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Design of a flow injection method for chlorophyll determination in in vitro plants

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Abstract

A flow injection (FIA) method was designed for the determination of chlorophylls a and b in small in vitro *Dieffenbachia maculata* "Sublime" plants. In the first step, the pigments from spinach leaves were separated, purified by solvent extraction and freeze–dried, to obtain standards for the FIA optimization. The sample extraction procedure was optimized. Four solvents were tested: diethyl ether, methanol, acetone and ethanol. The ethanol 96% was the optimal solvent for FIA purposes. It allows to the efficient extraction of the pigments and water can be used as carrier. The best FIA conditions found for the quasi-simultaneous quantification of chlorophylls a and b were a flow rate of 10.84 mL min^{-1}, a sample injection volume of 1.45 mL and a reactor length of 63 cm. The detection was performed with the automatic wavelength scanning Cintra 10e spectrometer, at 649 and 665 nm. The results obtained by the FIA method were compared to those obtained by the Arnon method. A deviation less than 5% was found between results for both methods. The concentration (mg g^{-1}) of chlorophylls a and b during three periods of the plants (in vitro, acclimatization, and adult) was determined to evaluate the whole in vitro procedure. It was found an increment of both pigment concentrations since the in vitro step till the adult stage, while the chlorophylls a to b ratio decreases. The designed method is suitable especially for the determination of the pigments at low concentrations in small samples with appropriate analytical quality.

Keywords: Flow injection; in vitro plants; Chlorophyll; UV–vis

1. Introduction

The chlorophyll concentration is one of the most important parameters to be determined in the studies involving plants for the evaluation of physiological processes [1,2]. The chlorophyll levels are influenced by soil conditions [3] and nutrients [4,5]. The determination of these variables becomes important in environmental studies [6,7]. The post-harvest evaluation of some fruits also requires the determination of the pigments, since the external color is a characteristic to be evaluated [8,9]. The quantification of chlorophylls a and b can be performed by non-destructive direct methods [9–11], by flourimetry [7], by chromatographic techniques [12] and frequently by UV–vis molecular absorption spectrometry using the Arnon equations [1,13,14]. In this last procedure, a previous extraction and separation is often required. It is necessary to take into account the solvent influence by means of the corrections established by Liechtenthaler et al. [13,14]. The method is absolute and must be modified for the FIA application since the use of standard calibration is necessary. On the other hand, agronomical studies demand the quantification of a large number of samples and, in some cases, the use of minimal amounts and low concentrations, as could be the evaluation of in vitro plants. In this work, a FIA method was designed for the determination of these pigments in small in vitro plants, taking into account the simplification of the analysis procedure and the use of minimal sample amounts at low concentration levels.

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2. Experimental

2.1. Reagents

Ethanol 96% (Riedel de Haen), Acetone (Riedel de Haen), petroleum ether (Merk), methanol (Riedel de Haen) and di-ethyl ether (Riedel de Haen). Deionized water (18 MΩ cm^{-1}) was used in the extraction and as carrier.

2.2. Standards

The methodology of Blanco et al. [2] was modified to obtain the chlorophylls a and b standards. The pigments from spinach leaves were separated, extracted by solvent, purified and dried to obtain standards for FIA optimization. The procedures were performed in dark room.

In a first step, approximately one gram of fresh spinach leaves is covered with acetone (about 10 mL) in a mortar and the pigments are extracted with the aid of clean sand. The mixture is then centrifuged for separation of the supernatant. The remaining sand is washed with acetone to ensure the quantitative removal of the pigments. After centrifugation the supernatant is mixed with the first extracted fraction. The use of sand helps for an easy, fast and efficient extraction in comparison to other methodologies, and independently of the solvent.

The supernatant is taken to a separation funnel and petroleum ether and water are added. After shaking the aqueous phase is discarded and the organic phase washed three times with deionized water. Methanol 92% (v/v) in water is added to the funnel. After shaking the methanol phase will contain chlorophyll b and xanthophylls, while the petroleum ether phase will contain the chlorophyll a and carotenoids fraction. Each fraction is purified with the addition of alcoholic KOH (30% (m/v)) in a separation funnel. In the case of methanol fraction, the pigments are separated to a diethyl ether phase in a previous step. The whole process is repeated several times in order to obtain significant amounts of the dried chlorophyll extracts. The purified chlorophylls in ether fractions are dried in nitrogen flow, to obtain solids, which can be redisolved in different solvents. It is important to point out that the procedure did not allow to a pure standard, but to enriched fractions of each pigment. The presence of the remaining pigments even after the purification step cannot be avoided and is observed in the spectra taken for the control of the process. Nevertheless, the standards can be characterized using the Arnon equations (the concentration values for both chlorophylls in the stock standard solutions are given in Table 1), and are necessary for the FIA quantitation. This procedure gave a solution to the alternative of pure commercial chlorophyll standards; whose are expensive, easily degradated and available only at few milligrams. On the other hand, the dried extracts permit the use of different solvents.

