Lab Documentation / Supplementary Material.

The chemistry of pigments in green plants has been studied since the first half of the nineteenth 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 pods are chlorophylls (Chl) a and b, and carotenoids (Car) *(4)*; when beans are blanched (prior to freezing) or stored in poor conditions, pigments include chlorophyll derivatives (pheophytins –Pheo- and allomers respectively from a and b forms). The group of carotenoids can be divided into hydrocarbon carotenoids (β- carotene and α-carotene, respectively β-Car and α-Car) *(6)* and oxidized carotenoids or xanthophylls (lutein Lut, violaxanthin Vio, neoxanthin Neo) *(6)*. In the early days of Car research, the terms xanthophyll and lutein were often confused *(1, 7)* probably because Lut is the main component of the xanthophyll carotenoid group *(6)*. Of course, this confusion should be avoided in chemical education, even though it remains in recent publications *(8)* or in documents given by chemical products suppliers[1].

In this paper, we propose to study pigments from green beans pods, even though spinach (*Spinacea oleracea L.*) leaves are a frequently used source of green vegetable pigments (our method however can be applied to other plant tissues). Chromatographic separation of plant pigments was proposed by Tswett *(9)* and variations of this initial method have been studied *(6, 8)*. Of course, complete separation of pigments through preparative liquid-liquid partition can also be achieved, but this method can be sometimes cumbersome and limited to fresh tissues *(11)*. High-performance liquid chromatography is also a routine method for analysis *(5)* but it is not always available in factories or in student laboratories. Thin layer chromatography (TLC),

being fast, easy and cheap, seems to very appropriate for chemical studies *(6, 8, 11)*. Spectroscopic techniques on directly extracted samples have been also set up to quantify the content of pigments in fresh plants and fruits but these methods have drawbacks. In particular UV-visible spectroscopy measurement and characterization are limited to the quantification of Chl a, Chl b and total Car *(12)*. Proposed methods of spectra analysis give no reliable results when Chl's and certain derivatives are mixed, nor do they give information on single Car *(12, 13, 14)*.

In this paper, we propose a direct quantification of all the pigments, through TLC separation, quantification of the TLC plates using a flatbed scanner and processing the digitised chromatogram.

Experimental Procedure

Chemicals

Acetone (99.8%) was from Carlo Erba (Ródano, Italy). Cyclohexane (analytical grade), Fontainebleau sand, sodium sulphate (99%) and hydrochloric acid (35.5%) were obtained from SDS (Peypin, France). Diethylamine (reagent grade) was from Merck (Darmstadt, Germany). Commercially pure β-Carotene, Chlorophyll a, Chlorophyll b and Lutein, were purchased from Sigma-Aldrich (β-Car, C4582; Chl b, C5878; Chl a, C5753, Lut, X6250). TLC plates (Silica Gel 60 F_{254} *Merck* 1.05735) and capillary tubes (borosilicate glass 20 µL micro-pipettes, *Corning* 7099S-20) were used for the chromatographic separation.

CAS Registry Numbers for all Chemicals

Acetone	CAS Number: [67-64-1]
β- Carotene	CAS Number: [7235-40-7]
Chlorophyll a	CAS Number: [479-61-8]

Chlorophyll b CAS Number: [519-62-0]

Cyclohexane CAS Number: [110-82-7]

Diethylamine(DEA) CAS Number: [109-89-7]

Hydrogen chloride CAS Number: [7647-01-0]

Lutein CAS Number: [127-40-2]

Sea sand CAS Number: [7631-86-9]

Sodium sulphate CAS Number: [7757-82-6]

Extraction of Pigments from Green Beans

Fresh green beans were purchased from a local market and frozen green beans (var. *Calisto*) were obtained from *Gelagri Inc.* (Loudéac, France). The method used for the extraction was modified from a previously published (*8*) extraction method. This modification consists in adding a liquid-liquid extraction step after the pigment extraction from the plant tissues. The reason for this modification was to concentrate the pigment extract. Under subdued light (in order to prevent pigment degradation), green beans (ca 1 gram, carefully weighted) were combined with ca 1 gram of anhydrous sodium sulphate and 5 grams of sand. The mixture was ground in a mortar with pestle until a light green powder was obtained (approximately 10 minutes). This powder was transferred to a 50 mL test tube containing 10 mL of acetone (this solvent can enter the plant tissues, but it dissolves water; hence the use of sodium sulphate). The tube was agitated with vortex mixer and the mixture was allowed to stand for 10 min, after which the green acetone solution was removed with pipette and transferred into a centrifugation tube. This extract was centrifuged 3500 x g for 15 minutes. The separation of soluble impurities from acetone and concentration of pigments are accomplished through liquid-liquid partition with 1 mL cyclohexane *(5)*. The separation of both phases is achieved adding 3 mL of distilled water. The upper organic layer (hypophase) was washed twice with

additional 3 mL of distilled water to remove acetone. A small quantity of the supernatant was taken in a capillary tube, weighted before and after filling in, in order to assess the quantity being analyzed. The plant extract was immediately spotted on the TLC sheet.

Separation of Pigments using TLC

The extract in the capillary tube was spotted immediately on the TLC sheet. The size of each TLC sheet was 3 x 10 cm. The extract was spotted at 1.5 ± 0.1 cm from the lower edge. The atmosphere of the developing chamber was pre-equilibrated with the developing solvents at least 15 min before the sheets were inserted.

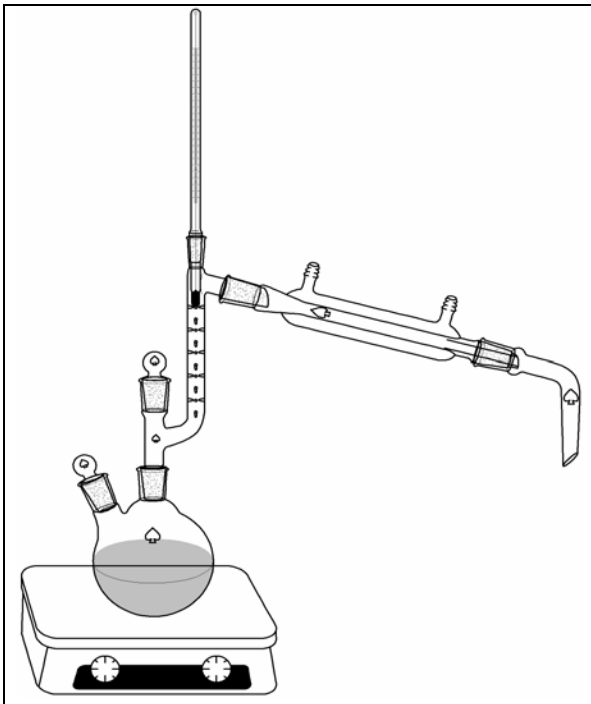
The developing solvent system used was cyclohexane-acetone-diethylamine (10:4:1, v/v/v) as in *(10),* but we substituted the petroleum ether mixture for cyclohexane, which is a chemically pure substance. In order to reduce background noise during TLC processes, it is recommended to use recently distilled diethylamine because it can easily degrade into yellow coloured derivatives *(15)*.

Distillation of diethylamine

DEA can be purified following the next experimental procedure carried out through fractional distillation.

As said above, DEA is used in TLC as part of the solvent system for developing the pigment extract. DEA degrades into oxidized nitro compounds which are yellow. This transformation should be avoided for two major reasons. If the DEA used is not pure, the development of spots is slightly different because the proportion of the solvents is not really 10:4:1. Also yellow compound formed (a nitro compound) is absorbed at the bottom of the TLC and this can make interferences when the TLC is scanned and image processed for pigment quantification.

The main properties of DEA are a molecular weight = 73.14 . Flammable, strongly alkaline liquid, melting point = -50º C, boiling point = 55.5 º C, d(20-4ºC) = 0.7074. Miscible in water (81.5 g in 100 mL). Keep well closed.

