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Article

Development of a Multicommuted Flow Analysis Procedure for Photometric Determination of Total *N*-ureide in Soybean Tissues

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No presente trabalho é descrito um procedimento automático para determinação fotométrica de *N*-ureídeos em tecidos de soja. O processo fotométrico baseou-se na reação dos *N*-ureídeo com hipoclorito, seguido da reação com fenol em meio alcalino, formando o composto azul de indofenol, monitorado a 660 nm. O sistema de fluxo múltiplo com base no processo multicomutado foi adaptado para permitir a sua integração com um fotômetro baseado em LED, de modo a formar uma unidade compacta. Após definição das condições apropriadas operacionais relacionadas com a configuração do instrumento e procedimento analítico, a eficácia global foi confirmada pela determinação dos *N*-ureídeos em tecidos de soja. A precisão avaliada através da aplicação do teste-*t* pareado entre os resultados obtidos utilizando um método de referência mostrou que não existe diferença significativa para um nível de confiança de 95%. Outras características úteis também foram obtidas, tais como resposta linear variando de *N*-ureídeos 30 a 260 μmol L^{-1} (r = 0,9995), limite de detecção de *N*-ureídeos 1,0 μmol L−1, um desvio padrão relativo de 3% (n = 15), consumo de 0,55 e 6,8 mg de hipoclorito e de fenol *per* determinação, respectivamente, com produção de resíduos de 3,3 mL *per* determinação, e amostragem ao longo de 36 determinações *per* hora.

In the current work, an automated procedure for photometric determination of *N*-ureide in soybean tissues is described. The photometric procedure was based on the reaction of *N*-ureide with hypochlorite, followed by its reaction with phenol in an alkaline medium, forming the indophenol blue compound, monitored at 660 nm. The flow system manifold based on the multicommuted process was tailored to allow its integration with a LED-based photometer in order to form a compact unit. After setting the appropriated operational conditions related with instrument setup and analytical procedure, the overall effectiveness was ascertained by the determination of *N*-ureide in soybean tissues. Accuracy assessed by applying the paired *t*-test between results obtained using a reference method showed that there is no significant difference at 95% confidence level. Other useful features were also achieved, such as a linear response ranging from 30 up to 260 μmol L^{-1} *N*-ureide (r = 0.9995); a detection limit of 1.0 μmol L^{-1} *N*-ureide, a relative standard deviation of 3% (n = 15), consumption of 0.55 and 6.8 mg hypochlorite and phenol *per* determination, respectively; waste generation of 3.3 mL *per* determination and sampling throughout 36 determinations *per* hour.

Keywords: multicommuted flow analysis; LED-based photometer, *N*-ureides; soybean; green chemistry

Introduction

Nowadays, there is a broad interest in the soybean because of its high protein content that is about 40% (m/m) in grains, thereby representing an important protein source for human and animal diets. In the last decade, Brazil has become a large producer of soybeans, and as a consequence, this has created a high demand for fertilizers based on nitrogen. The intensive use of this type of fertilizer is necessary in order to increase the grain productivity; nevertheless, contamination of groundwater sources caused by fertilizers lixiviating through the soil is considered inevitable.[1]

Ureides (allantoin and allantoic acids) preceded by microorganisms comprise the main forms of soluble

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nitrogen and are metabolized by the legumes (soybean) of tropical origin.[2] These ureides are predominant in nodulated plants, rather than those that are grown in media using fertilizers based on NO_3^- or NH_4^+,[3] whereby suggesting that there is an association between N_2 fixation by microorganisms and *N*-ureide levels in plant tissues.[4] In this sense, the use of biological nitrogen fixation (BNF) has become a beneficial alternative for nitrogen-based fertilizers. Benefits include cost efficiency and risk reduction for groundwater contamination.[2] In this sense, the determination of ureides in soybean tissues is an essential requirement in order to ascertain the BNF efficiency.

The concentration of *N*-ureides (allantoin and allantoic acids) has been determined using spectrophotometric tecnniques.[5-7] In the first case, the methodology involves extraction from soybean tissues and conversion to urea and glyoxylate using alkalinization and acid hydrolysis steps. Glyoxylate is converted to its phenylhydrazone, which is oxidized in a strong acidic medium with ferricyanide to produce an intensely colored formazan.[5] This method is specific, sensitive, and accurate; nevertheless, it is very laborious, including the addition of several reagents followed by warming and cooling steps, which were done by immersing the reaction vessel in boiling water and ice baths. High consumption of concentrated HCl, as well as toxic reagents, also requiring intense involvement of the analyst, impairs its selection for large scale routine analysis.

