

Flow injection system for hydrolysable tannin determination

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Abstract

A flow injection system was proposed to evaluate the transient product of a colorimetric reaction between hydrolysable tannin and potassium iodate (KIO₃) solution. The system optimization was accomplished by using statistical methods based on experimental design. Flow rate of KIO₃ solution, sample volume, carrier flow rate, and reaction coil were the selected factors for evaluation. On screening step, complete factorial 2⁴ was used and two levels for each selected factor were studied. For the optimization phase, a centered face composite design 2² + star was employed to evaluate sample volume and flow rate of KIO₃ solution, which were the factors identified in the screening phase as having more influence on the absorbance signal. After optimization, the proposed system was compared with batch determination. Some characteristics, such as analytical frequency, reagent consumption and chemical residues generation presented better results by the use of the proposed system if compared with batch method. The system presented good repeatability with standard deviation lower than 3%, for $n=10$, linearity ($R^2=0.9974$) for tannic acid standard, analytical frequency of 15 injections h⁻¹ and limit of quantification of 24 mg L⁻¹ of tannic acid. Good results were obtained when the proposed system was applied to hydrolysable tannin determination in *Stryphnodendron barbatimão*, *Eucalyptus citriodora* and *Phyllanthus niruri*, samples of plants commonly used in popular medicine.

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1. Introduction

Tannins are high molecular polyphenolic compounds produced by secondary plant metabolism. They are divided into two main classes: condensed and hydrolysable tannins [1,2].

Hydrolysable tannins are formed by a carbohydrate core, usually D-glucose, whose hydroxyl groups can present ester bonds with phenolic groups [3]. Natural hydrolysable tannins can be named as ellagitannins, when only ellagic acids are the polyphenolic groups bonded to carbohydrate core. Gallotannins occur when only gallic acids are bonded to the carbohydrate core, and mixed tannin when both, ellagic and gallic acids are bonded to the same carbohydrate core [4].

Natural hydrolysable tannins occur in wood, bark, leaves, fruits and galls [4–6] and they show different nutritional, eco-

logical and medicinal effects [7,8]. Considering that ecological and nutritional studies may require analysis of hundreds of samples, it is impractical to characterize each individual compound as ellagitannin or gallotannin [9]. On the other hand, total hydrolysable tannin determination can be very useful and provide enough information for situations such as nutritional, plant medicinal potential evaluation and ecological studies. Therefore, developments of simple methods for hydrolysable tannin determination can be very useful.

In 1965, Haslam described that the reaction between hydrolysable tannin and KIO₃ resulted in a colored product [10]. Otherwise, this reaction only appeared for analytical determinations in 1977, reported by Bate-Smith [11]. From this report, many other modifications have been proposed in an attempt to apply this reaction as analytical device for hydrolysable tannin colorimetric determination [12]. The reaction between hydrolysable tannins and KIO₃ produces a transient colored compound (Fig. 1). When the reagents are mixed together, a red colored product is developed. The color reaches their maximum

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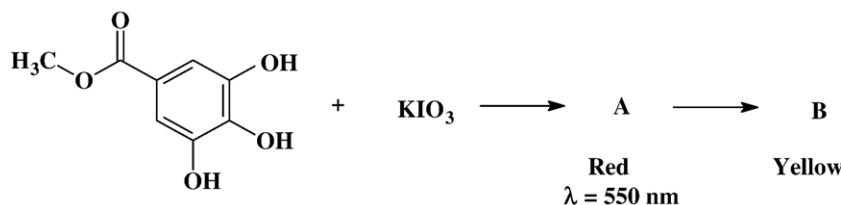


Fig. 1. Reaction between hydrolysable tannin and KIO_3 .

in a space of time and then it starts to disappear to generate a yellow product.

The previous traditional method for hydrolysable tannin determination based on the KIO_3 reaction recommends to chill reagents mixture before the measurement of the colored product, at 550 nm [13]. However, additional research in this area has indicated that, opposite to the chilling, high temperatures increase the reaction speed [11,12].