	-1 round flask -1 fractional distillation apparatus; column, thermometer and condenser. -1 adaptor for recovery of the distillate -2 round flasks; one for the head of distillation and the other one for the distillate product. -1 heater. -some pumice stones.

DEA is very miscible in water, so all distillation apparatus components were cleaned, rinsed with acetone and dried at 80º C before using.

The impure DEA solution is introduced in a round flask and a distillation column, thermometer and condenser are connected to it (see picture above). The pumice stones are added in the same flask to avoid overheating and to have a homogeneous heating. The heater is carefully placed underneath the round flask. The flask is heated and all products recovered by distillation at temperature lower than 55.5º C are discarded. The first drops of the distillation at 55.5º C are also discarded to ensure that the finally distillate is as pure as possible.

Once the temperature reaches 55.5 º C the distilled product is recovered in another flask until the temperature starts to rise again. Then the heater is turned off and the recovered product is kept at 4 º C until use.

The residue of the distillation is a sticky oilish yellow (the oxide of the amine) that is water soluble and can be cleaned quite easily once the distillation is finished.

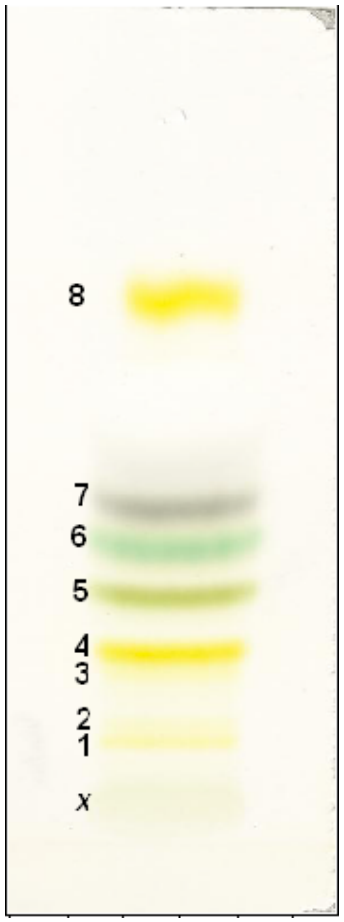
Preparation of Calibration Standards

Standards of Chl a, Chl b, β-Car and Lut were commercially available products. If these products are not available (eg because of their price), preparative layer chromatography can be used. Solutions of these pigments with concentrations from to 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 the addition of acid (HCl 25%) *(12)*. As Vio and Neo have UV-visible spectra similar as 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 digitised images were treated with image treatment software.

Scanning

The digitization of the TLC plates was done in a *Canon* CanoScan 5200F flatbed scanner. The TLC plates are placed in the flatbed scanner, siliceous side in contact with the glass of the scanner. The scanner lid must be closed during the scanning process because the scanner uses the whole scanning area for calibration. The parameters of the scanner drive, TWAIN scanning module and resolution of the scanning process (300 pixels per point) were set to installation defaults. The image was scanned and registered in JPEG (joint photographic experts group)

136 compressed files. Other formats of picture registration can be used: uncompressed BMP files
137 (Byte map) or TIFF (tagged image file format).
138
139 *Image processing.*
140 Either *Image J* (National Institutes of Health, USA, http://rbs.info.nih.gov/ij/) or the image
141 analysis package from *IGOR Pro* (Wavemetrics Inc, USA, http://wavemetrics.com) software
142 was used for the processing of scanned TLC plates. All the results presented here were
143 calculated using *IGOR Pro*, but image processing can be equally done with *Image J* free
144 software.
145



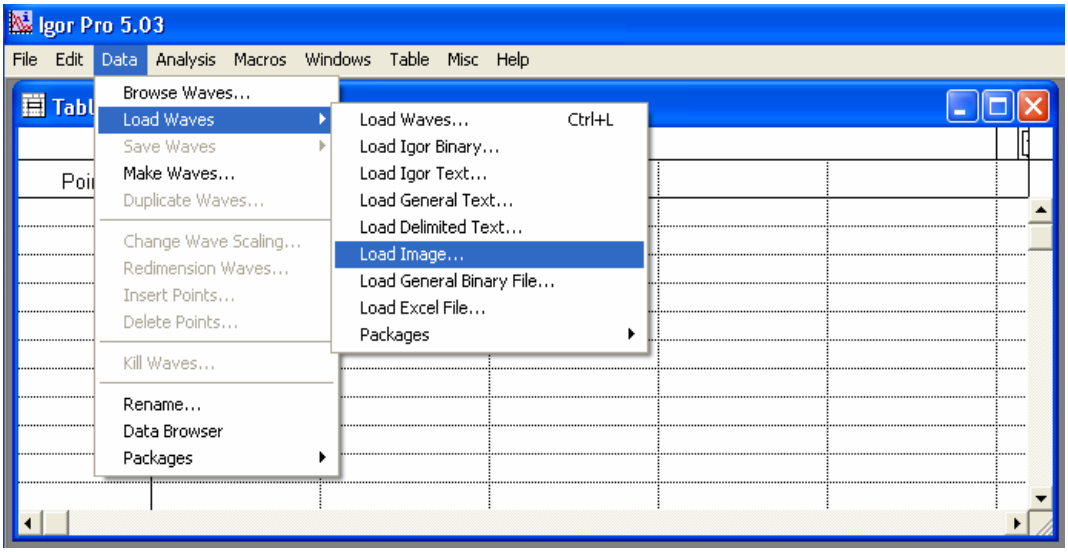
146
147 **Fig.1. Scanned TLC plate where the photosynthetic pigments were developed in**
148 **cyclohexane-acetone-diethylamine (10:4:1 ; v/v/v). (x) Origin, (1) Neoxantin, (2)**

Violaxanthin, (3) 5, 6-Epoxy Lutein, (4) Lutein, (5) Chlorophyll b, (6) Chlorophyll a, (7) Pheophytin a, (8) α and β- Carotenes.

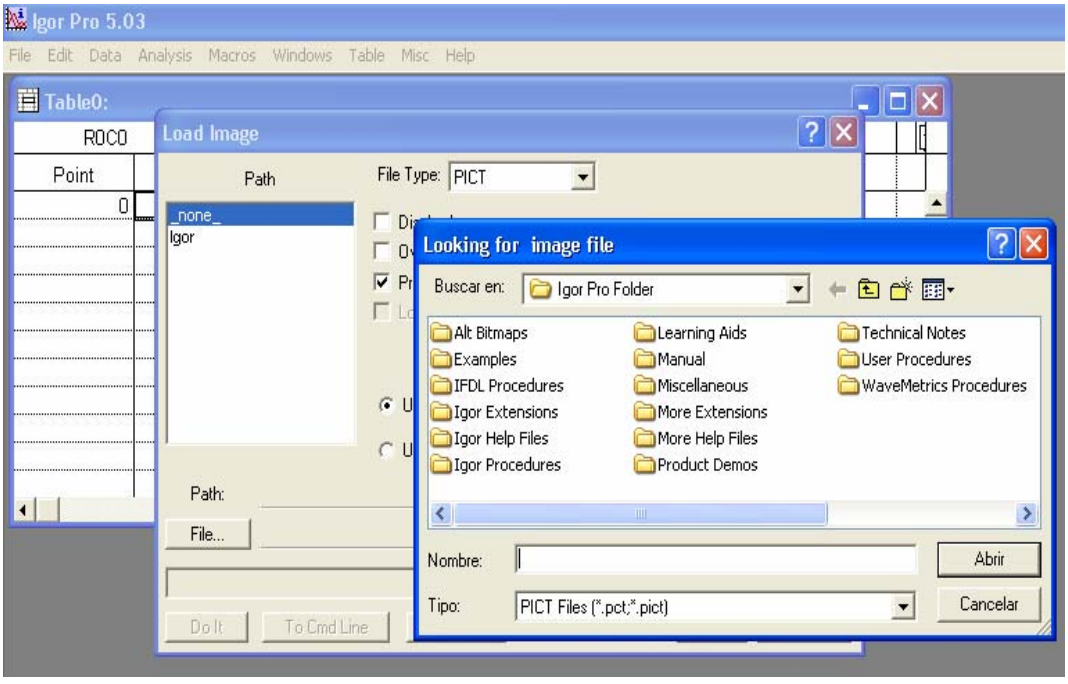
Experimental procedure for IGOR Pro 5.0 for Windows XP