Domnas[6] described a spectrophotometric method based on the reaction of amide nitrogen (allantoin) with hypochlorite followed by a reaction with phenol forming a compound monitored at 625 nm. The method is not specific for allantoin, owing to the free amino compounds present in the sample extract acting as interference. A modification of the Domnas method was proposed by Patterson *et al.*,[7] which included a sample treatment step to remove interfering compounds prior to analysis using a cationic exchange resin. The interfering effect was effectively overcome; nevertheless, the procedure generated a great volume of waste containing toxic compounds, impairing the environmental sustainability of the procedure, according to the Green Analytical Chemistry (GAC) guidelines.[8-10]

When the use of toxic reagents cannot be avoided, reduction of reagent consumption, as well as waste generation, are among the requisites recommended by the GAC in order to obtain greener analytical procedures.[11,12] This characteristic can be achieved through automation, employing either multicommuted flow analysis (MCFA),[13-15] or sequential injection analysis (SIA),[16,17] which enables facilities to handle low volumes of sample and reagent solutions.

In this work, we intend to develop a multicommuted flow analysis procedure for photometric determination of *N*-ureide in soybean tissues. The method is based on the formation of the indophenol blue compound, which occurs through oxidation of amide nitrogen with sodium hypochlorite followed by a reaction with phenol in an alkaline medium.[6] The indophenol compound is monitored at 625 nm using a LED-based photometer developed in the laboratory. In this case, the use of phenol as a reagent is inevitable. Therefore, the flow system will be designed to allow for an effective control of the reagent consumption, considering the GAC recommendation.[8,12]

Experimental

Reagents and solutions

All solutions were prepared with analytical grade chemicals. Purified water presenting an electric conductivity lower than 0.1 mS cm^{-1} was used throughout.

A 1500 μmol L^{-1} (237 ppm) allantoin stock solution was prepared by dissolving 0.0593 g of solid allantoin (Sigma, Germany) in 250 mL water. Working standard solutions with concentrations ranging from 30 up to 260 μmol L^{-1} (4.74 up to 41 ppm) allantoin were prepared daily by diluting the previous one with water. These solutions were stored in polyethylene bottles. A 1.5 mol L^{-1} sodium hydroxide solution was prepared by dissolving 6.3 g NaOH (Merck) in 100 mL water. A 0.2 mol L^{-1} potassium hydrogen phthalate solution (pH 4) was prepared by dissolving 10.2 g $C_8H_5KO_4$ (Merck) in 250 mL water. A sodium hypochlorite stock solution was prepared from a domestic bleach stock solution, which was standardized using the iodometric titration method. Sodium hypochlorite solutions with concentration of 5, 10, and 15% (v/v) active chlorine in alkaline medium (0.05, 0.075, 0.15 and 0.30 mol L^{-1} NaOH) were prepared by appropriate dilution from the stock solution.

A 1.6 mol L^{-1} phenol stock solution was prepared by dissolving 15 g of solid in 100 mL of water. A small portion of solid was observed in the bottom of vessel, indicating a saturated condition. This solution was stored in an amber bottle and maintained in a freezer. A 0.80 mol L^{-1} phenol solution in ethanolic medium was prepared by mixing equal volumes (50 mL) of phenol stock solution and ethanol, which was used to prepare working solutions with concentrations of 0.16, 0.32, 0.40, 0.48, 0.56 and 0.64 mol L^{-1} in alkaline medium (0.05, 0.075, 0.15, 0.30 and 1.0 mol L^{-1} NaOH). Each solution was prepared before use, maintaining the ethanol proportion, and was stored in an amber bottle.

A 0.5 mol L^{-1} sodium hydroxide solution was prepared by dissolving 1.0 g NaOH (Merck) in 50 mL of water. A 0.65 mol L^{-1} HCl solution was prepared by dilution from a 4 mol L^{-1} HCl solution. A 0.4 mol L^{-1} Potassium dihydrogenphosphate solution was prepared by dissolving 10.88 g KH_2PO_4 (Merck) in 200 mL of water. A 0.4 mol L^{-1} potassium hydrogen phosphate solution was prepared by dissolving 13.92 g K_2HPO_4 (Merck) in 200 mL of water. A 0.4 mol L^{-1} phosphate buffer solution (H_2PO_4/HPO_4^{2-}) at pH 7.0 was prepared by placing into a vessel 200 mL of HPO_4^{2-} and stepwising the H_2PO_4 solution up to pH 7.0. A 0.23 mol L^{-1} phenylhydrazine solution was prepared by dissolving 0.1663 g of solid in 50 mL of water. A 0.05 mol L^{-1} potassium ferricyanide solution was prepared by dissolving 0.8396 g of solid in 50 mL of water. This solution and 100 mL concentrated HCl to be used in the analytical procedures were maintained in the freezer at 0 ºC.

Preparation of samples

Leaves of soybean inoculated with *Bradyrhizobium japonicum* obtained from an experimental field were dried in an oven at 60 ºC. The dried material was ground and sieved using a 60 mesh screen.