The flow systems present an intrinsic kinetic characteristic [14]. All the measurements are made at the same time and dispersion conditions for analytical standards and samples, without requiring reaction completion. Moreover, flow system can be considered as a “quasi” closed system, reducing exposure to chemical solutions and environmental contamination problems, due to a reduction in generated residues [14].

Every proposed flow system for replacing a batch analytical method must be optimized. Experimental design for optimization is a chemometric tool used to examine systematically problems that arise within research, development and production. With experimental design interesting information can be obtained, because the experiment is not performed randomly, but is planned [15]. Chemometric techniques were introduced in the analytical chemistry almost at the same time as the flow injection analysis, and the increased usage of both has revealed similar tendencies [16]. Experimental design is a chemometric strategy that works with many factors simultaneously, which can influence a system response. Therefore, it is possible to evaluate the factors that have presented significant influences on the system response, and if these influences are negative or positive [17,18].

The aim of this work was to build and evaluate a suitable assay for hydrolysable tannin level determination in plants commonly used as medicines applying chemometric devices to proposed assay optimization.

2. Materials and methods

All solutions were prepared from analytical grade reagents and distilled–deionized water was used throughout.

The reagent KIO_3 solution (2.5% w/v) was prepared by dissolution of 25 g of KIO_3 in 1000 mL of water.

The extraction solution, used for sample preparation, was acetone 70% (v/v), which was prepared by diluting 70 mL of pure acetone in 100 mL of water.

The stock analytical solution of 5000 mg L^{-1} was prepared by solubilization of 0.25 g of tannic acid in 50 mL of extraction solution. The analytical standard solutions containing 0, 100, 200, 400, 600, 800 and 1000 mg L^{-1} of tannic acid were prepared by aqueous dilution of the stock analytical solution.

The evaluated samples were species of plants which have medicinal properties: *Stryphnodendron barbatimão*, *Eucalyptus citriodora* and *Phyllanthus niruri*. The plants were harvested and immediately washed in common water flow to remove dust particles. Afterwards, the samples were rinsed with distilled–deionized water, frozen in liquid nitrogen ($-196 \text{ }^\circ\text{C}$) and freeze dried in a lyophilizer (Savant model Novalyphe NL 150). In addition, dried samples were ground in a cryogenic mill (Spex Certiprep model 6750).

Moreover, a mass of 0.25 g of ground samples (dry matter) was mixed with 15 mL of extraction solution (acetone 70% v/v) and the mixture was shaken for 10 min in an ultrasonic bath (Unique, model USC 1400, 81 W of power and 40 KHz of frequency), followed by 10 min of centrifugation at 2000 rpm. The final extracts were five times diluted with water, before determination.

A basic flow system was developed to permit the on-line reaction between hydrolysable tannin and KIO_3 solution. The flow setup comprised a model ISM 761 peristaltic pump (Ismatec, Switzerland) with viton pumping tubes, commutate injector [19],

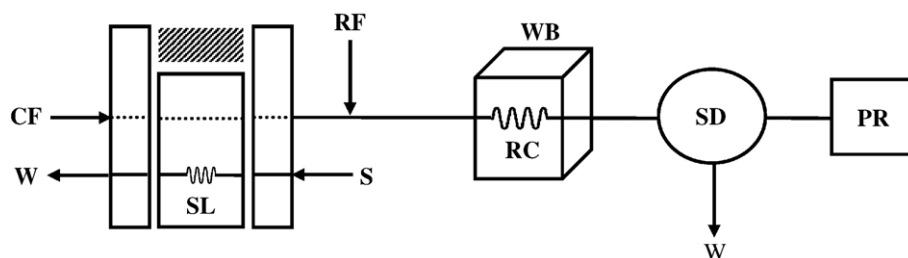


Fig. 2. Proposed flow system: S, Sample; SL, Sample Loop ($350 \mu\text{L}$); CF, Carrier Flow (water, 0.30 mL min^{-1}); RF, Reagent Flow (KIO_3 2.5% (m/v), 0.88 mL min^{-1}); RC, Reaction Coil ($500 \mu\text{L}$); WB, Water Bath ($T=30 \text{ }^\circ\text{C}$); SD, Spectrophotometric Detector ($\lambda=550 \text{ nm}$); W, Waste; PR, Potentiometric Recorder.

