Quantitative Determination of Photosynthetic Pigments in Green Beans Using Thin-Layer Chromatography and a Flatbed Scanner as Densitometer

Juan Valverde* and Hervé This**

Equipe INRA de Gastronomie Moléculaire, UMR 214 (INRA/AgroParisTech) Laboratoire de Chimie Analytique 16, rue Claude Bernard, 75005, Paris, France; **juan.valverde@agroparistech.fr*; ***herve.this@paris.inra.fr*

Marc Vignolle

Marie Groupe UNIQ, 13-15 Pont des Halles, 94526, Rungis, France

The chemistry of pigments in green plants has been studied since the first half of the 19th century, in particular with investigations by Berzelius *(1)*. These compounds are biologically important, in particular for their role in photosynthesis *(2)*. In food, they have organoleptic and nutritious properties *(3)*. The main pigments in green beans are chlorophylls (Chl) a and b and carotenoids (Car) *(4)*. When beans are blanched (prior to freezing) or stored in poor conditions, pigments include certain chlorophyll derivatives: pheophytins (Pheo) and allomers from the a and b forms. The group of carotenoids can be divided into (i) hydrocarbon carotenoids, β-carotene and α-carotene (β-Car and α-Car) *(5)*, and (ii) oxidized carotenoids or xanthophylls, lutein (Lut), violaxanthin (Vio), and neoxanthin (Neo) *(5)*.

In this article, we propose to study pigments from green beans but our method can be applied to other plant tissues. Chromatographic separation of plant pigments was proposed by Tswett *(6)* and variations of this initial method have been studied *(5, 6)*. Thin-layer chromatography (TLC) is fast, easy and inexpensive and thus useful for chemical studies *(5, 7–9)*. We propose a direct quantification of all the pigments through TLC separation and quantification of the TLC plates using a flatbed scanner and processing the digitized chromatogram.

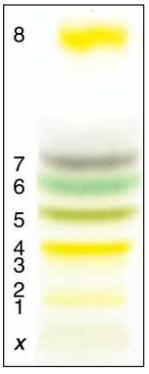

Figure 1. Scanned TLC plate where the photosynthetic pigments were developed in cyclohexane–acetone–diethylamine (10:4:1; v/v/v): (1) neoxantin, (2) violaxanthin, (3) 5,6-epoxy lutein, (4) lutein, (5) chlorophyll b, (6) chlorophyll a, (7) pheophytin a, (8) α- and β-carotenes, and (*x*) origin.

Experimental Procedure

Extraction of Pigments from Green Beans

Fresh green beans were purchased from a local market and frozen green beans were obtained from a local store. The method used for the extraction was modified from a previously published extraction method *(7)*. Under subdued light (to prevent pigment degradation), green beans were combined with anhydrous sodium sulfate and sea sand and ground in a mortar with pestle until a light green powder was obtained. The powder was transferred to a test tube containing acetone. The test tube was agitated and the green acetone solution was removed and centrifuged. The extract was concentrated with a liquid–liquid partition with cyclohexane *(10)*. A small quantity of the supernatant was taken in a capillary tube, weighed before and after filling, to assess the quantity being analyzed. The plant extract was immediately spotted on the TLC sheet.

Separation of Pigments Using TLC

The developing solvent system was cyclohexane–acetone–diethylamine (10:4:1, v/v/v) as in ref *8,* but cyclohexane, a chemically pure substance, was substituted for the petroleum ether mixture.

Preparation of Calibration Standards

Standards of Chl a, Chl b, β-Car, and Lut were prepared from commercially available products. If these products are not available (e.g., due to their price), preparative layer chromatography can be used. Solutions of these pigments with concentrations from 50 mg/L to 1000 mg/L in cyclohexane were used as test samples to prepare calibration plots. The Pheo a and b samples were prepared from Chl a and b, respectively, by addition of acid (HCl 25%) *(11)*. As Vio and Neo have UV–vis spectra similar to Lut, their quantification was carried out using the Lut calibration curve. All pigments were spotted at different concentrations on the same plate. The TLC plates were always scanned under the same conditions. The digitized images were treated with image treatment software.

