HPLC Determination of Major Pigments in the Bean *Phaseolus vulgaris*

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Major pigments in *Phaseolus vulgaris* L. beans were determined by high-performance liquid chromatography (HPLC) on a C_{18} reversed-phase column. Three classes of pigment were identified; in order of chromatographic elution, these were xanthophylls, chlorophylls, and carotenes. The most abundant xanthophyll was lutein; other xanthophylls were detected in very small quantities. Chlorophyll *a* and chlorophyll *b* were both detected, but chlorophyll derivatives were either not present or, in a few samples, present in trace quantities. The only carotene detected was β-carotene. Evaluation of the method used indicated high sensitivity and precision.

INTRODUCTION

The carotenoids, together with the chlorophylls, are present in most photosynthetic tissues. In green vegetables, the predominant carotenoids are lutein and β-carotene (Heinonen et al., 1989), and the predominant chlorophylls are chlorophylls *a* and *b*, which generally occur at a ratio of about 3:1 in higher plants (Belitz and Grosch, 1988). Interest in the carotenoids has increased in the past decade, since they have been identified not only as vitamin A precursors but also as possible inhibitors of cancer and ulcers (Peto et al., 1981; Colditz et al., 1985; Mozsik et al., 1984). Recently, various methods have been developed for the determination of carotenoids and chlorophylls in fruit and vegetables by high-performance liquid chromatography (HPLC) (Eskins et al., 1977; Khachik et al., 1986, 1991; Cano, 1991; Taylor and MacDowell 1991).

Here, we determined the major pigments in *Phaseolus vulgaris* L. beans sampled throughout July and August. We also report development of the method used and evaluate its suitability for this purpose.

EXPERIMENTAL PROCEDURES

Reagents. Chlorophyll *a*, chlorophyll *b*, *all-trans*-lutein, and *all-trans*-β-carotene standards were from Sigma-Aldrich, Spain. HPLC grade solvents (methanol, acetonitrile, dichloromethane, and hexane) were from Scharlau. Analytical grade solvents were from Scharlau (tetrahydrofuran stabilized with butylated hydroxytoluene) and Normasolv (acetone and petroleum ether).

Equipment. We used a Spectra-Physics liquid chromatography apparatus equipped with an SP8800 ternary pump, a 20-μL Rheodyne injection jet, a UV–vis forward optical scanning detector, and Spectra Focus software (all from Spectra-Physics).

Column. Separation was carried out on a 250 × 4.6 mm Spherisorb ODS2 column (C_{18}, 5-μm particle size), with a 20 × 4.6 mm guard column with the same packing (both from Sugelabor).

Chromatographic Procedure. Isocratic elution for 9 min with a mixture of methanol (15%), acetonitrile (75%), and 1:1 dichloromethane/hexane (10%) was followed until 16 min postloading (pl) with a gradient leading to a final composition with 15% methanol, 40% acetonitrile, and 45% dichloromethane/hexane 1:1; elution was continued isocratically with this mixture until 24 min pl. Flow rate was maintained at a constant 0.8 mL/min. Following each run the column was re-equilibrated with the initial eluent for 20 min at 2 mL/min.

Separation of the compounds under study was achieved in 22 min. Absorption in the visible region was measured at three wavelengths selected on the basis of spectra of the HPLC peaks.

Sampling and Sample Preparation. We analyzed a total of 16 samples of green beans (*P. vulgaris* L. var. Helda) planted in March and harvested in July and August. Samples of mature beans were collected twice a week throughout the harvesting period. Maturity was indicated by a high ratio of pod size to bean size. Fresh tissues (pod and bean) were homogenized; 10 g of homogenate was then ground with 1 g of calcium carbonate, 20 g of anhydrous sodium sulfate, and a small quantity of quartz sand. Small quantities of a 50:50 mixture of acetone and petroleum ether were added to this preparation and collected by filtration (Whatman No. 541) under vacuum, until the residue was colorless, and the pooled filtrates were then brought up to 100 mL. The solvent was evaporated from 10 mL of this extract in a water bath at 37 °C in a nitrogen stream, and the dry residue was redissolved in 2 mL of hexane; this solution was then filtered (Millipore 0.22 μm) to remove insoluble particles prior to injection. Each of the 16 homogenized samples was analyzed twice.

Pigment Identification and Quantification. Pigment identification was based on comparison of the retention times and visible absorbance spectra of samples with those of standards. For quantification, calibration lines for peak areas were constructed using a range of standard concentrations such that the concentrations of pigments in the sample lay in the middle of the range.

RESULTS AND DISCUSSION

Extraction Procedure. Two solvents were tested: tetrahydrofuran stabilized with butylhydroxytoluene and a 50:50 mixture of acetone and petroleum ether (Khachik et al., 1986). Since the former solvent extracted much less chlorophyll *a* than the second (though for the other pigments, no differences were observed) the acetone/petroleum ether mixture was selected. This solvent has the additional advantages of lower cost and lower toxicity.