Table 1
Concentration of chlorophylls a and b in the standards

Standard stock solution	Chlorophyll a (mg L^{-1})	Chlorophyll b (mg L^{-1})
Chlorophyll a	49.03 ± 0.01	83.51 ± 0.01
Chlorophyll b	13.1670 ± 0.0001	26.4539 ± 0.0001

Table 2
Comparative table of solvent characteristics for chlorophyll extraction in a poled leaf sample

Solvent	Chlorophyll a (%, $n = 5$)	Chlorophyll b (%, $n = 5$)	Toxicity
Di-ethyl ether	0.039 ± 0.007	0.012 ± 0.002	Toxic
Methanol	0.057 ± 0.005	0.024 ± 0.002	Toxic
Ethanol 96%	0.071 ± 0.003	0.024 ± 0.001	Non-toxic
Acetone	0.070 ± 0.007	0.020 ± 0.002	Irritant

The values are the % (w/w) on dry basis of the pigments in a poled sample.

2.3. Samples

Dieffenbachia maculata "Sublime" leaf samples were analyzed at three stages of the in vitro process: in vitro, acclimatization and adult. Representative leaf samples were taken by punching out disks of 5 mm of diameter as reported by Aspelmeier (2001) [15]. An amount of 100 mg of leaf sample was weighed. The pigments from samples were extracted using ethanol 96% and clean sand in mortar in a similar way as used in the preparation of purified standards explained in Section 2. The mixture in the mortar was centrifuged and the supernatants were quantitatively transferred to volumetric flasks for measurements, without pigment separation or purification steps.

In order to optimize the pigment extraction from samples four solvents were tested: di-ethyl ether, acetone, methanol and ethanol 96%. Spinach leaves were used as testing samples in a first step.

Optimal results were found when using ethanol 96% (see Table 2). It allows to the efficient extraction of the pigments and water can be used as carrier in the FIA system. Additionally, it must be noticed that ethanol is less expensive than the other solvents evaluated.

3. Instrumental

3.1. Apparatus

An ISMATEC peristaltic pump IPC model was used for sample and carrier introduction controlled by a Temporizer GrabLab model 900 and a control valve Cole-Palmer, model 625 E Bunker CT. The detection was performed with the automatic wavelength scanning Cintra 10e spectrometer, at 649 and 665 nm in a flow cell by high speed scanning between the two values, to obtain simultaneously chlorophylls a and b fiagrams, as shown in Fig. 1. The FIA manifold has two channels (carrier and sample) and was designed for low

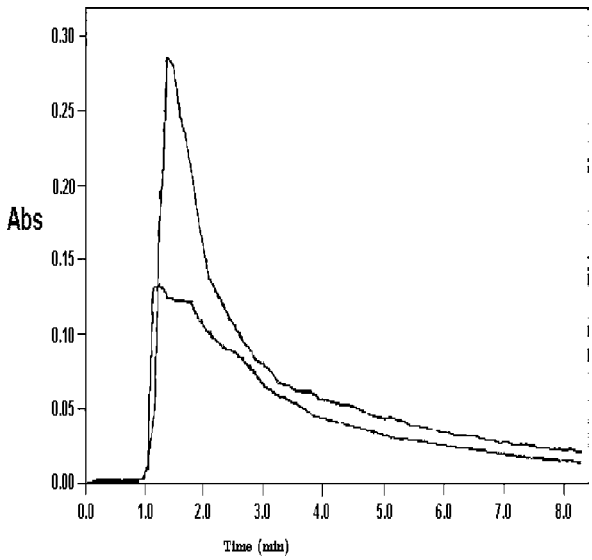

Fig. 1. Sample fiagrams for in vitro plants at 665 and 649 nm. The higher absorbance value correspond to chlorophyll a (665 nm) and below chlorophyll b (649 nm).

dispersion with Tygon tubing R-3606 of 1 mm diameter. The carrier is continuously passing through the flow cell in a first step. In a second step, the sample is injected through the second channel into the solvent stream and passes to the flow cell for the measurement (see Fig. 2). A non-coiled reactor was used with the minimal possible length. The FIA conditions for a low dispersion system were optimized in univariated way.