The ureide extraction was carried out as follows: a 0.5 g amount of the sample material was suspended in a solution medium containing 2.5 mL of ethanol plus 5.0 mL of 0.1 mol L^{-1} phosphate buffer solution (pH 7), which was then heated in a water bath at 80 ºC for 15 min. The mixture was centrifuged and the supernatant was filtered using a Whatman No. 1 paper.[3] Three replicates of 2.0 mL of the extract were selected and the volumes were completed to 15 mL with water and stored in polyethylene bottles.

Cation exchange resin column

The ion exchange resin preparation was done as described by Patterson *et al.*.[7] The column employed to pack the ion exchange resin was machined in acrylic as described elsewhere.[18] The hole for placing the resin presented an inner diameter of 3 mm and a length of 20 mm that was enough to contain a resin amount of about 1 g (Dowex 50WX4-200 hydrogen, Sigma-Aldrich).

Reference procedure

The reference method described below was implemented as proposed by Vogels and Van Der Drift.[5] A 50 µL of the sample extract was transferred to a glass vial (5 mL inner volume) using an automatic pipette. Afterwards, an aliquot of 250 µL NaOH solution (0.5 mol L^{-1}) plus 700 µL of water were added to the glass vial. This mixture was homogenized using a vortex stirrer and was warmed for 8 min using a water bath at 100 ºC. After cooling using an ice bath, 250 µL of a 0.65 mol L^{-1} HCl solution were added to the glass vial and heated again to 100 ºC for 4 min. After cooling using an ice bath, 250 µL of a 0.4 mol L^{-1} phosphate buffer solution (pH 7.0) plus 250 µL of a 0.23 mol L^{-1} phenylhydrazine solution were added to a glass vial, which was mixed using a vortex at room temperature for 5 min. After this step, the glass vial was placed in the ice bath and 1.25 ml concentrated HCl plus 250 µL of a 0.05 mol L^{-1} potassium ferricyanide solution were added to them and mixed for 2 min. Afterwards, the glass vial was removed from the ice bath, and its absorbance read at 535 nm after a delay time of 10 min.

Apparatus

The equipment set up consisted of a microcomputer equipped with a PCL 711S electronic interface card (American Advantech Corp., San Jose, CA, USA), affording facilities to generate the control signals; an IPC-8 Ismatec peristaltic pump furnished with a Tygon pumping tube; a homemade LED-based photometer constituted by a high brightness red LED (λ_{max} = 660 nm) and a photodiode (0PT301, Burr-Brown); a homemade water bath with temperature stabilized at 40 ºC; a homemade boron-silicate glass flow cell with an 100 mm optical path-length and a 1.2 mm inner diameter; seven three-way solenoid valves (HP225T031) NResearch, West Caldwell, NJ, USA); reaction coils; an ion exchange column;[18] joint device machined in acrylic; a homemade bubble-removing device machined in acrylic with an inner volume of 20 µL; an electronic interface to drive the solenoid valves;[19] and homemade regulated power supplies (–12 V, +12 V) 0.5 A and (12 V) 2.0 A to feed the photometer and solenoid valves, respectively. The reaction coil, sampling loop, and flow lines were of Teflon tubing, 0.8 mm inner diameter.

The equipment and accessories used to perform the reference method included a water bath of controlled temperature (Marconi, TE-159), a mini-microcentrifuge (Benchmark, BSC1006-B), a vortex shaker (Tecnal, AP-56), a pH meter (Tecnal, TEC 2mp), an electromagnetic stirrer (TE-085), and a spectrophotometer (FEMTO, 700 Plus).

Flow system assembling and procedure

The photometer was assembled by attaching the radiation source (LED) and the photodetector (Det) to

the observation windows of the flow cell. As depicted in Figure 1, the radiation beam (I) emitted by the LED is transmitted through the flow cell toward the photodetector (Det). After crossing the flow cell window, the radiation beam (II) is collected by the photodetector, which generates a potential difference (mV) that presents a linear relationship with the intensity of the radiation beam II. When a solution into the flow cell absorbs radiation, the intensity of the radiation bean II becomes lower than that of the radiation beam I. Under this condition, the signal generated (mV) by the photodetector is a function of the concentration of the chemical specie into the flow cell.

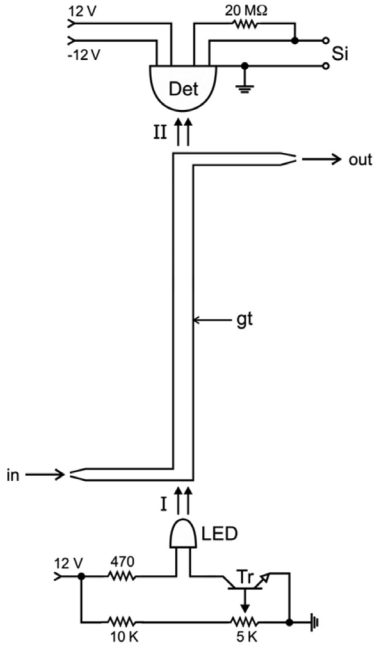

Figure 1. Diagram of the photometer. Tr = transistor (BC547); LED = light emitting diode, λ_{max} = 660 nm; gt = flow cell body, borosilicate glass tube, inner diameter of 1.2 mm; I = radiation beam emitted by the LED; II = radiation beam after cross the flow cell window; Det = photodetector (0PT301); Si = generated sinal (mV); in and out = input and output of solution, respectively.