Table 1
Factors levels studied on screening phase (2^4 factorial design)

Factors	Low level (-1)	High level (+1)	Unity
KIO ₃ flow rate	0.88	6.0	mL min ⁻¹
Carrier flow rate	0.30	3.4	mL min ⁻¹
Sample volume	50	250	μL
Reaction coil	500	1500	μL

Table 2
Factors levels studied on optimization phase (2^2 +star design)

Factors	Low level (-1)	Central level (0)	High level (+1)	Unity
KIO ₃ flow rate	0.62	0.88	1.14	mL min ⁻¹
Sample volume	150	250	350	μL

a model 432 spectrophotometer (FEMTO, S. Paulo, Brazil) with a tubular flow cell (inner volume *ca* 80 μL, optical path 12 mm), a model BD111 stripchart recorder (Kipp and Zonen, Delft Holland), and a water-bath (Fanem, Brazil). The manifold was built up with 0.8 mm i.d. polyethylene tubing of a non-collapsible wall type and perspex Y-shaped connectors. The basic performance of the proposed flow system, depicted in Fig. 2, was optimized using an experimental design.

For system optimization a tannic acid solution of 500 mg L⁻¹ was used. In the screening phase, a 2^4 factorial design was applied to explore the KIO₃ and carrier stream flows rate, sample volume and reaction coil factors. The two levels studied for each factor are showed in Table 1.

During optimization phase, a centered face composite design 2^2 + star was applied to evaluate sample volume and KIO₃

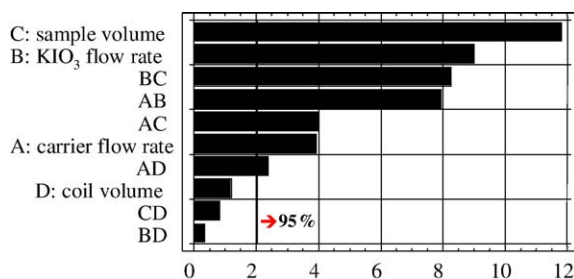


Fig. 3. Pareto's chart for absorbance values determined after reaction between KIO₃ and 500 mg L⁻¹ of tannic acid.

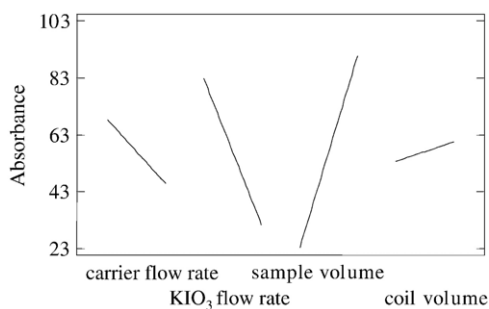


Fig. 4. Main effects plot for absorbance value determined after reaction between KIO₃ and 500 mg L⁻¹ of tannic acid.

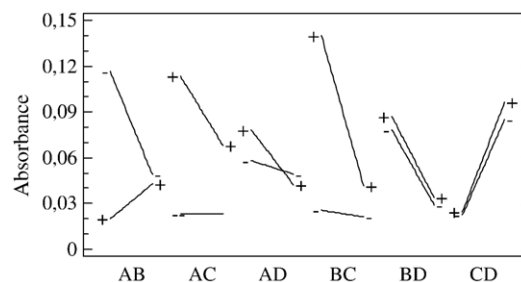


Fig. 5. Interaction plot for absorbance value determined after reaction between KIO₃ and 500 mg L⁻¹ of tannic acid (A: carrier flow rate, B: KIO₃ flow rate, C: sample volume, D: coil volume).

solution flow rate factors. The studied levels are showed in Table 2.