Scanning

The digitization of the TLC plates was done on a Canon CanoScan 5200F flatbed scanner (Figure 1). The image was scanned and registered in JPEG compressed files.

Image Processing

Either ImageJ *(12)* or the image analysis package from IGOR Pro *(13)* software was used for the processing of the scanned TLC plates. All the results presented here were calculated using IGOR Pro, but image processing can be equally done with ImageJ free software.

To achieve pigment quantification, scanned plates are first transformed into grayscale pictures and then into intensity profiles. The intensity profile of one lane of the plate is obtained from the grayscale picture by selecting a lane with the same width as the largest spot of the lane (in this way, the area and intensity are both considered together, which means integration and better quantification of pigments). Gaussian fitting of the peaks on intensity profiles is performed to remove baseline variations and to separate peaks when they overlap.

Two Dimensional-TLC Analysis (2D-TLC)

Two dimensional TLC was performed on bigger sheets (10 × 10 cm) to check that the pigments are not degraded during the experiment (through chemical interaction with silica).

Hazards

The solvents (acetone, cyclohexane, and diethylamine) used in this experience are volatile and highly flammable; contact with skin or eyes should be avoided. As inhalation may produce drowsiness and dizziness, the experiment should be carried out under a fume hood. In addition diethylamine is strongly alkaline and can cause severe burns.

Results and Discussion

TLC Separation of Pigments

As it has been reported that pigments are susceptible to degradation with siliceous adsorbents *(5)*, two tests were carried out with the material used. First different samples of β-Car and Lut (ca. 50 mg/L to 1000 mg/L each) were applied on the plates in both water-free solvent (cyclohexane) and water-miscible solvent (acetone). The ratio to front (R_f) was calculated for each spot and compared with those previously published in literature *(8)*. Then an epoxy test *(5, 11)* was carried out to identify the pigments. As this test was negative in both cases, no epoxy rearrangement was observed confirming that no oxidation of the samples into epoxy groups was produced during the TLC separation of pigments. This validated the materials and method used. It also confirmed that 2D-TLC could be done without risk of pigment degradation.

Image Processing

A transformation (ImageLineProfile in IGOR software) produces a grayscale profile from the grayscale picture. This transformation can be done along vertical or horizontal lanes. The profiles calculated through this transformation show the intensity of reflected light along the considered lanes (Figure 2).

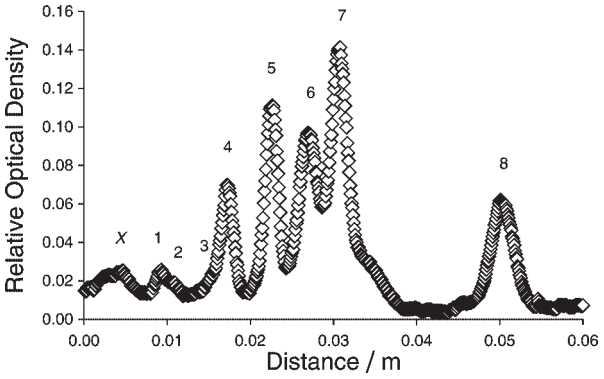

Figure 2. The plot profile of the scanned TLC plate after development with cyclohexane–acetone–diethylamine (10:4:1; v/v/v): (1) neoxantin, (2) violaxanthin, (3) 5,6-epoxy lutein, (4) lutein, (5) chlorophyll b, (6) chlorophyll a, (7) pheophytin a, and (8) α- and β-carotenes. The large quantity of pheophytin a in this sample is due to poor (realistic) storage conditions.

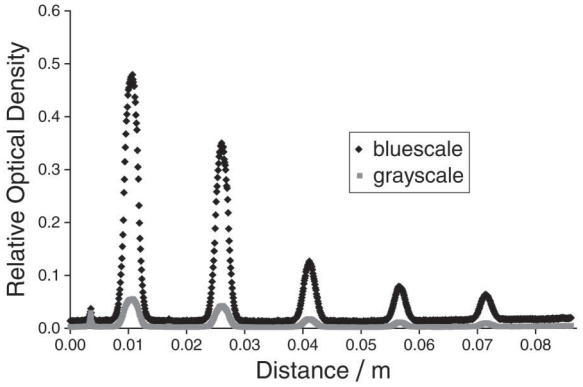

Figure 3. Plotted profiles of β-Car samples in grayscale and in bluescale. Because of the specific absorption of β-Car, more intense signal is obtained using a bluescale.