The flow system manifold shown in Figure 2 was designed to be an active hardware, which, when controlled by a microcomputer furnished with essential electronic interfaces and software, was able to carry out all of the steps of the analytical procedure. The software written in Quick BASIC 4.5 afforded the facilities to individually control the time interval to maintain the ON/OFF switch for all solenoid valves, thus allowing the insertion of the volumes of sample and reagent solutions into the analytical path.

The photometer calibration was the first step to be carried out when the control software was run. The LED was turned off using the variable resistor coupled to the

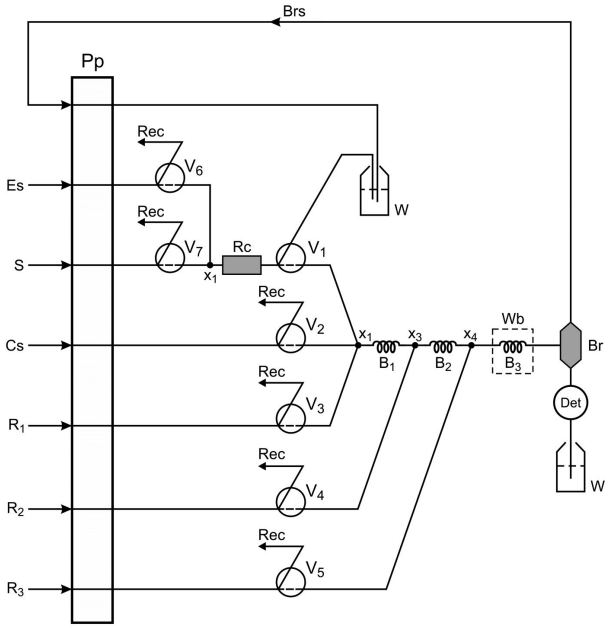

Figure 2. Diagram of flow system. Pp = peristaltic pump; Cs = carrier solution; S = sample or standard solution; R_1, R_2 and R_3 = phthalate solution (0.2 mol L^{-1}, pH 4.0), sodium hypochlorite solution (10 %), phenol solution (0.48 mol L^{-1}), respectively; Es = eluent solution, 0.5 mol L^{-1} NaCl; W = waste; V_1, –V_7 = three way solenoid valves; Rec = circulating solution through their storing vessels; Rc = resin column; B_1, B_2 and B_3 = reaction coil of Teflon tubing, 0.8 mm inner diameter and 25, 50 and 150 cm long, respectively; x_1, x_2 and x_3 = jointing devices machines in acrylic; Wb = water bath at 40°C; Det = LED-based photometer; Br = bubble removing chamber, 20 μL inner volume; Brs = bubble removing stream. Continuous and doted lines in the valves symbols indicate the fluid pathway when valves were switched OFF and ON, respectively. Arrows indicate the pumping direction.

base of the transistor (Tr), and the potential difference (Si) generated by the photometer was read by the microcomputer through the analog input of the PCL711 interface card, which was converted to digital and saved as the dark measurement (Dkm). In the next step, the LED was powered by turning on the variable resistor, and its emission intensity was increased to the signal (full scale measurement, Fsm) generated by the photometer, which attained a value of around 2000 mV. The Dkm and Fsm measurements were saved to be used in the absorbance calculation.

Prior to beginning the solution handling steps, the microcomputer showed on the screen the last values of the control variables, which have been summarized in Table 1. In this stage, their values can be changed. Afterwards, the microcomputer carried out all steps of the analytical procedure following the sequence indicated in the valve timing course shown in Figure 2.

In the flow diagram illustrated in Figure 2, all valves were switched OFF, so that all solutions were pumped toward their storing recipient, except the carrier solution (Cs), which flowed through the reaction coils and detector

Table 1. Sequence of the analytical run

Step	Event	V_1	V_2	V_3	V_4	V_5	V_6	V_7	time / s	Cycle
1	washing column	0	0	0	0	0	0	1	10	–
2	inserting sample	1	1	0	0	0	0	1	$t_1(0.4)^a$	
3	inserting R_1	0	1	1	0	0	0	0	$t_2(0.4)^a$	$n_1(16)^a$
4	inserting R_2	1	1	0	1	0	0	1	$t_3(0.4)^a$	
5	inserting R_3	0	1	0	0	1	1	0	$t_4(0.3)^a$	$n_2(16)^a$
6	displacing sample	0	0	0	0	0	1	0	$t_5(0.4)^a$	
7	insertig eluente	0	0	0	0	0	1	0	100	–