The software Statgraphics plus for Windows 3.0 was used to analyze the data from experimental designs.

The same analytical standards and sample solutions submitted to optimized flow system were also submitted to the Willis and Allen batch method, only changing the temperature proposed by them [12]. The batch method consisted of heating 5 mL of KIO₃ solution (2.5% w/v) for 7 min at 30 °C, followed by the addition of 1 mL of sample solution. The mixed solution was then submitted to 2 min more of heating at 30 °C, before spectrophotometric determination at 550 nm.

3. Results and discussions

The main aim of this work was to develop an easy, simple and low cost method for hydrolysable tannin determination, which could be useful in nutritional, medicinal and ecological studies. The colorimetric reaction between hydrolysable tannin and KIO₃ solution, already used by Willis and Allen [12] in a batch method, appeared to be perfect for this aim. Moreover, the reaction between hydrolysable tannin and potassium iodate is considered the most suitable method for ellagitannin and gallo-tannin determinations [8].

The automation of the reaction was determined as the first step of these studies. In the preliminary step, temperature reaction was evaluated. According to Willis and Allen [12], temperatures higher than 25 °C could be advantageous if an automated system is used. Thus, 30 °C was applied as the starting point of this study, as it was already used by Hartzfeld et al. [9]. Other temperatures further 30 °C were evaluated, but during the experiments

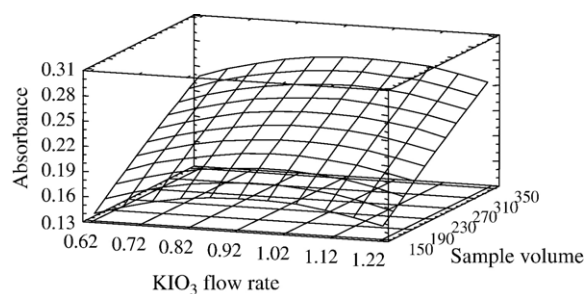


Fig. 6. Estimated response surface for absorbance value determined after reaction between KIO₃ and 500 mg L⁻¹ of tannic acid.

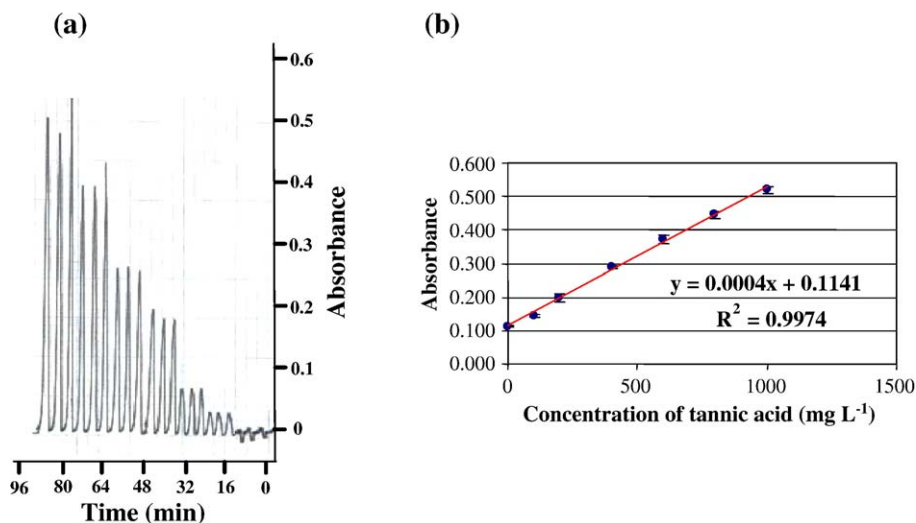


Fig. 7. (a) Recorder output for analytical curve: from the right to left triplicates injection of solution, 0, 100, 200, 400, 600, 800 and 1000 mg of tannic acid per liter of solution. (b) Analytical curve plot and its fit linear.

temperatures higher than 30 °C produced bubbles in the polyethylene tubes, especially in the reaction coil. Consequently, it was observed as a disturbance in analytical signal stability. Therefore, the temperature was fixed at 30 °C.