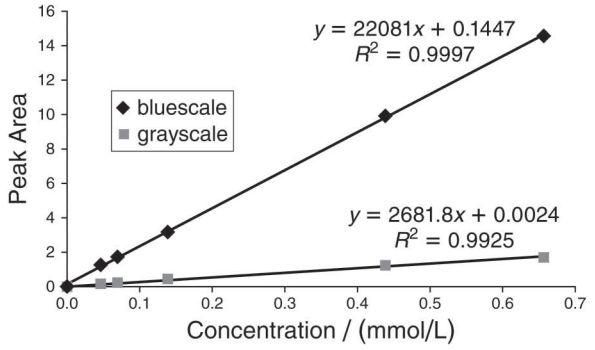

Figure 4. Comparison of sensitivity for calibration plots of β-Car using grayscale and bluescale. As it can observed the sensitivity and precision for bluescale is bigger than for grayscale.

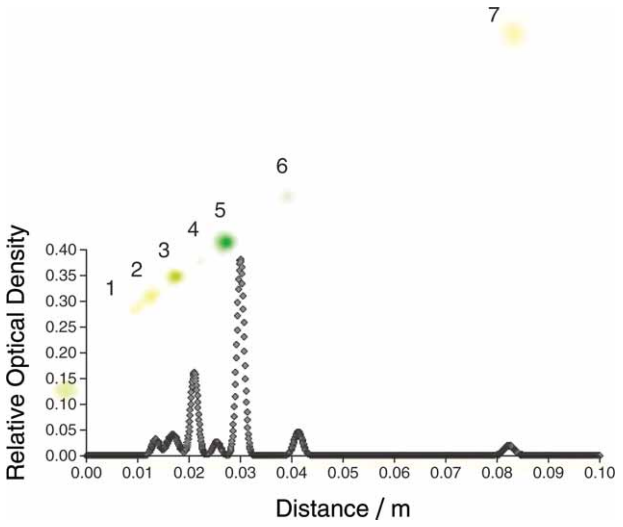

Figure 5. Scanned 2D-TLC plate. The developing solvent system in both dimensions was the same (cyclohexane–acetone–diethylamine; 10:4:1; v/v/v). The alignment along the main diagonal shows that there is little degradation of pigments between the first and the second developments (about 10 min). Superposed densitogram was obtained with a Canon flatbed scanner and transformed with IGOR Pro. The processed lane is the main diagonal. Spot identification is (1) 5,6-epoxy lutein, (2) lutein, (3) chlorophyll b, (4) pheophytin b, (5) chlorophyll a, (6) pheophytin a, and (7) α- and β-carotenes.

Comparison of Two Image Processing Methods

The sensitivity of the method can be improved by changing image processing. Instead of using a grayscale signal, RGB channels can be kept and analyzed separately. This operation is especially useful for yellow pigments, which can be barely detectable in grayscale pictures. To compare the two methods (grayscale processing versus color processing), calibration curves were performed for β-Car in bluescale and grayscale (R and G channels are not useful because β-Car does not absorb noticeably in these two channels). Results of comparison are given on Figures 3 and 4.

Resolution and 2D-TLC Analysis

With 2D-TLC (Figure 5) the complete resolution of all peaks was achieved. 2D-TLC was especially useful for Pheo b, which could not be resolved completely in one-dimensional TLC (Figure 5).

Conclusions

Students are exposed to a fast, straightforward, inexpensive, and consistent method to separate plant pigments. Time required for the whole experiment (extraction of pigments, chromatographic separation, and scanning and image treatment) is approximately 50 min. The experiment has the additional advantage that a large (up to 10) number of samples can be analyzed at one time. The use of color channels RGB separately can be useful because it increases sensitivity of the method and is more precise. The method could be used for the characterization of complex mixtures of pigments or dyes.

Acknowledgments

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[W]Supplemental Material

A more detailed verison of the article is available in this issue of *JCE Online.*

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