[a]Selected values. The "1" and "0" indicated that the corresponding valve is switched ON and OFF, respectively; n_1 and n_2 number of sampling cycles.

toward waste (W). In the first step, the solenoid valve V_7 was switched ON and the sample solution flowed through the column toward the waste (washing step). Second, third, and fourth steps were performed by sequentially switching the valves V_1, V_3 and V_4 while valve V_2 was maintained switched ON. Under this condition, carrier solution (Cs) circulated through its storing vessel, and aliquots of sample and reagent solutions R_1 and R_2 were inserted into the analytical path. This valve switching sequence is named here as a sampling cycle (n_i), which was repeated several times to insert the required volumes of the sample and reagent solutions.

The insertion of the reagent solution R_3 was performed by switching alternately valves V_2 and V_5. Afterwards, valve V_6 was switched ON for a preset time interval in order for the elution of the cationic specie retained while the sampling step was in course. After ending the seventh step, the software returned to the first step in order to begin the next analytical run.

The volume of each aliquot of solutions inserted into reaction coils was to function at both the pumping flow rate and the time interval preset to maintain the selected solenoid valve switched ON. Flow rates were maintained throughout, and assays to find the appropriated solution volumes to be used were carried out by varying the time intervals (Δt_i) to maintain switched ON the solenoid valves.

The signal generated by the photometer (Si) was read by the microcomputer one time while each valve (V_1, V_3, V_4 and V_5) was maintained switched ON. While the seventh step proceeded, the signal was read maintaining a frequency of 0.5 Hz. The signal was converted to digital by the PCL711 interface card, which was used for the absorbance calculation using the following equation:

$$\text{Absorbance} = \log[(\text{Fms} - \text{Dks})/(\text{Si} - \text{Dkm})] \quad (1)$$

where: Si = signal in mV; Fms and Dks = full scale and dark measurements (mV), respectively, as previously described.

The absorbance was saved in the ASCII standard to allow for further processing. While the analytical run proceeded, a plot of the signal was displayed on the microcomputer screen as a function of time to allow for its visualization at real time.

Results and Discussion

General comments

Because the reaction to form the blue indophenol compound is very slow, a heating step was employed in order to improve the kinetic reaction. Previous assays were carried out using a blank solution and a 200 μmol L^{-1} allantoin standard solution. When the temperature of the bath water was varied from 26 to 40 °C, the blank measurement presented an increase of about 87%, while for the allantoin solution the augment was of 158%. When a temperature higher than 40 °C was tested, an excessive delivering of gas bubbles occurred, merging into the flow cell and impaired the signal. Based on these assays, the temperature of the bath water was maintained at 40 °C.

Variables that exert influence on the sensibility, such as reagent concentration, length of reaction coil (B_3), number of sampling cycles, and time interval for inserting reagent solutions, were investigated in order to find the best operational condition. The results are presented and discussed in the next sections.

Alkaline effect on the reaction development

Since the reaction to form the compound to be detected occurred in alkaline medium,[20] the concentration of sodium hydroxide was the first parameter studied. This was done using hypochlorite and phenol solutions prepared in sodium hydroxide medium. The assays were carried out using both blank and 160 μmol L^{-1} allantoin standard solution, maintaining phenol and hypochlorite solutions

with concentrations of 0.30 mol L^{-1} and 10% (v/v), respectively. Considering the net absorbance (subtracting the blank measurement) as the parameter of interest, the results achieved are shown in Table 2.

Table 2. Alkalinity effect on the analytical signal

NaOH concentration / (mol L^{-1})	Hypochlorite absorbance	Phenol absorbance
0.05	0.142	0.100
0.08	0.176	0.116
0.15	0.432	0.331
0.30	0.012	0.434
1.00	–	0.088

Results average of 5 consecutive measurements.

Analyzing the data shown in Table 2, we can observe that the best results were obtained with sodium hydroxide concentrations of 0.15 and 0.30 mol L^{-1} for hypochlorite and phenol solutions, respectively; therefore, these concentrations were selected to perform further experiments.

Effect of the sodium hypochlorite concentration

In the first stage of the method, ureide (allantoin) reacted with hypochlorite to form chloroamine.[20] In the proposed manifold (Figure 2), this reaction proceeded while the sample zone flowed through the reaction coil B_1. Since the amount of hypochlorite must be enough to allow for a reaction development, assays to find the appropriate concentration were performed, and yielded the results shown in Table 3.

Table 3. Effect of the sodium hypochlorite concentration

Hypochlorite solution / % (v/v)	Absorbance	
	Blank solution	Allantoin solution (160 μmol L^{-1})
5	0.1182 ± 0.0015	0.2956 ± 0.0163
10	0.1334 ± 0.0036	0.5624 ± 0.0522
15	0.1925 ± 0.0031	0.4872 ± 0.0178

Results are average of 5 consecutive measurements.

