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A Simple and Accurate Procedure for the Determination of Tannin and Flavonoid Levels and Some Applications in Ethnobotany and Ethnopharmacology

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ABSTRACT

In this work, we present protocols for the determination of tannin and flavonoid levels in plant extracts and their application in ethnobotanical and ethnopharmacological studies, developed from the investigations of our research group. In addition, we test the application of these protocols to provide analysts with an understanding of the variables that might affect the quality of their results, from literature data and from our researches, for example, the effect of temperature and solvent in the flavonoid content in *Bauhinia cheilantha*. We also discuss the necessity of validating of these methods in order to obtain robust and reproducible results. We exemplify this with the procedure for flavonoid validation for the cited species.

Keywords: Folin-Ciocalteu, phenolic compounds, validation

INTRODUCTION

Spectrophotometric methods for the determination of tannin and flavonoid levels in plant extracts are very popular for their speed and practicality, making them useful for various studies and techniques. Although, there is a vast literature in which these methods are presented, the reproduction of protocols often becomes difficult for a number of reasons: the authors do not explicitly discuss the difficulties and the adjustments that need to be made, the stages are not presented in a step by step manner or clearly enough to permit reproduction, and the interpretative limits (theoretical and methodological), are not discussed in the articles. Even under the best conditions, the implementation of a protocol might result in the loss of precious time during the investigation, as well as a waste of resources. Additionally, the plants used in these experiments present intra and inter-specific differences in their chemical constitution, and different factors such as seasonality, circadian rhythm, radiation, temperature, altitude and humidity, might modify their secondary metabolism, interfering quali and/or quantitatively in the production of active compounds (Gobbo-Neto and Lopes 2007). Because of these factors, the analytic methodologies should be adjusted and properly evaluated, keeping in mind the multitude of possible interferers in the results.

Spectrophotometric methods are highly sensitive, which often limit their robustness and reproducibility. In this work, we present the investigative protocols that our research group has adapted for the study of tannin and flavonoid levels. The originality of our contribution is to point out some of the main obstacles that analysts may face in the implementation of similar protocols in their laboratories, as well as to provide information that is not readily available in the vast literature. This manuscript is divided into three parts: preparation of the samples for analysis, protocols for the determination of tannin and flavonoid levels, and method validation. To ensure the clarity of our explanation, we have chosen data from investigations that our group has conducted in the semi-arid region of the Brazilian northeast. Additionally, we comment on how the protocols presented here might be used in ethnobotanical and ethnopharmacological studies.

TANNIN AND FLAVONOID LEVELS IN ETHNOBOTANICAL STUDIES

Phenolic compounds might be responsible for a wide range of therapeutic indications attributed to plants by local communities. Some of these activities include anti-inflammatory, cicatrizing, antifungal and antioxidant actions (Zuanazzi and Montanha 2004). Many plants are widely collected because of the presence of these compounds, especially the tannins. The stem barks of Brazilian semi-arid plants, for example, are commonly extracted in order to meet a demand from both the local communities and the small and medium pharmaceutical industries. The following protocols might find use in ethnobotanical investigations applied to biodiversity conservation. We indicate them as follows:

1. Identification of local succedaneum, with good levels of these extractives in order to propose collection and management strategies through, the substitution of species that suffer any kind of threat. Ethnobotanical investigation might be useful in the revelation and indication of these plants, as much as in the evaluation of the receptivity of possible succedaneum in the researched communities or cultures.
2. Effects of the extraction techniques and management strategies upon the levels of these compounds. For instance, after the removal of a plant's stem barks, will the regenerated tissue present the same levels as it did before the extractive damage?
3. Evaluation of the possible substitution of parts of the plants. For instance, the rural communities of Caatinga (Northeast of Brazil) tend to extract stem bark for the

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Protocols Paper

Table 1 Medicinal plants collected in the Caatinga region of the Brazilian Northeast and analyzed with respect to their tannin (T) and flavonoid (F) levels (% w/w), modified from Araújo *et al*. (2008). Average ± Standard deviation