1. Loading an image.

Data/Load Wave/Load Image [ENTER]

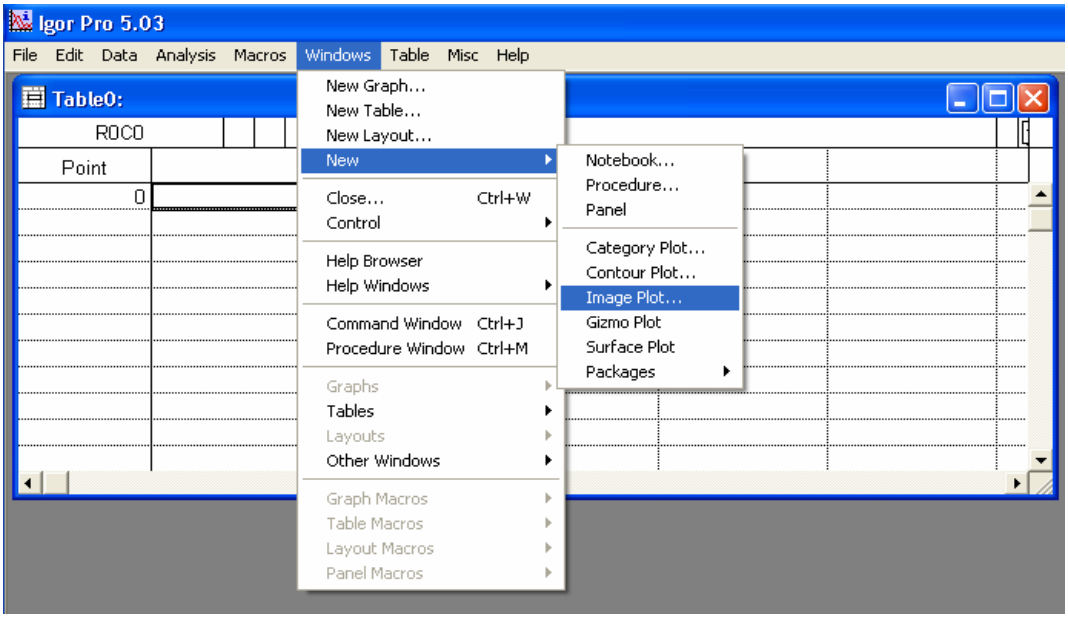


Show the path to the image (*image.jpg* for example)



2. Creating an image plot.

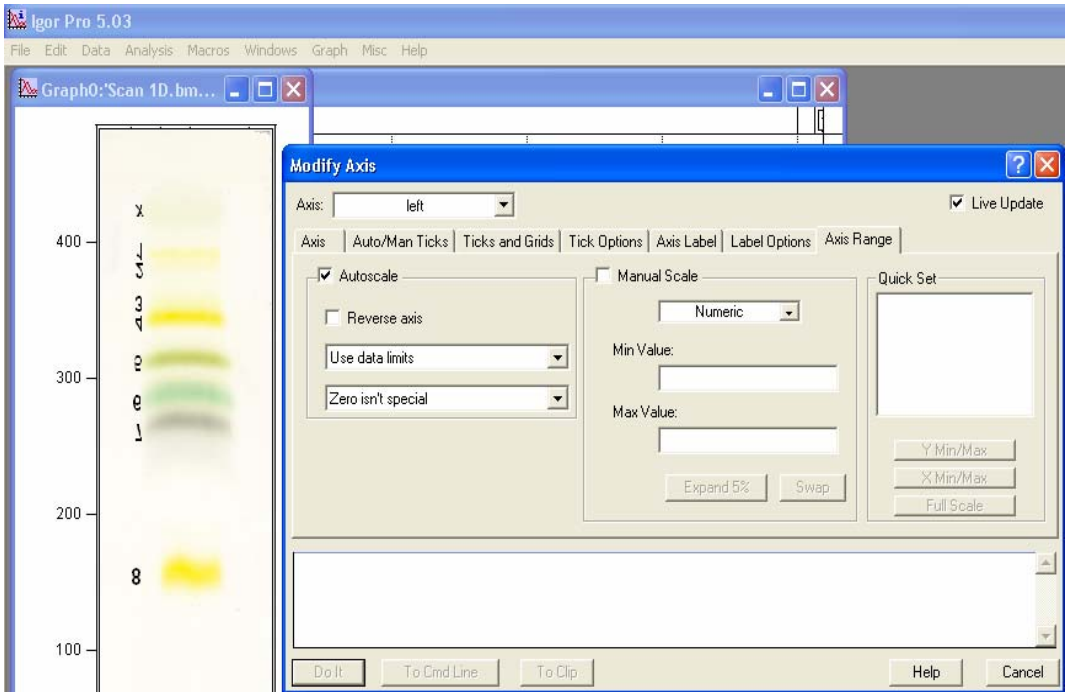
Windows/New/New image plot [ENTER]



3. Image Orientation. By default the plot is reversed. The image can be flipped vertically by reversing the Y axis.

Graph/Modify axis [ENTER]

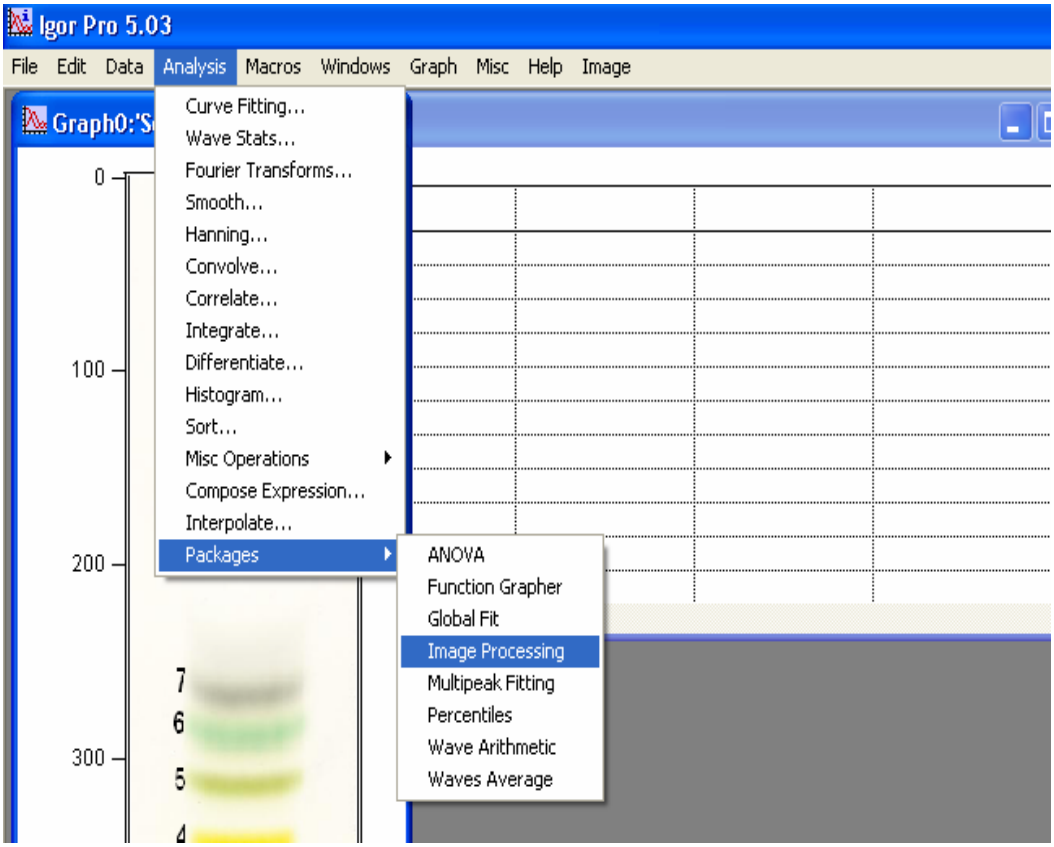
Axis Range tab/ mark the check box to reverse axis.