By analyzing these results, we can deduce that when hypochlorite concentrations varied from 5 to 10%, the corresponding measurements presented augments of 13 and 90% for blank and allantoin standard solutions, respectively. Comparing the results obtained using 10 and 15% hypochlorite solutions, we can deduce that while blank measurement underwent an increasing of 44%, the signal of allantoin standard solution presented a decreasing of 20%, thus indicating that higher hypochlorite concentration impaired the results. Therefore, a 10% hypochlorite solution was selected to carry out further experiments.

Effect of the phenol concentration

The assays to verify the effect of phenol concentration were carried out using the hypochlorite solution concentration selected in the previous section and maintaining the phenol solutions in a medium of 0.30 mol L^{-1} NaOH, yielding the results shown in Figure 3. As we can see, a curve related to the blank solution presented insignificant variation up to a phenol solution concentration of 0.48 mol L^{-1}, while for the allantoin solution, a continuous increasing occurred.

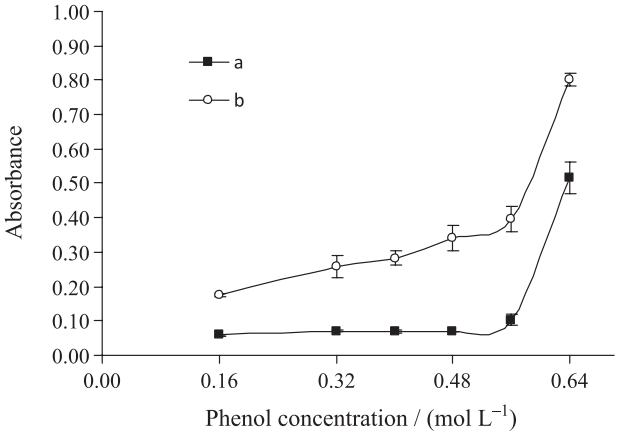

Figure 3. Effect of the phenol concentration. Blank solution (a); 160 μmol L^{-1} of allantoin standard solution (b). Results are average of 5 consecutive measurements.

We can also observe that when a phenol solution with a concentration of 0.64 mol L^{-1} was used, the blank measurement underwent an abrupt increasing that was higher than that generated by the allantoin standard solution. Based on these results, the 0.48 mol L^{-1} phenol solution was selected for further experiments.

Effect of the phenol solution volume

The assays to study the effect of the phenol solution volume on the signal were carried out using time intervals of 0.2, 0.3, and 0.4 s to maintain switched ON valve V_4 (Figure 2) and setting 22, 15, and 11 sampling cycles, respectively, yielding the results shown in Table 4. The total volume of the phenol solution constituting the sample zone was maintained. The slug of sample solution was maintained at 13.2 μL; thus, in the first case (third line), the reagent volume was 50% lower than that of the sample volume. In the third case, the volumes were equal, but the absorbance shown in the third line is 36% lower that

observed in the first case. While the slug volume of the reagent solution increased, the slug volume of the allantoin standard solution was constant and, as a consequence, underwent dilution. In this sense, we could suppose that the dilution effect caused the reduction of the signal.

Table 4. Effect of the phenol solution volume

Phenol solution / μL		Absorbance	
Slug volume	Total volume	Blank solution	Alantoin solution[a]
6.6	145.2	0.065 ± 0.001	0.647 ± 0.028
9.9	148.5	0.057 ± 0.002	0.625 ± 0.020
13.2	145.5	0.064 ± 0.015	0.414 ± 0.024

[a]Alantoin standard solution 200 μmol L^{-1}. Results average of 5 consecutive measurements. The slug volumes of alantoin standard solution and hypochlorite solution both were maintained at 13.2 μL.

Effect of the coil length

The reaction to form the indophenol blue compound to be detected is very slow,[20] thus requiring a long interval time for reaction development. In the flow system, the sample residence time is a function of both flow rate and length of reaction coil (B_3, Figure 2). In the current work, the flow rate was constant, and assays were carried out using reaction coils with lengths varying from 50 up to 400 cm, yielding the results shown in Table 5.