Given the instability of the pigments under study, the extraction process should be as short as possible. The extraction procedures used by other authors include transfer of the pigments to petroleum ether (Khachik et al., 1986) or diethyl ether (Cano, 1991); in the present study, this step (whether using petroleum ether or diethyl ether) had no effect on results (data not shown) and was therefore omitted.

Pigment Distribution. The chromatograms corresponding to a mixture of standards and to a bean sample are shown in Figures 1 and 2, respectively. Lutein was the predominant xanthophyll in all samples. Small quantities of other xanthophylls were also present (Figure 2, peaks 1–4); these were identified as such by comparison with previously published spectra (Khachik et al., 1986, 1988)

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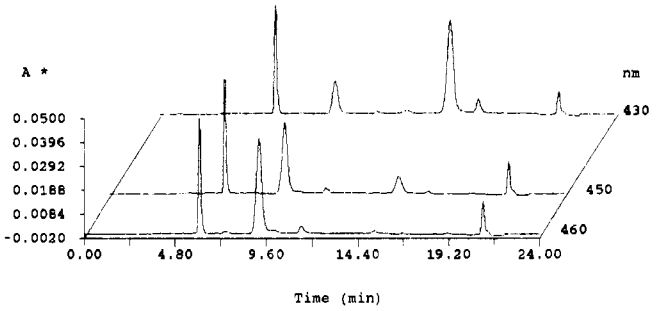

Figure 1. HPLC chromatogram of a standard solution of the four compounds under study: (1) *all-trans*-lutein; (2) chlorophyll *b*; (3) chlorophyll *a*; (4) *all-trans*-β-carotene.

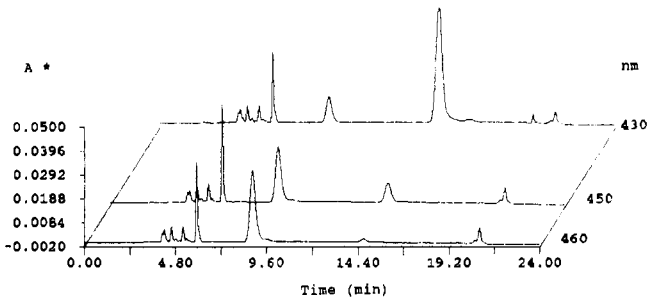

Figure 2. HPLC chromatogram of green bean extract. Peak identifications are given in Table I.

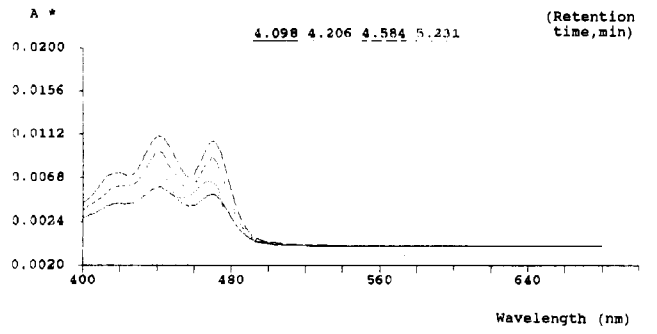

Figure 3. Visible absorption spectra of xanthophylls (peaks 1–4) in the HPLC solvent.

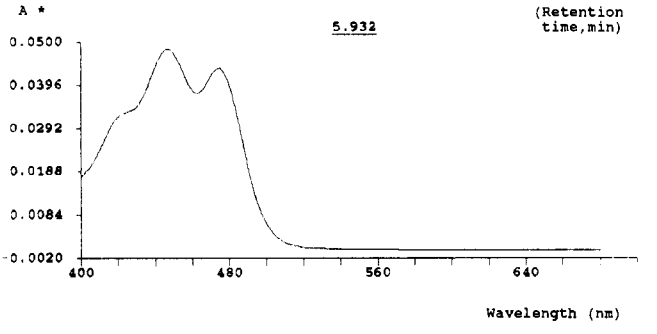

Figure 4. Visible absorption spectra of the major xantophyll (*all-trans*-lutein) in the HPLC solvent.

but were not quantified. β-Carotene was the only carotene identified. Both chlorophyll *a* and chlorophyll *b* were found, but chlorophyll derivatives were present in only a few samples. Visible absorption spectra of sample peaks are shown in Figure 3 (minor xanthophylls), Figure 4 (*all-trans*-lutein), Figure 5 (chlorophylls *a* and *b*), and Figure 6 (*all-trans*-β-carotene); in all cases they coincide closely with the spectra of the corresponding standards (results not shown). Retention times and maximum absorbance wavelengths for each component are shown in Table I. The wavelengths selected for pigment quantification were

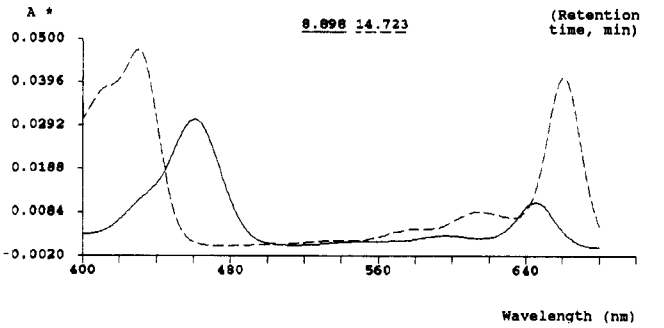

Figure 5. Visible absorption spectra of chlorophylls (*b* and *a*) in the HPLC solvent.