Scientific name/Family	Local name/organ	T (%)	F (%)
Myracrodruon urundeuva Allemão / Anacardiaceae	aroeira/bark	13.58 ± 0.30	0.75 ± 0.02
Anadenanthera colubrina (Vell.) Brenan / Mimosaceae	angico/bark	13.46 ± 0.13	0.34 ± 0.02
Ocimum campechianum Mill. / Lamiaceae	alfavaca/aerial parts	5.04 ± 0.03	2.21 ± 0.09
Spondias tuberosa Arruda / Anacardiaceae	umbu/bark	4.82 ± 0.04	0.88 ± 0.10
Ocimum gratissimum L. var. *macrophyllum* Briq. / Lamiaceae	louro/leaves	3.51 ± 0.10	1.99 ± 0.04
Maytenus rigida Mart / Celastraceae	bom nome/bark	3.27 ± 0.10	0.12 ± 0.03
Hyptis suaveolens (L.) Poit. / Lamiaceae	alfazema/leaves	2.71 ± 0.20	2.56 ± 0.05
Tabebuia impetiginosa (Mart. ex DC.) Standl. / Bignoniaceae	pau d'arco roxo/bark	1.18 ± 0.09	0.13 ± 0.03
Plumbago scandens L./ Plumbaginaceae	louco/leaves	0.81 ± 0.02	2.95 ± 0.07
Tephrosia purpurea (L.) Pers./ Fabaceae	sena/aerial parts	0.52 ± 0.01	8.78 ± 0.20
Cereus jamacaru DC / Cactaceae	mandacaru/cladode	0.15 ± 0.01	0.12 ± 0.08

preparation of local medicines. Could the stem barks be substituted for other parts of the plants?

Other questions could certainly be broached, but the ones mentioned above illustrate the potential of using simple techniques in order to test hypotheses in ethnobotany, ethnopharmacology and chemical ecology. The reader can learn more about these approaches by consulting the works of Monteiro *et al*. (2006a, 2006b) and Araújo *et al*. (2008). The following sections present the protocols used in the above-mentioned processes.

PREPARATION OF THE SAMPLES

Delineation of sample concentration

The first step in a analysis is the construction of a calibration curve to represent the proportional relationship between the concentration of the analyte in the sample and, the response of the equipment. This curve must have at least six determinations. After a visual linear relation of the graphically plotted data, a statistical analysis must be performed in order to define the coefficient of determination and the regression equation. In the analysis, we used the concentration range cited in the experimental protocol for the construction of the calibration curve comprising the concentrations of the samples studied by our group. The extracted aliquot, for both total and residual phenols, may vary from species to species (**Table 1**), and may not be found within the limit of the analytic curve. We suggest the utilization of lower extract aliquots for species with high tannin levels, regarding the dilution used for the final calculation. In **Table 1**, the tannin and flavonoid levels for different studied species are presented, illustrating the individual variations.

Stabilization

An important step that precedes the quantitative analysis is the process of sample stabilization; that is, after collection the plant material must receive a treatment to stop the enzymatic action and, thus, avoid the structural modification of substances originally present in the plant. Material stabilization by heating is the most adequate for posterior quantification because, in addition to dehydrating and blocking the hydrolysis reaction and the microbial growth, it allows storage for long periods, leaving the constituents unchanged (Falkenberg *et al*. 2004). The out of doors drying is more economic, nevertheless, it requires more attention to produce homogeneity during sample drying. The use of stoves is a more expensive option, but beneficial for process efficiency and control.

A study performed with *Bauhinia cheilantha* (Bongard) Steudel, showed that different drying temperatures (25, 40, 60 and 80°C), had a strong influence on flavanoid levels. Although, drying at temperatures up to 60°C is a viable method, there might be a reduction in the flavonoid content at higher temperatures (**Fig. 1**).

Extraction process

Several factors need to be carefully studied before extraction can take place. These include the granulometric standardization of the sample and the plant organ collected, as well as the methodologies and solvents, that will be employed for the extraction. The lower the sample granulometry, the higher the efficiency of the extraction, as a lower granulometry will increase the contact area between the solvent and the particle (Falkenberg *et al*. 2004). The diverse organs of the plant have very different anatomic structures, mainly regarding the degree of cellular organization. In stems and roots that have lignified tissues, the extraction process is harder than in leaves and flowers that had smaller

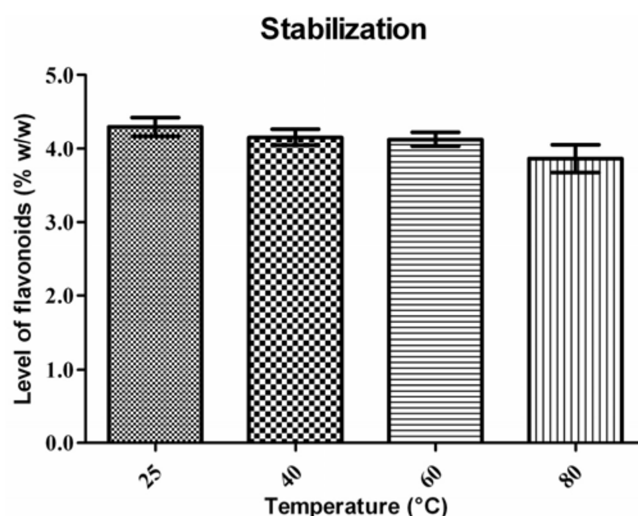

Fig. 1 Levels of foliaceous flavanoids of *Bauhinia cheilantha* (Bongard) Steudel, submitted to different drying temperatures.