Table 5. Effect of the reaction coil length

Coil length / cm	Absorbance		Signals / ratio (Al / Bl)[b]
	Blank solution	Alantoin solution[a]	
50	0.098 ± 0.003	0.407 ± 0.018	4.17
100	0.052 ± 0.003	0.585 ± 0.032	11.34
150	0.052 ± 0.003	0.633 ± 0.015	12.19
250	0.055 ± 0.002	0.711 ± 0.011	12.95
350	0.055 ± 0.003	0.750 ± 0.033	13.60
400	0.058 ± 0.002	0.623 ± 0.020	10.81

[a]Alantoin standard solution 200 μmol L^{-1}. [b]Ration of signals between Alantoin and Blanck solutions. Results are average of 5 consecutive measurements

Analyzing these results, we can observe a signal increasing up to the reaction coil length of 350 cm. Considering the signals related with reaction coils with lengths of 150 and 250 cm, we can deduce that the signal augment was of 10%, and thus, we can suppose that there was a tendency to attain an equilibrium condition. The decrease observed in the result related to the reaction coil of 400 cm would be attributed to the dispersion effect. The result obtained with the reaction coil of 250 cm was 5% lower than the result obtained with that of 350 cm. Subsequently, this result was chosen in order to increase the sampling frequency.

Removing of the interfering compounds

The phenol reaction with *N*-ureides is not specific; therefore, compounds such as amino acids asparagine, glycine, aspartic acid, arginine, lysine, and histidine that were naturally existent in the soybean tissues[21] become potential interference that could impair the results. Since *N*-ureides did not bind to cationic exchange resin,[7] a column packed with an appropriated ion exchange resin could be used to retain the interfering compounds. Intending to verify this possibility, four samples of plant extracts were treated outside the analytical path using a cation exchange resin. Samples were processed employing the proposed procedure and a reference method, yielding the results shown in Table 6.

It is evident that the results obtained employing the proposed procedure using treated and untreated samples are quite different. Nevertheless, results obtained using treated samples presented a good agreement with those obtained employing the reference method,[5] which was proven by applying the paired *t*-test, and no significant difference at 95% confidence level was observed. Thereby, cationic resin can be considered as an effective resource to be used for interfering removal.

Performance of the proposed procedure

The assays previously described were carried out to select the working variables, which are summarized in Table 7. Intending to verify the performance of the

Table 6. Removal of interfering compounds

Sample	Proposed procedure		Reference method[5]	
	Untreated	Treated	Untreated	Treated
	Removal / (μmol L^{-1})			
A1	132.2 ± 4.8	70.4 ± 1.6	71.7 ± 9.1	71.5 ± 6.9
A2	115.8 ± 6.6	65.2 ± 1.1	65.7 ± 10.5	64.7 ± 6.3
A3	125.5 ± 1.2	55.6 ± 1.5	55.3 ± 6.3	56.7 ± 7.8
A4	110.3 ± 1.5	74.9 ± 2.7	72.0 ± 10.0	73.2 ± 8.1

Results are average of 5 consecutive measurements.

Table 7. Experimental variables

Parameter	Range assayed	Selected value
Phenol solution concentration / (mol L^{-1})	0.16-0.64	0.48
Hypochlorite solution concentration / %	5-15	10
Sodium hydroxide concentration in hypochlorite solution / (mol L^{-1})	0.05-0.30	0.15
Sodium hydroxide concentration in phenol solution / (mol L^{-1})	0.05-1.00	0.30
Phenol solution slug volume / µL	6.6-13.2	9.9
Length of reaction coil / cm	50-400	250

Table 8. Performance comparison

Parameter	Proposed procedure	Reference[7]
Linear range / (µmol L^{-1})	30-260	–
Linear coefficient / r	0.9995	–
Relative standard deviation / %	< 3 (n = 15)	–
Limit of detection / (µmol L^{-1})[a]	1.0	–
Phenol consumption / mg[b]	6.8	22.9
Hypochlorite consumption / mg[b]	0.530	0.434
Phthalate consumption / mg[b]	8.6	10.0
Waste generation / mL[c]	3.3	10.8
Sampling throughput / h^{-1}	36	35

[a]Limit of detection estimated according to the 3σ criterion.[22] The labels [b,c]indicate consumption and waste generation *per* determination, repectively.

proposed procedure, a set of allantoin standard solutions was processed using these variables, yielding the results summarized in Table 8.

Analyzing the results shown in this table, we observed that they are very favorable to the proposed procedure, including the environmental sustainability, considering the use of small amounts of dangerous reagents and generation of a waste volume 70% lower than that of the preferred method.

Results comparison

Once the better operational condition was established (Table 7), a set of soybean tissue extracts was analyzed in order to prove the effectiveness of the proposed procedure. Aiming for accuracy assessment, samples were also analyzed employing an independent method,[5] yielding the results showed in Table 9. Applying the paired t-test between results for a 95% confidence level, the calculated value is $t_{exp} = 0.0343$, while the theoretical value is $t_{tab} = 2.160$, therefore indicating that there is no statistically significant difference between the results.

