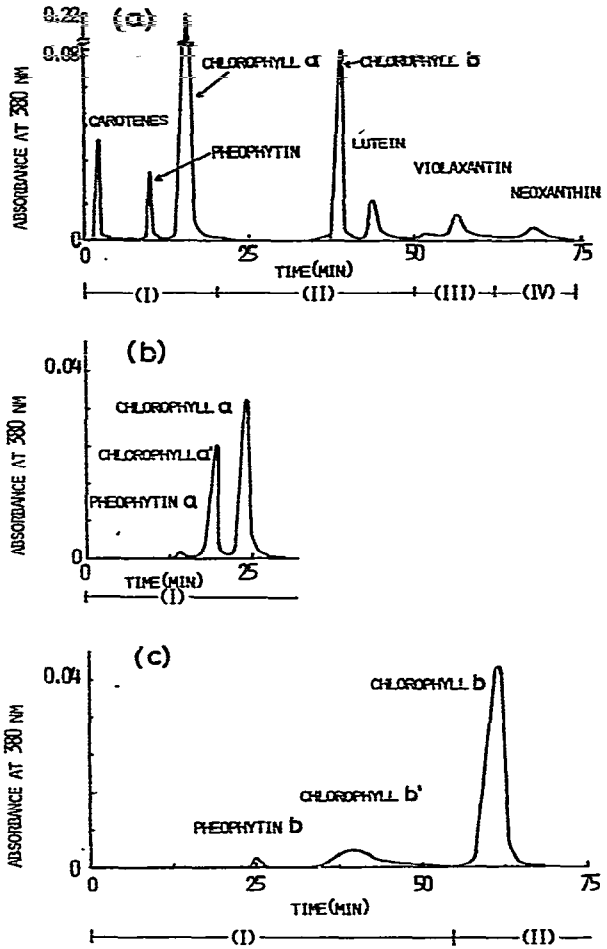

Fig. 1. Chromatograms (a) for the acetone extract, (b) for the mixed pigments (pheophytin *a*, chlorophyll *a'* and chlorophyll *a*), (c) for the mixed pigments (pheophytin *b*, chlorophyll *b'* and chlorophyll *b*). (I) 1%, (II) 2%, (III) 5% and (IV) 10% isopropyl alcohol in hexane were eluted at a flow-rate of 16 μl/min.

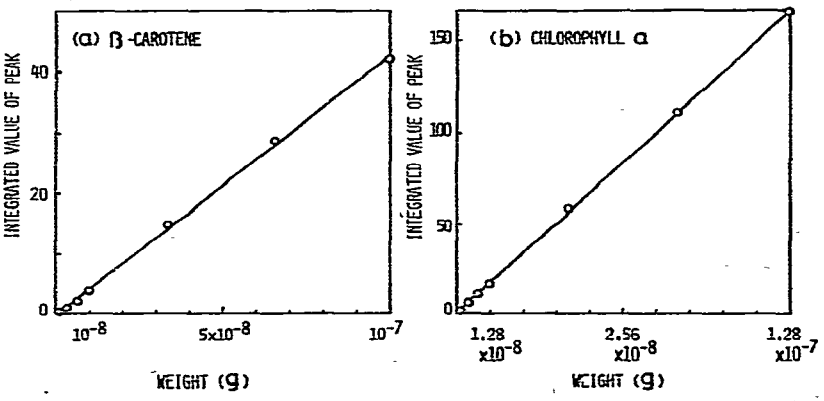

Fig. 2. The relationship between the integrated value of a peak and the weight of pigment (β-carotene or chlorophyll *a*) contained in 1 μl of injected solution. The integrated value of a peak was calculated by multiplying the peak height by the width at half the height.

values and the weight of pigments injected was obtained. The results of this calibration study show that quantitative analysis of these pigments down to 10^{-9} g and qualitative detection down to 10^{-10} g can be achieved. These sensitivities could be higher if the various substances were detected at the wavelengths of the absorption maxima. Each pigment spotted on the silica gel sheets was detected visually by daylight down to 10^{-8} g.

In *in vitro* studies of chlorophylls, such as studies of their physicochemical properties, prepared or purchased chlorophylls should be checked for possible contamination by yellow pigments and the chemical degradation products of chlorophylls. It is generally recognized that chlorophylls are decomposed by acids, alkalis, oxidizing agents, oxygen, heat and intense light. Therefore the purity of chlorophylls should also be checked after experiments, as the existence of the contaminants may have induced erroneous experimental results. The method described above is suitable for such checking.

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