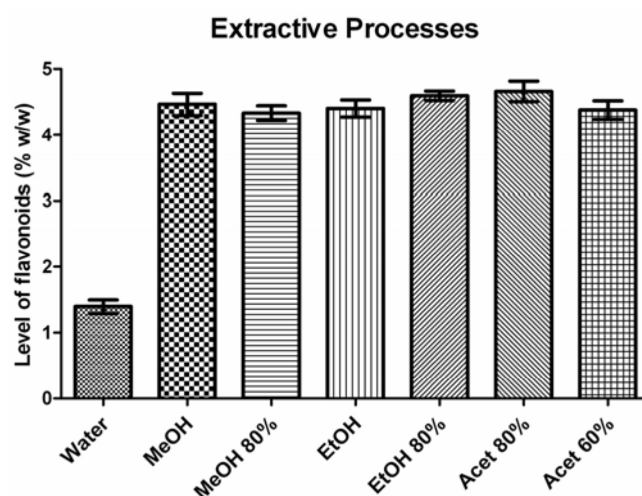

Fig. 2 Representation of the efficiency of different extractive processes for foliaceous flavanoids of *Bauhinia cheilantha* (Bongard) Steudel.

amounts of lignin (Falkenberg *et al*. 2004).

Perhaps the main dilemma faced in an extraction process is the choice of the solvent or the most adequate system to carry it out. Generally, for phenolic compounds, the extraction is made through organic solvents (ethanol, methanol and acetone), or through a mixture of organic solvents with water, in order to optimize the process and increase the profit (Oliveira 2005). However, there is great difficulty in establishing the most efficient solvent or extractive system, as many factors, such as the analyzed species, the physico-chemical properties of the substances, the extraction time and temperature, the toxicity and the solvent availability and cost, are involved (Falkenberg *et al*. 2004; Andreo and Jorge 2006).

The results obtained for extraction through flavonoid decoction of the pulverized leaves of *Bauhinia cheilantha* (Bongard) Steudel, by different extractive systems are shown on the **Fig. 2**. The extraction method with 80% acetone was more efficient than with 80% ethanol. Nevertheless, the use of acetone was more expensive.

DETERMINATION OF TOTAL TANNINS AND PHENOLS

Present in the majority of the plants, the tannins are phenolic compounds that are soluble in water and have a molecular weight between 500 and 3000 Daltons. They are also able to form water-insoluble complexes with proteins and alkaloids (Santos and Mello 2004). Because of this capacity, these compounds are responsible for the astringency of many plant products (Santos and Mello 2004; Monteiro *et al*. 2005).

The tannins are classified, according to their biosynthetic origin, into two groups: the hydrolysable tannins, found in woody and herbaceous dicotyledons, which are characterized by an esterified glycosidic nucleus with gallic acids (gallotannins), and ellagic acids (ellagitannins), formed from the shikimate metabolic route and the condensed tannins or proanthocyanidins, that occur mostly in gymnosperms and angiosperms and are polymers of the flavan-3-ol and/or flavan-3,4-diol, derived from phenylpropanoid metabolism (Bruneton 1991; Santos and Mello 2004).

We can divide the analytic methods of these compounds into colorimetric and protein precipitation. According to Hagerman (1987), the first has the disadvantage of being very specific, like the proanthocyanidin and valine assays, or not being selective for tannins, like the Folin-Denis assay. For the protein precipitation assays, the same author reports that despite being specific, they require many steps, request special material or use volatile or thermosensitive substances that change the concentration of the analyzed extract.

Many methodologies for dosing tannins have been developed (see, for example, Verza *et al*. 2007). Reviews and traditional methods about tannin compounds can be found in Mueller-Harvey (2001) and Schofield *et al*. (2001). Among the related methods, the Folin-Ciocalteu is the most used, as it is more practical and less expensive, despite being chemically complex and reacting with phenols.