In order to achieve pigment quantification, scanned plates are first transformed into greyscale pictures and then into intensity profiles.

4. Image Processing. In order to process the image properly a macro (image processing package) must be opened.

Analysis/Packages/Image Processing

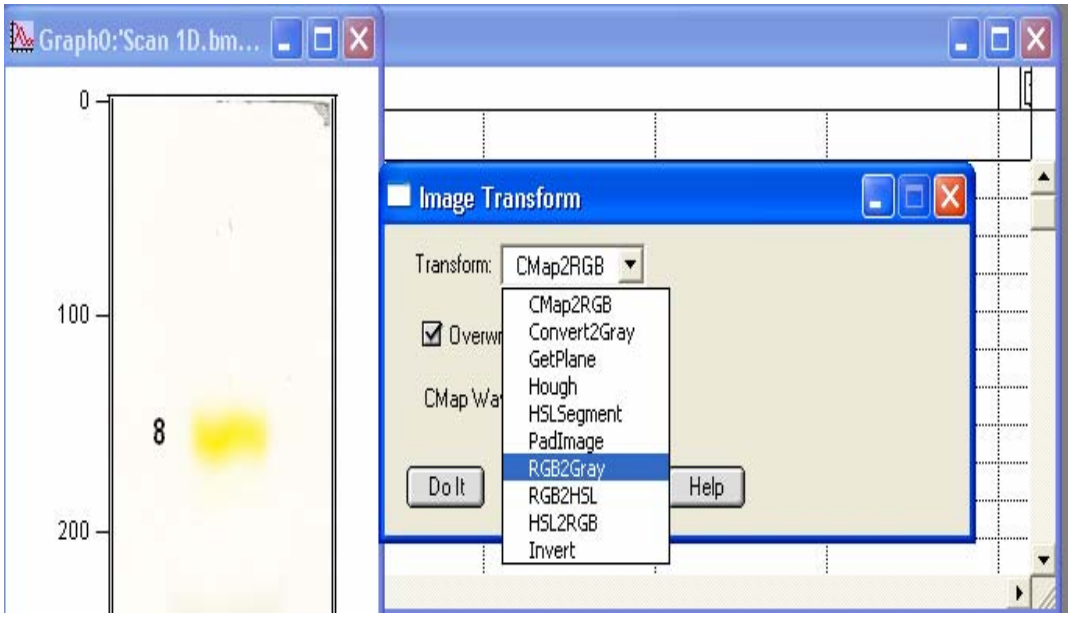


Once the macro has been opened the plot profile can be done in RGB (Red, Green, Blue) or in Greyscale. For doing it in RGB go directly to Line Profile.

5. Image Transformation. The original image is transformed into a greyscale image.

Image/ Image Transformations [ENTER]

1. The image transformation chosen for this purpose is: RGB2Gray.
2. Check “Overwritesource wave” checkbox.
3. Press Do it

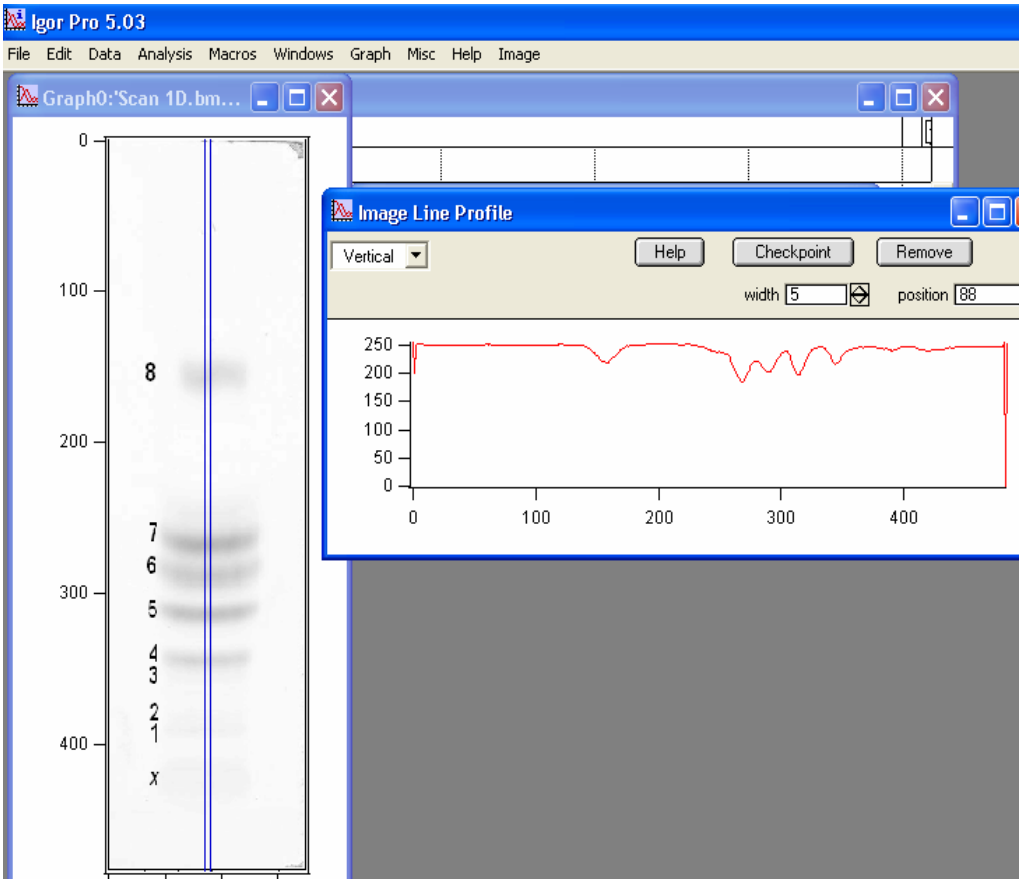


The intensity profile of one lane of the plate is obtained from the greyscale picture by selecting a lane with the same width as the largest spot of the lane (in this way, the area and intensity are both considered together, which means integration and better quantification of pigments).

6. Plotting the Line Profile

Image/Image Line Profiles [ENTER]

1. Select the way line profile must be done (vertical or horizontal), chose a suitable position and width to include the whole spot in the line profile.
2. Press “Checkpoint” Button



A new wave is generated. This wave is called *image.jpg_Prof*

For each lane, the two "lower" edge (the one near with the spots are deposited) and "upper" edge (opposite side toward which the solvent is moving) are not considered *(16)*.

7. Calculation of the Relative Optic Density

The calculation of the ROD is done in the next way:

Analysis/Compose Expression [ENTER]

Choose the wave *(image.jpg_Prof*) and insert the function, the wave, and operation to convert the plotted profile into ROD signal. The Expression box must contain:

Log(255/'image.jpg_Prof').

Once the signal has been transformed the fitting of the ROD curve must be done. For that purpose a new specific "package" is opened. But before the extreme data points must be eliminated to avoid error in the fitting.