The non-destructive determination of total chlorophyll in *Dieffenbachia* "Sublime" leaves was performed with the SPAD-502 chlorophyll meter, as reported in reference [15].

3.2. Flow rate

The flow rate for low dispersion must be higher than 2 mL min^{-1} and for this work it is not a limitation since the carrier is water. The flow rate tested were 6.50, 8.67 and 10.84 mL min^{-1}, while fixing the sample volume in 1.45 mL and the reactor length at 63 cm. The optimal flow rate was 10.84 mL min^{-1}, since allows to the higher sample throughput and minimal dispersion. Higher flow rates were not available, since 10.84 mL min^{-1} correspond to the limit of the peristaltic pump for the manifold.

3.3. Sample volume

This parameter was adjusted for a flow rate of 10.84 mL min^{-1} and a reactor length of 63 cm. The injection times tested were 5, 8 and 10 s, equivalent to 0.90, 1.45 and 1.81 mL, respectively. The optimal sample volume corresponded to an injection time of 8 s or 1.45 mL. The dispersion was high for 5 s of injection time. Using 10 s or 1.81 mL, reliable results were obtained, but this injection time implies higher sample consumption, which is a limitant when small samples with low chlorophyll content are analyzed.

3.4. Reactor length

Two reactor lengths were tested; 63 and 126 cm. Lower reactor lengths are not possible to test due to the geometrical restrictions of the FIA manifold. The length of 63 cm was the minimal distance available from valve to flow cell without producing tension on the tubing. For 1.45 mL of sample volume and flow rate of 10.84 mL min^{-1} the optimal length was 63 cm. The fiagrams obtained using the larger reactor show, as expected, a higher dispersion and the base line is allowed after 10 min, while using the 63 cm reactor, the base line is obtained in 8 min, and other sample can be injected for a higher sample throughput.

4. Results

4.1. Quantification by FIA

In the common batch procedure, the chlorophylls are determined by an absolute method of quantification using the Arnon equations. In the case of the FIA method, the dilution of the pigments in the manifold and flow cell must be taken into account by calculation of the system dispersion. To solve this difficulty, in a first step of the quantification the concentration of chlorophylls a and b in the

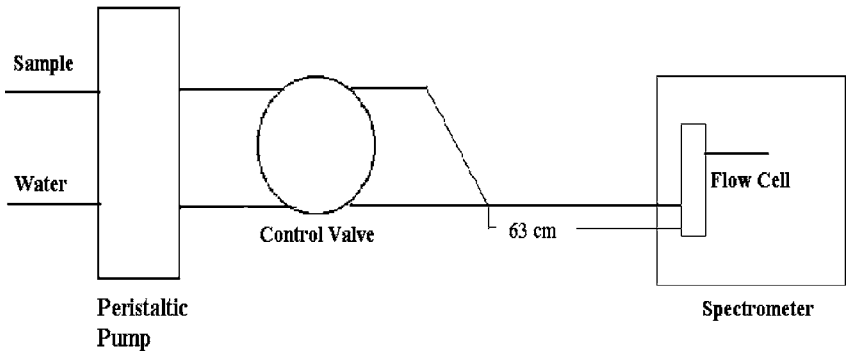

Fig. 2. FIA manifold for simultaneous chlorophylls a and b determination.

Table 3
Concentration of standard solutions of chlorophyll b by Batch and FIA methods

Dispersed concentration-FIA ($mg\,L^{-1}$) ($n = 5$)	Non-dispersed concentration-Batch ($mg\,L^{-1}$) ($n = 5$)
0.482 ± 0.002	0.534 ± 0.002
0.977 ± 0.002	1.067 ± 0.002
1.603 ± 0.002	1.600 ± 0.002
1.925 ± 0.002	2.134 ± 0.002