Experimental protocol for the Folin-Ciocalteu method

Equipment, glasswear, reagents and reference compound

In order to determine total phenols and tannins, the analyst must have: an analytic balance, a hot plate, a spectrophotometer, quantitative filter paper, a pulverizer, volumetric balloons of 25, 50 and 100 mL, an Erlenmeyer flask of 50 mL, glass funnels, graduated pipettes of 1, 2 and 5 mL, volumetric pipettes of 1, 5 and 10 mL, distilled water, 80% methanol (v/v), an aqueous solution of sodium carbonate (Na_2CO_3), 75.0 mg/L, an aqueous solution of 10% Folin-Ciocalteu phenol reagent (v/v) and tannic acid.

Pattern preparation for tannic acid

Prepare an aqueous solution of tannic acid, 0.1 mg/mL. Remove six aliquots of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL and transfer to 100 mL volumetric balloons. Add 5 mL of the Folin-Ciocalteu phenol reagent, 10 mL of the aqueous solution of sodium carbonate and complete the volume with distilled water. Wait 30 min (with standard temperature and lighting) and read each sample in the spectrophotometer at 760 nm. After the dilutions, the final concentrations will be 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µg/mL, respectively. Use distilled water as a blank solution.

Extract preparation

Weigh 500 mg of the dry plant sample (amount optimized by our group), pulverized and standardized; transfer to a 50 mL Erlenmeyer flask, add 25 mL of 80% methanol and heat through mild ebullition for 30 min. Filter into a 50 mL volumetric balloon. To avoid loss, the residue must be washed with 25 mL of 80% methanol. Complete the volume with 80% methanol. Repeat until triplicate measurements are made.

Total phenol quantification

Transfer 1 mL of the sample, recently prepared, with a volumetric pipette to a 100 mL volumetric balloon containing 50 mL of distilled water. In a manner similar to that employed for the construction of the curve, add 5 mL of the Folin-Ciocalteu phenol reagent, 10 mL of the aqueous solution of sodium carbonate and complete the volume with distilled water. Wait 30 min (with standard temperature and lighting) and read the samples in the spectrophotometer at 760 nm.

Residual phenol quantification

Weigh 1.0 g of casein and transfer to a 50 mL Erlenmeyer flask, adding 6 mL of the sample and 12 mL of distilled water. Keep under mechanical agitation in an agitation table for 3 h at room temperature and filter into a 25 mL volumetric balloon. Remove aliquots from the filtered sample and transfer to a 100 mL volumetric balloon. In our analysis, we used aliquots of 3 mL, once we verified that this volume allowed an adequate concentration for the reading. Repeat the described protocol for total phenol quantification.

Determination of tannin levels

In this methodology, the tannin level is calculated as the difference between the total phenol level and the non-complex residual phenol level (Folin and Ciocalteu 1927; Santos and Mello 2004), once the tannins are removed from the medium through complexation with caseine.

Foundation of chemical methods

In alkaline medium, the total phenols reduce the mixture of phosphotungstic and phosphomolybdic acids that are present in the Folin-Ciocalteu phenol reagent in blue colored tungsten and molybdenum oxides, proportional to the concentrations of the phenolic compounds that characterize the method.

FLAVONOID DETERMINATION

Flavonoids comprise a class of secondary metabolites with a high structural diversity and, until now, about 9000 substances belonging to this group have been identified (Martens and Mithöfer 2005). They are composed of a simple skeleton with two phenol rings interconnected by a propionic chain, that can be associated with carbohydrates (heterosides), not associated (aglycones), or polymerized (anthocyanins), and can be differentiated by their substituent

groups (Zuanazzi and Montanha 2004). Ring A is generated from the acetate route (Malonyl-CoA), while ring B, together with the three carbon bridges, comes from the shikimate route (*p*-Coumaroyl-CoA), (Zuanazzi and Montanha 2004; Martens and Mithöfer 2005).

Many techniques can be employed for flavonoid detection and quantification in plant samples, such as High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), Liquid Chromatography Mass Spectrometry (LC-MS), and Thin Layer Chromatography (TLC), among others (Li *et al*. 2004; Aguilar-Sánchez *et al*. 2005; Matysik and Wójciak-Kosior 2005). However, spectrophotometry is a highly accessible, practical and less onerous technique. Due to the double bonds present in the aromatic rings, flavonoids can be analyzed in ultraviolet or visible ranges (Mabry *et al*. 1970; Markhan 1982).