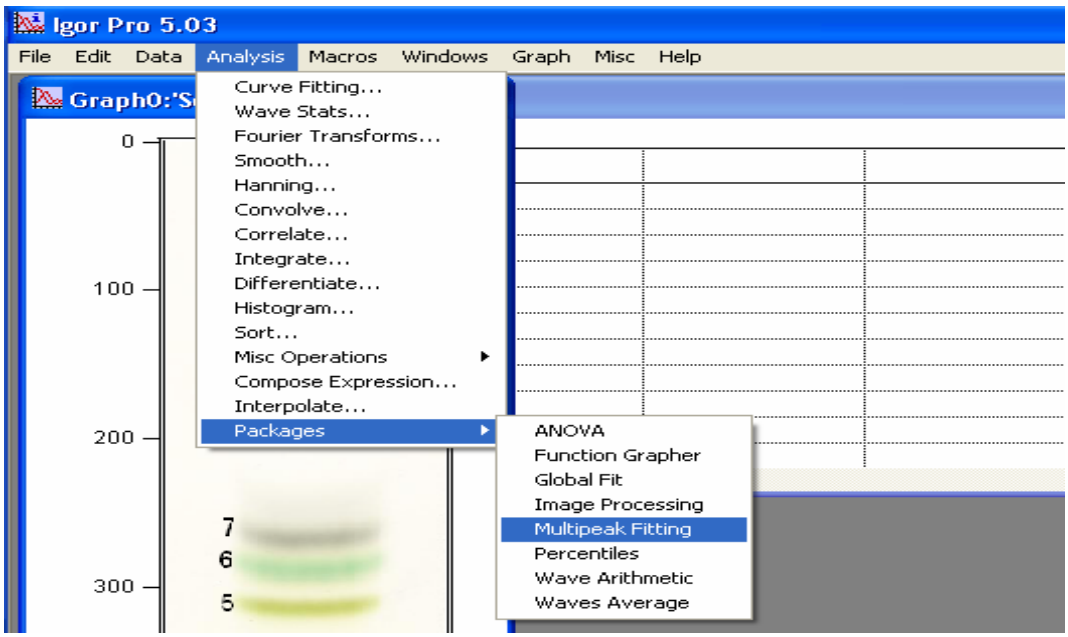
Data/Delete Points [ENTER]

1. The wave is chosen *image.jpg_Prof*.
2. The range of at least the first and last 100 points is deleted.

Gaussian fitting of the peaks on intensity profiles is performed in order to remove baseline variations and to separate peaks when they overlap. With this fit, experiments showed that good results are obtained when the signal is considered to be the sum of Gaussian peaks plus a polynomial baseline whose degree is chosen through fitting. This leads to a better appreciation of the signal due to pigments, which can be integrated and compared to a standard.

8. Fitting the ROD signal

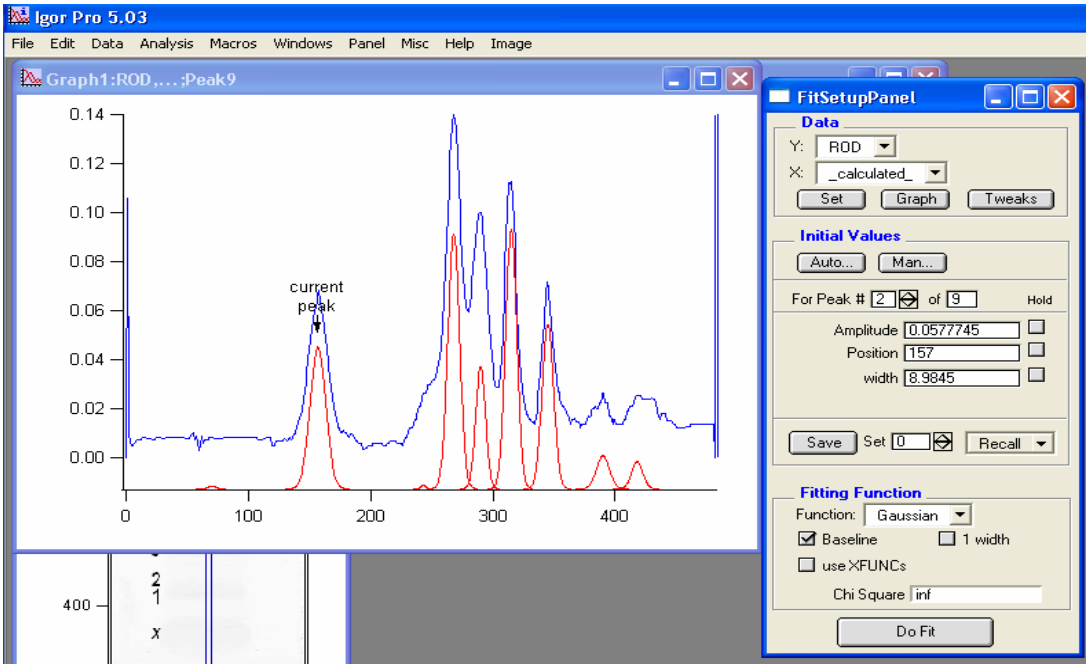
Analysis/Multi Peak Fitting [ENTER]



Choose Y wave (*image.jpg_Prof.*) and X wave (*_calculated_* by default)

[SET]

1. Introduce the initial values for each peak in the corresponding boxes.
2. Choose the kind of peaks (Gaussian, Lorentzian, Voight…): Choose Gaussian.
3. Check the baseline checkbox for a polynomial fitting of the baseline.
4. Press [Do it].



When you are satisfied with a fit, choose PrintPeakParams from the Macros menu. A new table is opened displacing values giving the peak position and area with estimated errors. A number of other values that depend on the specific function is also displaced.

For the "**Calibration curve**" the same procedure is done but the values for the peaks are correlated with the mass of pigment spotted in the TLC layer. This is calculated multiplying the concentration of the solution spotted (mg/L) by the volume spotted (L). The best way to measure this volume is weighting the capillary tube before and after solution uptake.

The linear regression will have an acceptable value when the correlation coefficient $R^2 \geq 0.99$ (this can be easily done in Excel worksheet but IGOR Pro is recommended).

Two dimensional-TLC analysis (2D-TLC).

2D TLC was performed in bigger sheets (ca 10 x 10 cm) in order to check that the pigments are not degraded during the experiment (through chemical interaction with silica). The sample is applied on the corner of the sheet. The plate is then developed in one direction. After completion the plate is allowed to dry, rotated 90º and developed using the same solvent system and same the conditions as described above.

Hazards

The solvents (acetone, cyclohexane and diethylamine) used in this experience are volatile, highly flammable; contact with skin or eyes should be avoided with gloves and laboratory goggles. 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.

Complete information regarding potential hazards for all chemicals to students and instructors and appropriate safety warnings is given below:

Acetone CAS Number: [67-64-1]

EMERGENCY OVERVIEW

Flammable (USA) Highly Flammable (EU). Irritant. Irritating to eyes. Repeated exposure may cause skin dryness or cracking. Vapours may cause drowsiness and dizziness.

Target organ(s): Liver. Kidneys. Nerves.

FLAMMABLE HAZARDS

Flammable Hazards: Yes

EXPLOSION HAZARDS

Vapour may travel considerable distance to source of ignition and flash back. Container explosion may occur under fire conditions.

FLASH POINT

1 °F - 17.0 °C Method: closed cup

EXPLOSION LIMITS

Lower: 2 % Upper: 13 %

AUTOIGNITION TEMP

465 °C

FLAMMABILITY

N/A

EXTINGUISHING MEDIA

Suitable: For small (incipient) fires, use media such as "alcohol" foam, dry chemical, or carbon dioxide. For large fires, apply water from as far as possible. Use very large quantities (flooding) of water applied as a mist or spray; solid streams of water may be ineffective. Cool all affected containers with flooding quantities of water.

FIREFIGHTING

Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes. Specific Hazard(s): Flammable liquid. Emits toxic fumes under fire conditions.

HANDLING

User Exposure: Do not breathe vapour. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep tightly closed. Keep away from heat, sparks, and open flame.

β- Carotene CAS Number: [7235-40-7]

EMERGENCY OVERVIEW

Risk of explosion if heated under confinement.

EXPLOSION DATA

Dust Potential: This material, like most materials in powder form, is capable of creating a dust explosion.

HANDLING

User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure. Open carefully.

STORAGE

Suitable: Keep tightly closed. Handle and store under nitrogen. Store at -20°C

SPECIAL REQUIREMENTS

Handle and store under inert gas. Air and light sensitive.

Chlorophyll a CAS Number: [479-61-8]

EMERGENCY OVERVIEW

Caution: Avoid contact and inhalation.