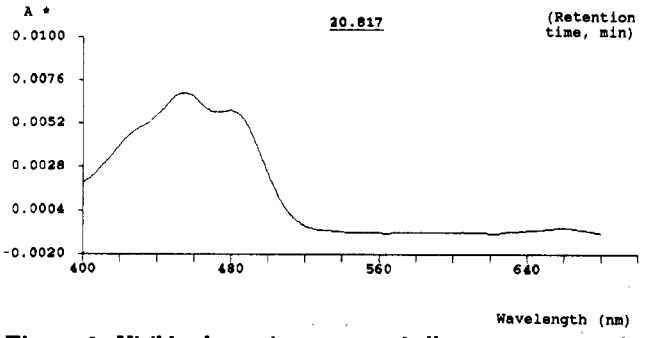

Figure 6. Visible absorption spectra of *all-trans*-β-carotene in the HPLC solvent.

Table I. HPLC Peak Identification of Major Pigments in the Green Bean Extracts

pigment class	peak	compound	retention time, min	λ_{max}, nm (HPLC solvent)
xanthophylls	1	(minor xanthophyll)	4.09	418, 441, 470
	2	(minor xanthophyll)	4.20	415, 438, 466
	3	(minor xanthophyll)	4.58	418, 451, 470
	4	(minor xanthophyll)	5.23	417, 441, 470
	5	*all-trans*-lutein	5.93	423, 447, 475
chlorophylls	6	chlorophyll *b*	8.89	461, 645
	7	chlorophyll *a*	14.72	430, 660
carotenes	8	*all-trans*-β-carotene	20.81	428, 455, 480

as follows: 450 nm for lutein and β-carotene, 430 nm for chlorophyll *a*, and 460 nm for chlorophyll *b*. At these wavelengths, detection limits determined in accordance with ACS (1980) guidelines were 0.17 μg/mL for lutein, 0.18 μg/mL for chlorophyll *b*, 0.75 μg/mL for chlorophyll *a*, and 0.12 μg/mL for β-carotene.

The pigment contents of each of the bean samples are listed in Table II, as the means of the two determinations performed for each sample. In no case did these two determinations differ by more than 2%. In 10 samples, the method described was compared with that of the Association of Official Analytical Chemists (1990) for the determination of chlorophylls *a* and *b*; in both cases, the results were very closely correlated (results not shown).

Measurement and Method Precision. To determine measurement precision, a series of six aliquots of the same ultrafiltered solution of extraction were analyzed. The interaliquot coefficients of variance (CVs) obtained were 1.80% for lutein, 2.30% for chlorophyll *b*, 2.55% for chlorophyll *a*, and 1.70% for β-carotene. To determine method precision, a series of 10 aliquots of the same homogenized saple were each subjected to the complete procedure. The CVs were 2.45% for lutein, 3.30% for chlorophyll *b*, 3.27% for chlorophyll *a*, and 2.32% for β-carotene.

Recovery. Known concentrations of standard solutions were added to samples. Recovery values of 99.2%, 98.7%,

Table II. Quantitative Distribution of Xanthophylls, Chlorophylls, and Carotenes in Green Beans (Means of Two Replicate Determinations)

	pigment content, mg/100 g of fresh weight			
sampling date	lutein	chlorophyll *b*	chlorophyll *a*	β-carotene
July 2 1992	0.48	1.01	1.56	0.13
July 7 1992	0.42	1.25	1.82	0.10
July 9 1992	0.46	1.24	2.06	0.11
July 14 1992	0.54	1.50	1.90	0.21
July 16 1992	0.48	1.31	1.70	0.14
July 21 1992	0.45	1.40	1.70	0.13
July 29 1992	0.64	1.48	4.04	0.22
July 30 1992	0.40	2.40	1.19	0.10
Aug 4 1992	0.52	1.29	2.38	0.23
Aug 6 1992	0.65	1.74	3.66	0.26
Aug 11 1992	0.61	2.00	1.13	0.18
Aug 13 1992	0.47	1.03	2.60	0.17
Aug 18 1992	0.40	0.85	1.06	0.16
Aug 21 1992	0.59	1.15	2.46	0.22
Aug 25 1992	0.64	1.48	3.68	0.28
Aug 27 1992	0.61	1.43	2.66	0.26

98.3%, and 99.8% were obtained for lutein, chlorophyll *b*, chlorophyll *a*, and β-carotene, respectively.

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