Moreover, the other factors considered in the study, which could interfere with the red color intensity of the reaction product were sample carrier flow rate (A), KIO₃ solution flow rate (B), sample volume (C), and volume of coil reaction (D). For optimization of these parameters, multivariate methodology was used because this kind of optimization methodology permits the evaluation of which factors present significant influences on the system response, and if the influence is negative or positive. The simultaneous variation of factors also permits evaluation of interaction effects among the studied factors. It is impossible to evaluate interacting factors using one-factor-at-a-time methodology for a system optimization [17,18]. In this way, the parameters were simultaneously varied, in a total of 16 experiments carried out in triplicates, considering two levels for each one. The absorbance value was the parameter monitored as system response. The Pareto chart (Fig. 3) shows that at 95% confidence level the individual factors sample volume (C), KIO₃ flow rate (B) and carrier flow rate (A), were, in this order, the most important factors on system response.

It is also clear that the interactions between sample volume and KIO₃ flow rate (AB), KIO₃ flow rate and carrier flow rate (BC), carrier flow rate and sample volume (AC) and carrier flow rate and coil reaction volume (AD), were the most influential factor interactions on system response. To evaluate the influence of individual factors and interactions, the plot of the main effects and the interactions plot were considered (Figs. 4 and 5 respectively).

In the main effects plot (Fig. 4) it can be observed that, however carrier flow rate (A) and KIO₃ flow rate (B) had a considerable effect on the absorbance value, these are negative influences, that is, in so far as A or B increases, the absorbance value decreases. As the minimum level studied for A was small (0.30 mL min⁻¹), it was judged inappropriate to study the lower

flows rate, considering the damages to analytical frequency. In this way, the optimal condition for factor A was fixed at its minimum level studied (0.30 mL min⁻¹). However, it was judged very important to evaluate the factor B (KIO₃ flow rate) again.

The interaction plot (Fig. 5) shows the interactions between the two factors when the first was fixed in the center of the studied levels. The negative bar represents the minimum level, while the positive bar represents the maximum level studied for the second interacting factor. In AB interaction, the factor A was fixed in 1.85 mL min⁻¹, in the center of 3.4 and 0.30, maximum and minimum studied levels respectively. As can be observed, in this situation, factor B (KIO₃ flow rate) has a great influence on absorbance value, at its minimum level, owing the negative bar is greater than the positive one. It confirms the negative influence of factor B with regard to the absorbance value observed in the main effects plot (Fig. 4). As discussed earlier, factor B needed to be evaluated again, so that this new evaluation may

Table 3
Hydrolysable tannin content determined in *Stryphnodendron barbatimão*, *Eucalyptus citriodora* and *Phyllanthus niruri* plants species by batch and flow system proposed method

Sample	Hydrolysable tannin (g kg ⁻¹)	Hydrolysable tannin (g kg ⁻¹)	F-test	t-test
	Batch method	Proposed flow method		
<i>Eucalyptus citriodora</i> (wood)	199 (±21) ^a	191 (±17)	1.5 NS ^b	0.42 NS
<i>Eucalyptus citriodora</i> (leaves)	93 (±22)	107 (±24)	1.2 NS	0.61 NS
<i>Stryphnodendron barbatimão</i> (wood)	70 (±8.5)	46 (±3.5)	5.9 NS	3.7 NS
<i>Phyllanthus niruri</i> (leaves)	134 (±1.6)	154 (±2.6)	2.6 NS	9.1 NS

^a Standard deviation for n=2.