HANDLING

User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep tightly closed. Store under nitrogen. Store at -20°C

SPECIAL REQUIREMENTS

Store under inert gas. Light sensitive. Air sensitive.

Chlorophyll b CAS Number: [519-62-0]

EMERGENCY OVERVIEW

Caution: Avoid contact and inhalation.

HANDLING

User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep tightly closed. Store under nitrogen. Store at -20°C

SPECIAL REQUIREMENTS

Store under inert gas. Light sensitive. Air sensitive.

Cyclohexane CAS Number: [110-82-7]

EMERGENCY OVERVIEW

Flammable (USA) Highly Flammable (EU). Harmful. Dangerous for the environment. Irritating to skin. Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment. Harmful: may cause lung damage if swallowed. Vapors may cause drowsiness and dizziness. Target organ(s): Lungs. Central nervous system.

FLAMMABLE HAZARDS

Flammable Hazards: Yes

EXPLOSION HAZARDS

Vapour may travel considerable distance to source of ignition and flash back. Container explosion can occur under fire conditions. In advanced or massive fires the area should be evacuated and the fire should be fought from a remote explosion-resistant location.

FLASH POINT

- 0.4 °F - 18.0 °C Method: closed cup

EXPLOSION LIMITS

Lower: 1 % Upper: 9 %

AUTOIGNITION TEMP

260 °C

FLAMMABILITY

N/A

EXTINGUISHING MEDIA

Suitable: For small (incipient) fires, use media such as "alcohol" foam, dry chemical, or carbon dioxide. For large fires, apply water from as far as possible. Use very large quantities (flooding) of water applied as a mist or spray; solid streams of water may be ineffective. Cool all affected containers with flooding quantities of water.

FIREFIGHTING

Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes. Specific Hazard(s): Flammable liquid. Emits toxic fumes under fire conditions. Specific Method(s) of Fire Fighting: Use water spray to cool fire-exposed containers.

HANDLING

User Exposure: Do not breathe vapor. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep container closed. Keep away from heat, sparks, and open flame. Store in a cool dry place.

Diethylamine CAS Number: [109-89-7]

EMERGENCY OVERVIEW

Flammable (USA) Highly Flammable (EU). Corrosive. Harmful by inhalation, in contact with skin and if swallowed. Causes severe burns. May cause sensitization by inhalation and skin contact. Lachrymator.

FLAMMABLE HAZARDS

Flammable Hazards: Yes

EXPLOSION HAZARDS

Vapour may travel considerable distance to source of ignition and flash back. Container explosion may occur under fire conditions.

FLASH POINT

- 9.0 °F - 23.0 °C Method: closed cup

EXPLOSION LIMITS

Lower: 1.8 % Upper: 10.1 %

AUTOIGNITION TEMP

312 °C

FLAMMABILITY

N/A

EXTINGUISHING MEDIA

Suitable: For small (incipient) fires, use media such as "alcohol" foam, dry chemical, or carbon dioxide. For large fires, apply water from as far as possible. Use very large quantities (flooding) of water applied as a mist or spray; solid streams of water may be ineffective. Cool all affected containers with flooding quantities of water.

FIREFIGHTING

Protective Equipment: Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes. Specific Hazard(s): Flammable liquid. Emits toxic fumes under fire conditions.

HANDLING

User Exposure: Do not breathe vapour. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep tightly closed. Keep away from heat, sparks, and open flame.

Hydrogen chloride CAS Number: [7647-01-0]

EMERGENCY OVERVIEW

Toxic. Toxic by inhalation. Causes burns. Irritating to respiratory system.

INHALATION EXPOSURE

If inhaled, remove to fresh air.

DERMAL EXPOSURE

In case of skin contact, flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes.

EYE EXPOSURE

In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers.

HANDLING

User Exposure: Do not breathe vapor. Do not get in eyes, on skin, on clothing.

STORAGE

Suitable: Keep tightly closed.

SPECIAL REQUIREMENTS

May develop pressure. Open carefully.

Lutein CAS Number: [127-40-2]

HANDLING

User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep tightly closed. Store at -70°C

SPECIAL REQUIREMENTS

Air sensitive. Store under inert gas.

Sea sand CAS Number: [7631-86-9]

Not consider Hazardous (specified in directive 67/548/EEC).

Sodium sulphate CAS Number: [7757-82-6]

Not consider Hazardous (specified in directive 67/548/EEC).

HANDLING

User Exposure: Avoid inhalation. Avoid contact with eyes, skin, and clothing. Avoid prolonged or repeated exposure.

STORAGE

Suitable: Keep tightly closed.

SPECIAL REQUIREMENTS

Hygroscopic.

Results and Discussion

Extraction of Pigments from Green Beans

The method of pigment extraction from previously reported techniques were compared *(4, 5, 6, 8, 10, 12, 18)*. Direct extraction in a non polar solvent (chloroform, methylene-chloride, benzene, diethyl ether or petroleum ether) can be performed from dried plant or algae material *(12, 17)* but the composition of pigments in extracts is not representative of the plant tissue and the process is slow.

Polar solvents such as acetone, methanol or dimethylformamide (DMF) are often used *(4, 5, 6, 8, 10, 12, 17, 20)*. In particular, extraction of the pigments using primary alcohols can be achieved with a good recovery yield but some chl degradation occurs by opening of the *iso* cyclic ring, especially in alkaline conditions *(18, 19)*. Acetone is commonly used for extraction procedure with good results (*4, 5, 6, 8, 10, 12, 17)*. Extraction with DMF gives more consistent results *(6)* but it is seldom used, probably because of safety considerations due to toxicity; moreover, the recovery through the liquid-liquid partition should be carefully considered if the pigments of concern are very soluble in DMF and less in non polar solvents (the various pigments are extracted differently, so there is a bias in analysis) *(21)*.

Extraction with acetone was chosen here *(8)* because it is safe for students and gives reliable results.

TLC separation of Pigments

As it has been reported that pigments are susceptible to degradation with siliceous adsorbents *(6),* 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 ratios to front (R_f) were

459 calculated for each spot and compared with those previously published in literature *(10)*. Then
460 epoxy test *(6, 10)* was carried out to identify the pigments. As the epoxy test was negative in
461 both cases, no epoxy rearrangement was observed, confirming that no oxidation of the samples
462 into epoxy groups was produced during the TLC separation of pigments. This validated the
463 materials and method used. It also confirmed that 2D-TLC could be done without risk of
464 pigment degradation.
465
466 *Scanning*
467 Digitization was done using a flatbed scanner, which is basically composed by a lamp
468 (CGFL, xenon, fluorescent) and a light sensitive sensor (Charged Coupled Device array) (*23)*.
469 The lamp emits light towards the plate where the document is placed; the reflected light is
470 collected on the light sensitive sensor (CCD). During all the scanning process light is emitted at
471 a constant intensity (this was checked in our experiments).
472 The CCD array of the scanner transforms the reflected light into an electrical signal
473 expressed as a collection of binary units (bits) *(23)*. The dynamic range of the imaging system
474 depends on the quantity of bits that are used to express each light measurement (in our
475 experiments 8-bit resolution -256 values- was used).
476 During the recording process reflected light is transformed into a set of RGB-space
477 intensity values (RGB is a colour model based on the additive combinations of red, green and
478 blue light to create all other colours). Each channel of primary colour is expressed on a scale of
479 256 values. In order to interpret the values given by the scanner, a signal = f (light intensity)
480 function has to be known. In particular, one should be aware that the sensor has a limited
481 sensitivity and can be saturated above a certain threshold. This range between the minimal
482 detectable light intensity and the saturation value is called the *dynamic range* of the sensor. If
483 the signal is too concentrated the signal can be saturated. This can be adjusted varying the

detection limit threshold of the scanned image. Following the conditions described above no adjustment is needed.