Experimental procedure

Equipment, glasswear, reagents and reference compound

For flavonoid determination, the analyst requires an analytical balance, a hot plate, a spectrophotometer, quantitative filter paper, a titrator, 25 mL and 50 mL volumetric flasks, a 50 mL Erlenmeyer flask, a glass funnel, 1 mL and 5 mL graduated pipettes, purified water, 80% methanol (v/v), 99.5% glacial acetic acid (v/v), a 20% methanol solution of pyridine (v/v), a methanol solution of aluminum chloride 50.0 mg/L, and rutin (standard).

Preparation of the standard

Prepare the methanol solution of rutin with a concentration of 0.5 mg/mL. Take six aliquots of 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 mL, to create final concentrations of 6.0, 8.0, 10.0, 12.0, 16.0 and 20.0 μg/mL of rutin, respectively. Add 0.6 mL of glacial acetic acid, 10 mL of the pyridine solution and 2.5 mL of the aluminum chloride reagent. Complete the volume with purified water, wait 30 min (with standard temperature and lighting). Take readings in the spectrophotometer at 420 nm utilizing purified water as the blank solution.

Extract preparation

Weigh 500 mg of the dried, triturated and standardized plant sample (amount optimized by our group), transfer to a 50 mL Erlenmeyer flask, add 25 mL of 80% methanol and warm under gentle ebullition for 30 min. Filter to a 50 mL volumetric flask. To avoid losses, the residues should be washed with 25 mL of 80% methanol. Complete the volume with 80% methanol. Repeat all steps in triplicate.

Quantification procedure

Transfer 1 mL of the plant extract with a volumetric pipette to the 25 mL volumetric flask. As in the construction of the standard curve, add 0.6 mL of glacial acetic acid, 10 mL of pyridine solution at 20% and 2.5 mL of aluminum chloride reagent. Complete the volume with purified water and wait 30 min (with standard temperature and lighting) take readings in the spectrophotometer at 420 nm. It is important not to change the sequence of the reagents when adding them to the reaction.

Foundation of chemical methods

Aluminum (Al^{3+}) reacts with OH groups of the flavonoids from the sample, establishing a stable flavonoid-Al^{3+} complex with a yellow coloring whose intensity is proportional to the concentration of flavonoids in the sample. This reaction promotes a dislocation to larger wavelengths in UV-Vis spectroscopy and an intensification of the absorption, making the sample able to be quantified without suffering from the influence of other phenolic compounds that are present in the sample (**Fig. 3**).

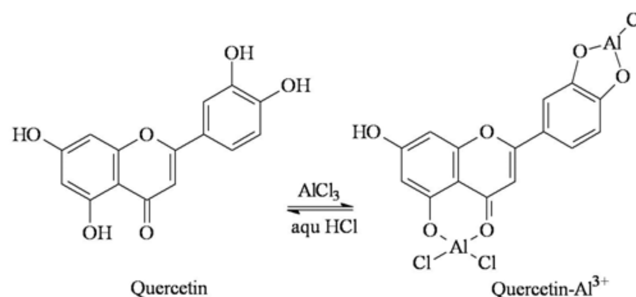

Fig. 3 Illustrated Scheme of the reaction between flavonoid and aluminum chloride ($AlCl_3$), forming a stable flavonoid-Al^{3+} complex. Source: Mabry *et al*. (1970).

METHODS VALIDATION – EXAMPLES FOR FLAVONOIDS

Although, it is a necessity for the execution of chemical and pharmaceutical analysis, validation is still not common in studies with plant extracts. However, all analytic methodology shall be validated since it is the only way to assure the reliability of the results. The universal parameters for validation are specificity, recovery, robustness, range, linearity, detection limit, quantification limit, precision and accuracy, all of which can be evaluated for plant samples. The official regulation of the country is used as reference literature to validate the method, which, in general, follows the Q2(R1) norm that is recognized by the International Conference on Harmonization (ICH 1996). We will exemplify these procedures from the study that we developed with *Bauhinia cheilantha* (Bongard) Steudel (Peixoto Sobrinho *et al.* 2008).