Table 9. Result comparison. Determination of Allantoin in soybean tissues

Sample	Proposed procedure (n = 3)	Reference method[5] (n = 2)
	µmol of *N*-Ureide	
A1	67.55 ± 0.02	68.22 ± 0.28
A2	27.72 ± 0.03	27.58 ± 0.50
A3	24.55 ± 0.05	25.09 ± 0.55
A4	15.39 ± 0.25	15.11 ± 0.61
A5	31.97 ± 0.12	32.41 ± 0.88
A6	59.64 ± 0.36	59.90 ± 2.71
A7	13.84 ± 0.26	13.41 ± 0.59
A8	47.13 ± 0.26	47.05 ± 0.90
A9	45.12 ± 1.00	44.42 ± 0.67
A10	30.09 ± 0.52	29.45 ± 0.28
A11	40.76 ± 0.10	40.69 ± 0.72
A12	72.00 ± 0.24	72.65 ± 0.55
A13	47.52 ± 0.49	47.41 ± 0.88
A14	40.74 ± 0.10	40.55 ± 1.55

Results corresponding by gram of dry material.

Conclusion

The quality of the results summarized in Tables 7, 8, and 9 allows us to conclude that the overall performance of the proposed system is excellent, thereby indicating that the equipment setup and control software worked very well throughout.

The system operation is fully controlled by the software, thus requiring a low involvement of the analyst. Therefore, this feature could be considered as an advantage, mainly when the use of a hazardous reagent is inevitable.

The proposed procedure presented sensitivity enough to be used for determination of ureide in soybean tissues in laboratories conducting research in soil microbiology and biological nitrogen fixation.

Low consumption of reagents and low volume of waste generation allows us to conclude that the condition should

be considered as a green method, according to the Green Chemistry guidelines that were achieved.

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References

1. Hungria, M.; Franchini, J. C.; Campo, R. J.; Crispino, C. C.; Moraes, J. Z; Sibaldelli, R. N. R.; Mendes, I. C.; Arihara, J.; *Can. J. Plant Sci.* **2006**, *86*, 927.
2. Reynolds, P. H. S.; Boland, M. J.; Blevins, D. G.; Schubert, K. R.; Randall, D. D.; *Plant Physiol*. **1982**, *69*, 1334.
3. Pate, J. S.; Atkins, C. A.; White, S. T.; Rainbird, R. M.; Woo, K. C.; *Plant Physiol*. **1980**, *65*, 961.
4. Sheoran, I. S.; Luthra, Y. P.; Kuhad, M. S.; Singh, R.; *Plant Physiol*. **1982**, *70*, 917.
5. Vogels, G. D.; Van Der Drift. C.; *Anal. Biochem*. **1970**, *33*, 143.
6. Domnas, A.; *J. Biochem*. **1961**, *50*, 46.
7. Patterson, T. G.; Glenister, R.; Larue, T. A.; *Anal. Biochem*. **1982**, *119*, 90.
8. Rocha, F. R. P.; Nóbrega, J. A.; Fatibello-Filho, O.; *Green Chem.* **2001**, *3*, 216.
9. Armenta, S.; Garrigues, S.; de la Guardia, M.; *TrAC, Trend Anal. Chem.* **2008**, *27*, 497.
10. Garrigues, S.; Armenta, S.; de la Guardia, M.; *TrAC, Trend Anal. Chem.* **2010**, *29*, 592.
11. Soto, N. O.; Horstkotte, B.; March, J. G.; de Alba, P. L. L.; Martinez, L. L.; Martin, V. C.; *Anal. Chim. Acta* **2008**, *611*, 182.
12. Melchert, W. R.; Rocha, F. R. P.; *Talanta* **2010**, *81*, 327.
13. Rocha, F. R. P.; Reis, B. F.; Zagatto, E. A. G.; Lima, J. L. F. C.; Lapa, R. A. S.; Santos, J. L. M.; *Anal. Chim. Acta* **2002**, *468*, 119.
14. Borges, S. S.; Peixoto, J. S.; Feres, M. A.; Reis, B. F.; *Anal. Chim. Acta* **2010**, *668*, 3.
15. Lavorante, A. F.; Morales-Rubio, Á.; de la Guardia, M.; Reis, B. F.; *Anal. Chim. Acta* **2007**, *600*, 58.
16. Infante, C. M. C.; Masini, J. C.; dos Santos, A. C. V.; *Microchem. J.* **2011**, *98*, 97.
17. Ruzicka, J.; Marshall, G. D.; *Anal. Chim. Acta* **1990**, *237*, 329.
18. Zarate, N.; Perez-Olmos, R.; Reis, B. F.; *J. Braz. Chem. Soc*. **2011**, *22*, 1009.
19. Rodenas-Torralba, E.; Rocha, F. R. P., Reis, B. F.; Morales-Rubio, A.; de la Guardia, M.; *J. Autom. Method Manag. Chem*. **2006**, 1.
20. Searle, P. L.; *Analyst*, **1984**, *109*, 549.
21. McClure, P. R.; Israel, D. W.; *Plant Physiol*. **1979**, *64,* 411.
22. Long, G. L.; Winefordner, J. D.; *Anal. Chem*. **1983**, *55*, 712.

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