Image processing

The analysis is carried out after transformation of the "digitised picture" of the plate into a "greyscale picture" because the reduced information obtained in this way is enough in many cases, but other channels can also be used for more sensitivity (see below). The greyscale is composed of 256 discrete steps (from 0 corresponding to black to 255 corresponding to white). This greyscale represents the luminosity and is obtained following equation:

$$\text{Grey scale} = 0.3R + 0.59G + 0.11B \quad \textbf{Eq(1).}$$

(Where R, G and B are the discrete values for the red, green and blue respectively.)

A transformation ("*ImageLineProfile*" in IGOR software) produces a "greyscale profile" from the greyscale 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. A parameter called opacity is defined as *(24)*:

$$Opacity = \left(\frac{incident\_light}{reflected\_light} \right) \qquad \textbf{Eq(2).}$$

The relative optical density (ROD) is the color of some point of the plate relative to the color of the uncoloured substrate. More precisely, ROD is defined as the decimal logarithm of the opacity and it has been shown to be proportional to the concentration; it's another way of expressing the Beer-Lambert law *(24)*.

$$ROD = \log(Opacity)$$ **Eq(3).**

The function ROD=f(Rf) is the "plot greyscale profile".

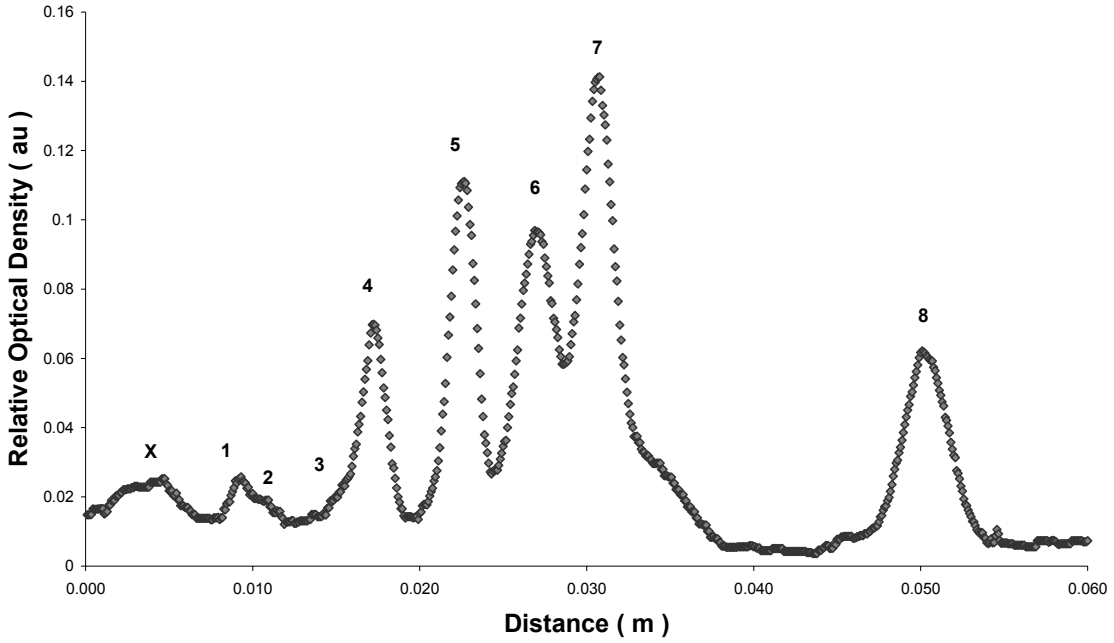
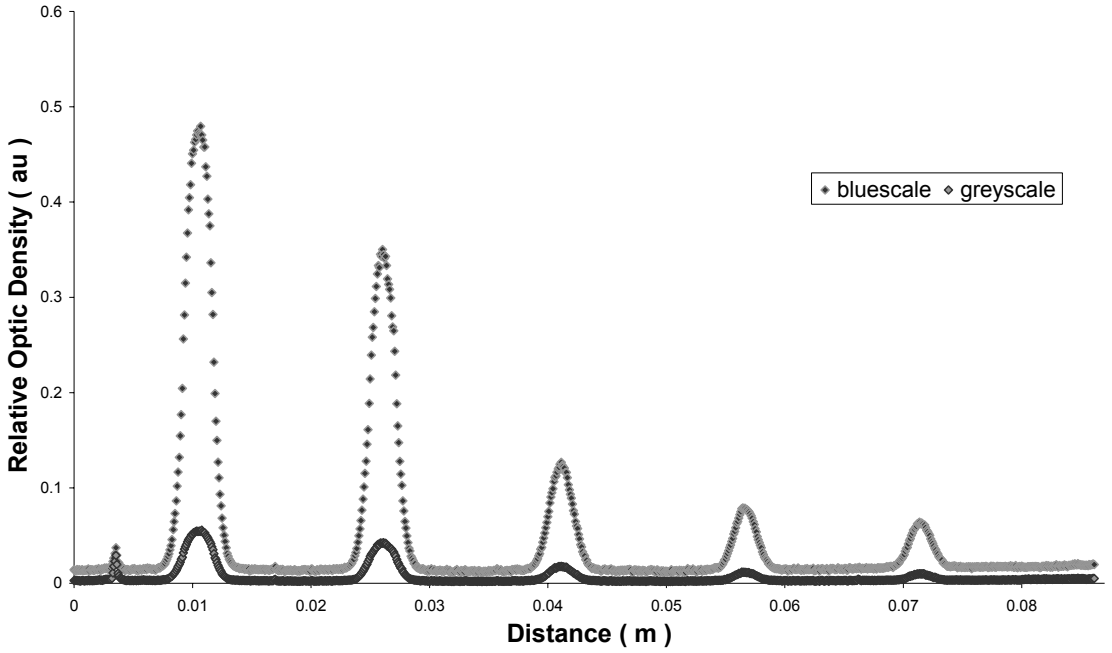


Fig.2. The plot profile of the scanned TLC plate after development with cyclohexane-acetone-diethylamine (10:4:1; v/v/v). (x) Origin, (1) Neoxantin, (2) Violaxanthin, (3) 5, 6-Epoxy Lutein, (4) Lutein, (5) Chlorophyll b, (6) Chlorophyll a, (7) Pheophytin a, (8) α and β- Carotenes. The large amount of Pheophytin a in this sample is due to poor (realistic) storage conditions.

In order to subtract a baseline and eliminate experimental noise, the signal is fitted using a sum of Gaussian peaks plus a polynomial. The assumption of a Gaussian distribution of pigments can be based on theoretical grounds: the many processes responsible for this dispersion along the lane generate a Gaussian peak. The degree of the polynomial can be obtained through

524 fitting, but in practice, it was always found to be equal to 5. The obtained Gaussian peaks are
525 integrated to get the concentrations of the pigments.
526 Two integrations are carried out. First, a lateral integration is done during lane definition, since
527 the plotted profile is function of lane width. Then vertical integration is performed from the
528 calculated peaks.
529
530 The value of the integral of each peak of the chromatogram is compared with a calibration
531 curve in order to determinate pigment concentration.
532
533 *Comparison of two image processing methods*
534 The sensitivity of the method can be improved by changing the image processing details.
535 Instead of using a greyscale signal, RGB channels can be kept and analyzed separately. This
536 operation is especially useful for yellow pigments, which can be barely detectable in greyscale
537 pictures (according to Eq. (1) the blue channel contributes less in the greyscale calculation).
538 In order to compare the two methods (greyscale processing *versus* colour processing),
539 calibration curves were performed for β-Car in blue scale and greyscale (no R and G channels
540 are not useful because β-Car does not absorb noticeably in these two channels). Results of
541 comparison are given on Figures 3 and 4.
542