Table 4
Chlorophyll concentration in in vitro plants by Batch and FIA methods

Chlorophyll	Batch method ($mg\,g^{-1}$) ($n = 5$)	FIA method ($mg\,g^{-1}$) ($n = 5$)	Relative deviation (%)
a	0.905 ± 0.006	0.949 ± 0.006	4.87
b	0.505 ± 0.006	0.482 ± 0.006	4.64

standards were determined by batch using the Arnon equations for ethanol 96% (Eqs. (1) and (2)) as calculated by Lichtenthaler and Wellburn [13,14]. Then, the concentrations of the pigments in the FIA cell were determined using also these equations. The procedure was performed for a series of standards prepared by dilution of the dried extract as shown in Table 3 for chlorophyll b to obtain the Eqs. (3) and (4) and elsewhere the dispersion after the application of a linear regression to the data.

$$C_a = 13.95A_{665} - 7.34A_{649} \quad (1)$$

$$C_b = 24.96A_{649} - 7.32A_{665} \quad (2)$$

$$C_a = 1.172C_{a\,FIA} - 0.237\left(\frac{mg}{L}\right) \quad (3)$$

$$C_b = 1.062C_{b\,FIA} + 0.010\left(\frac{mg}{L}\right) \quad (4)$$

The Eqs. (3) and (4) allows to the quantification of chlorophylls a and b in samples by FIA for the designed set up. The chlorophyll concentration of the samples in the FIA cell is calculated using the Eqs. (1) and (2). Then, the real concentration in sample is determined applying Eqs. (3) and (4) for the dispersion correction.

The results obtained by the FIA method were compared to those obtained by the Arnon batch method, for each chlorophyll in samples. A deviation less than 5% was found between the methods for both chlorophylls. The Table 4. shows the results. The precision evaluated with five independent replicates was similar by both procedures (Batch and FIA).

4.2. Simultaneous determination of chlorophylls a and b in in vitro Dieffenbachia "Sublime" plants

The procedure was applied in the simultaneous evaluation of the chlorophylls a and b levels of the in vitro plants of *Dieffenbachia* "Sublime" at three stages: in vitro, acclimatization and adult. Table 5 shows the mean values of these parameters and the chlorophylls a to b ratio. It was observed an increment of both chlorophyll levels since the in vitro stage till the adult stage but a diminution of the chlorophylls a to b ratio. This fact is in agreement with the tendency reported in the literature for shade plants [16]. It was expected a low concentration for both chlorophylls during the in vitro step, since the plantlets are under heterotrophic conditions (there is not photosynthetic function), and a continuous increment in the chlorophyll levels during the acclimatization till the adult stage. The tendency of the total chlorophyll concentrations was compared to the values obtained by means of direct measurement with the SPAD. The relative increment in total chlorophyll observed by the SPAD measurements was similar to the spectrometric data. This fact implies that both data could be correlated though this fact was not demonstrated and was not in the scope of this work. It is necessary to take into account the conversion between the units of both methods (SPAD and FIA), since SPAD measurements expressed in arbitrary SPAD units are related to the foliar area [15]. It must be determined the specific foliar mass per area (this variable changes to higher values during the process of acclimatization). Although the developed method was destructive (compared to the non destructive SPAD determination), it gives important information that is not possible to obtain by SPAD measurements, as could be the chlorophylls a to b ratio, in a simple way if compared to the traditional batch method for UV–vis determination of chlorophylls and using minimal sample amounts (Table 5).

5. Conclusions

The FIA simultaneous determination of chlorophylls a and b can be performed if the dispersion is calculated by means of correlation of standard concentration as a function of dispersed concentration. The ethanol is the most adequate solvent for the sample preparation and extraction of the pigments. The designed method is suitable especially for the determination of the pigments at low concentrations in small samples with appropriate analytical quality. The levels of both chlorophylls (a and b) and total chlorophyll

Table 5
Chlorophyll concentration in the in vitro plants of Dieffenbachia "Sublime" at three stages of development (in vitro, acclimatization and adult)

Stage	Chlorophyll a ($mg\,g^{-1}$) ($n = 3$)	Chlorophyll b ($mg\,g^{-1}$) ($n = 3$)	Chlorophyll a/chlorophyll b	Total chlorophyll ($mg\,g^{-1}$)	Total chlorophyll (SPAD units) $n = 3$
In vitro	0.617 ± 0.003	0.224 ± 0.002	2.75	0.841	26 ± 2
Acclimatization	1.301 ± 0.007	0.522 ± 0.002	2.49	1.823	35 ± 1
Adult	1.400 ± 0.007	0.642 ± 0.003	2.18	2.042	46 ± 3

increment during the in vitro process while the chlorophyll a to b ratio decreases, as expected in the shade plants of *Dieffenbachia* "Sublime".

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