^b Not significative.

consider lower levels, rather than the minimum studied in this experimental design.

The BC interaction (Fig. 5), plotted for factor B (KIO_3 flow rate) fixed at 3.44 mL min^{-1} , demonstrated that factor C (sample volume) influences greatly the increase of absorbance value, at its maximum studied level (the positive bar is greater than the negative). Therefore, the factor C also needed to be evaluated again. For AC interaction, it can be observed that when factor A was fixed at 1.85 mL min^{-1} , factor C (sample volume) influence is greater for the maximum level of C ($250 \mu\text{L}$). It confirms again the need to evaluate factor C in higher levels than the maximum studied here.

The last interaction highlighted as relevant at 95% confidence level was AD. When A (carrier flow rate) was fixed in 1.85 mL min^{-1} , factor D (reaction coil volume) did not present great influence at its maximum level ($1500 \mu\text{L}$). However factor A was studied again and, as factor D did not reveal influence as individual factor, it was fixed at its minimum level, aiming to increase the analytical frequency of the system.

Based on previous results, factors B (KIO_3 flow rate) and C (sample volume) were optimized. A surface response methodology, based on centered face composite design $2^2 + \text{star}$ was used for this aim. In Fig. 6, a response surface obtained by absorbance values from 10 experiments carried in triplicates is depicted.

The results show the best conditions for the evaluated factors. These are obtained by fixing as 0.88 mL min^{-1} KIO_3 flow rate (B) and $350 \mu\text{L}$ the sample volume inputted on system (C). Therefore, with these experiments it was possible to establish the system configuration, described in the legend of Fig. 2.

The optimized flow system was then evaluated, and it seemed suitable to be applied for hydrolysable tannin determination in plants. This system presented an analytical frequency of 15 injections per hour, a linear range up to 1000 mg L^{-1} of tannic acid ($R^2=0.9974$), good reproducibility and repeatability (RSD 3%, $n=10$) without memory effects (Fig. 7), and a limit of quantification of 24 mg L^{-1} of tannic acid.

Besides the common characteristics of flow system outlined by Ruzicka and Hansen [14], such as reagent economy and lower residues generation, the ability to get all measurements in the same period of time after reaction, was the key point of this method. As KIO_3 and hydrolysable tannin reaction presents a transient colored product [12], in the batch method it is very difficult to control the time for each measurement. Therefore, errors are always introduced [20]. These errors can cause decrease in linear range and repeatability due to the minimal differences of time among each determination.

The proposed method validation was made by use of addition and recovery procedure. With an addition of 200 mg L^{-1} of tannic acid to a sample, 97% of recovery was obtained, after determination in proposed flow system.

After optimization and characterization, the proposed flow system was applied to determine hydrolysable tannin concentration in three species of plants, commonly used in popular medicine, *S. barbatimão*, *E. citriodora* and *P. niruri*. The results obtained using the proposed flow system were compared with those obtained by batch method, described by Willis and Allen

[12], where the temperature employed was $30 \text{ }^\circ\text{C}$ instead of $25 \text{ }^\circ\text{C}$. This modification was done with the aim of maintaining the same conditions for the two compared methodologies and the obtained results are described in Table 3.

As can be observed, the paired *F*-test shows that at 95% confidence level, there is accordance between the results obtained by both, batch and proposed flow method. To compare the results pointed out as not significative by *F*-test, the medium comparison *t*-test was applied. The *t*-values also show that the results obtained by both methods agree at 95% confidence level.

4. Conclusions

The proposed system presented good characteristics to be applied to hydrolysable tannin determination in plants. The possibility of on-line determination increases the analytical frequency and produces results free of errors related to time of measurements, characteristic of batch methods. The use of chemometric approach for optimization allows a better system manifold with relative small number of experiments.

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