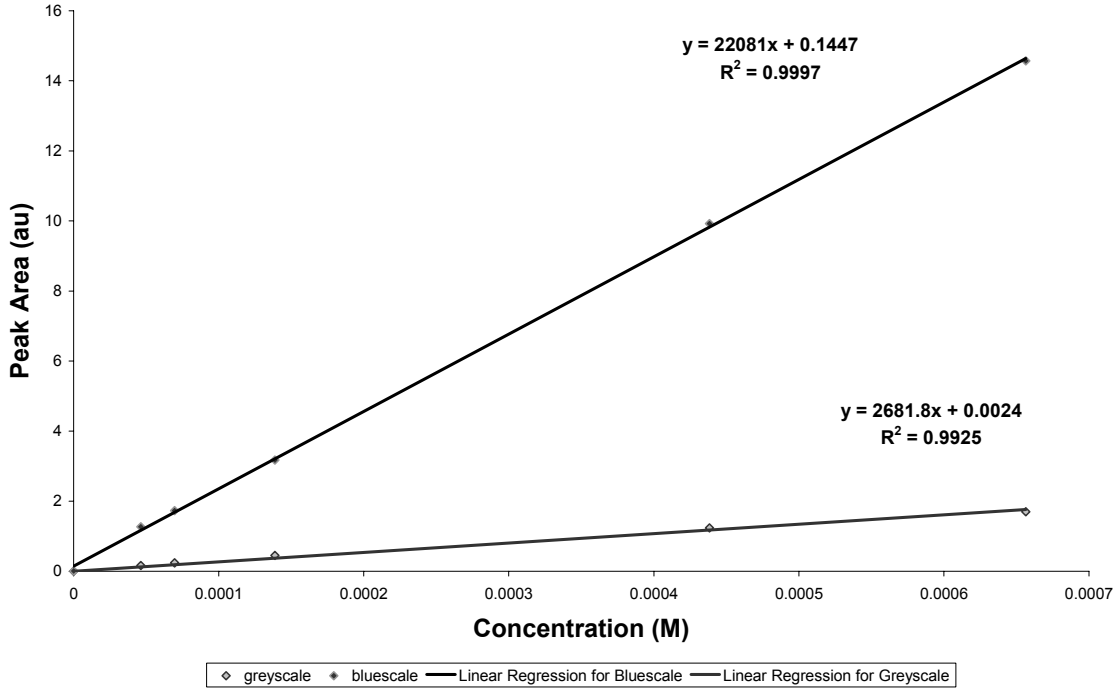
543

544

545 **Fig.3. Plotted profiles of β-Car samples in grey scale and in blue scale. Because of the**

546 **specific absorption of β-Car, more intense signal is obtained using a blue scale.**

547



548

Fig.4. Comparison of sensitivity for both calibration plots using greyscale and blue scale. Dark line corresponds to the linear regression for bluescale and light grey line to the linear regression for greyscale. As it can observed the sensitivity and precision for bluescale is bigger than for the greyscale.

Resolution and 2D-TLC analysis

The resolution of TLC analysis is defined as *(11):*

$$R_s = \frac{\Delta d}{W}$$

Where Δd represents the distance between spot centres for adjacent chromatographic spots and W represents the average spot width of two consecutive spots along the flow direction of the developing solvent).

For R_s values smaller than 0.5, both peaks are considered to be fused and for values bigger or equal to 1.5 separation is considered to be complete (baseline resolved). With 2D-TLC (Fig.5.) the complete resolution of all peaks was achieved. The spot resolution was improved for all spots in comparison to one-dimensional TLC. 2D-TLC was especially useful for Pheo b which could not be resolved completely in one-dimensional TLC (Fig.5).

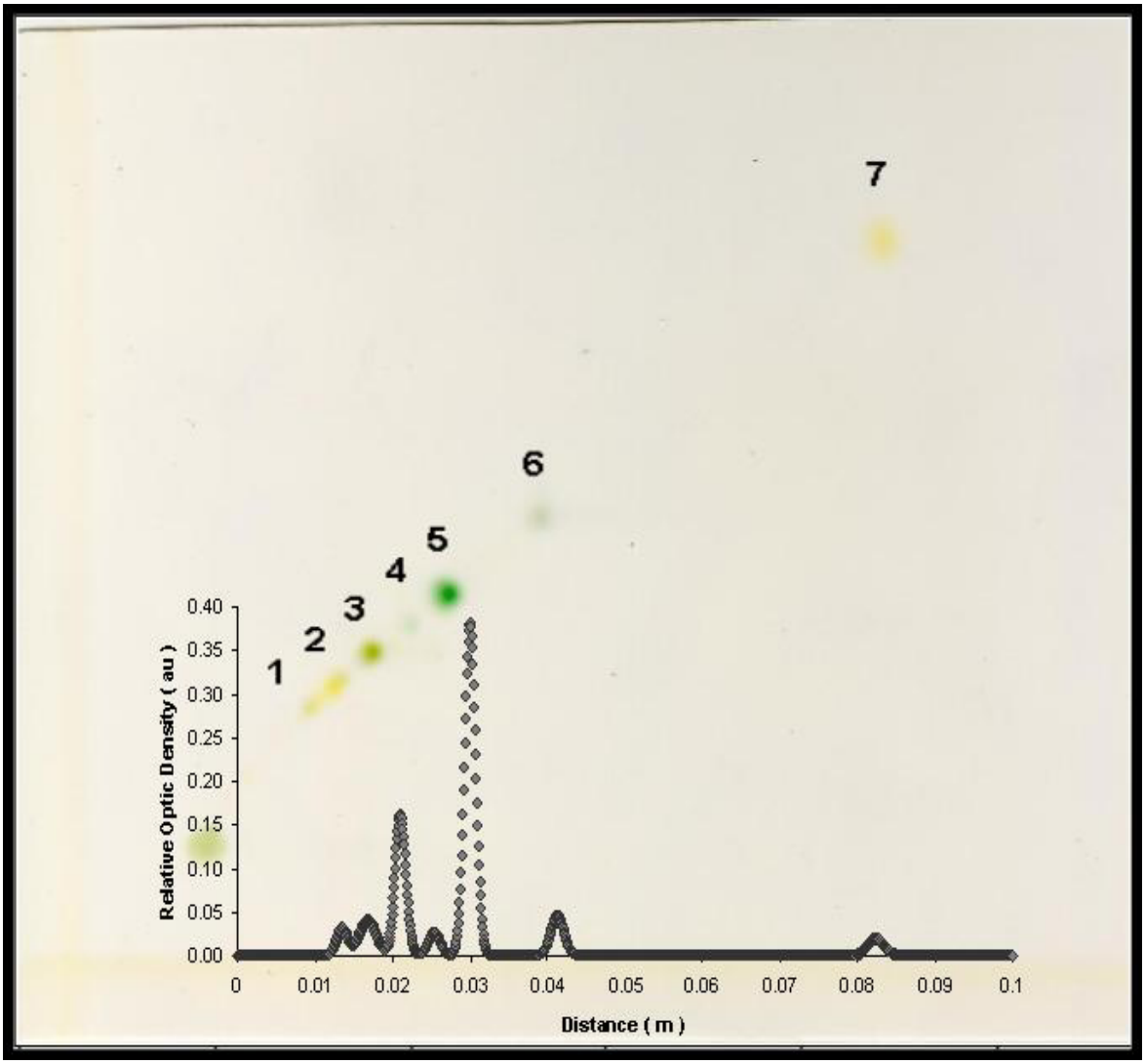


Fig.5. Scanned 2D TLC plate. The developing solvent system in both dimensions was the same (cyclohexane-acetone-diethylamine; 10:4:1; v/v/v). The alignment along the main diagonal shows that there is little degradation of pigments between the first and the second developments (about 10 min).

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, (7) α and β- Carotenes.

Conclusions

Results show that this method is fast, straightforward, cheap and consistent. The method does not require expensive analytical instrumentation. Time required for the whole experience (extraction of pigments, TLC separation and scanning and image treatment) is approximately 50 min. It has the additional advantage that a large (up to 10) number of samples can be analysed at one time.

The use of colour channels RGB separately can be very 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 and/or dyes.

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Notes

[1] MSDS, Sigma Aldrich: Product Name: Xanthophyll from Alfalfa, X6250. Sigma Aldrich at www.sigma-aldrich.com

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