Analyzed parameters

Specificity

Specificity is the ability of exactly and specifically measuring the substance of interest when other components are present. In the case of plant extracts, this point is crucial, given that a large number of substances are extracted from

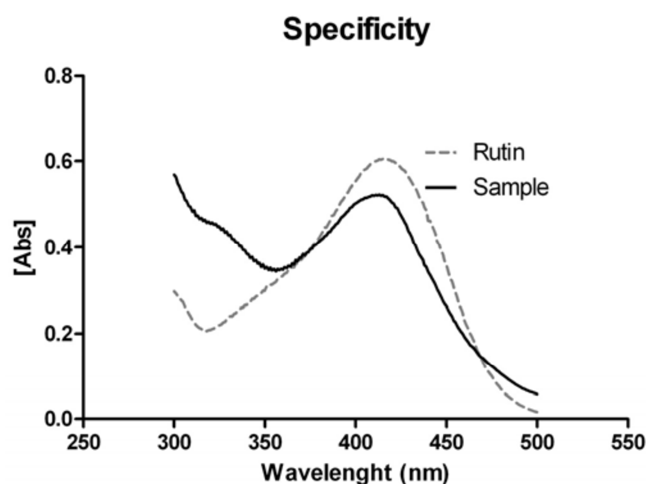

Fig. 4 Specificity of the method using rutin and *Bauhinia cheilantha* (Bongard) Steudel, plant extract scanning from 300 to 500 nm, with the greatest absorbance observed at 420 nm. From: **Peixoto Sobrinho TJS, Silva CHTP, Nascimento JE, Monteiro JM, Albuquerque UP, Amorim ELC** (2008) Validação de metodologia espectrofotométrica para quantificação dos flavonóides de *Bauhinia cheilantha* (Bongard) Steudel. *Revista Brasileira de Ciências Farmacêuticas*, in press, ©2008, with kind permission from Revista Brasileira de Ciências Farmacêuticas, São Paulo, Brasil.

the plant during the process and will be present during the analysis. Even if a better wavelength for a given metabolite is chosen, this does not prevent other mixture constituents from interfering with the reading. This fact makes it difficult or even impossible to establish the specificity in a spectrovolumetric method, since the apparatus response reflects the sum of the responses from all present substances that are able to absorb in the established wavelength.

Most authors have solved this problem by scanning over a large range of wavelengths, and adopting a value from which the greatest reading is obtained for both the extract and the standard (when it exists). Normally, scanning responses are similar, but not totally superposed. However, it is possible to predict when interference from other components will be low enough so that it does not compromise the values of the expected answer. When the wavelength is established in a compendium, the execution of this step is not necessary.

In our study, the specificity assay was conducted with the extract of *Bauhinia cheilantha* (Bongard) Steudel, with rutin as the flavonoid standard, both with a concentration of 18 μg/mL, over a range between 300-500 nm, for the verification of possible interference. When comparing responses from the standard and the extract, it is noted that the greatest absorbance occurred at 420 nm (**Fig. 4**).

Recovery

Recovery measures the efficiency of the analyte extraction process and is also useful to evaluate losses resulting from use of the technique, such as that of solvents, reactions, warming evaporation, filtration, pipetting, etc. In studies with plant extracts, when the analyte proportion is not known, the extracted quantity is considered to be 100% of the extraction, in which case, we have to insure that the maximum substance will be extracted. For the method to be reproducible, it is necessary to standardize the titration and powder granulometry techniques.

To verify the analyte recovery ($R_{(\%)}$), in an extractive process, it is possible to determine the absorbance of three samples: a sample composed of the pulverized plant added to the standard in a known concentration (AR), another sample only composed of the pulverized plant (A), and a third sample containing only the standard (R) - the last not being submitted to the extraction process. Recovery is calculated by Equation 1.

$$R_{(\%)} = \frac{AR - A}{R} x100 \qquad \text{(Equation 1)}$$

In the example for validation to *B. cheilantha*, recovery was of 98.36%, and this shows that the method has a high efficiency in the extraction (Peixoto Sobrinho *et al.* 2008).

Robustness

Robustness is defined as the capacity of the method to be unaffected by a small and deliberate modification of its parameters. In the official compendiums, the obligatory tests are: stability of analytical solutions, extraction time, variation in solution pH, variation temperatures, and different solvent fabricants.

Solvent impurities can influence the spectrophotometer response, but they are easy to control. Other tests are a direct reflection of analyte chemical structure preservation. Different pH values, can modify the ionization degree or, even completely invert the solubility of the present molecules. In addition, when an acid or base is added to produce pH variations, it can unleash or accelerate oxidation, hydrolysis or complexations, which can substantially change the results. This test should be applied rigorously, and the variation produced should be minimal since, by its nature, the method is very sensible. Robustness tests should be conducted using the standard and the extract.

Analytical responses to the robustness test with leaf extracts from *Bauhinia cheilantha* (Bongard) Steudel, showed low variations related to suppliers of solvent (Mark 1 and Mark 2), time and extraction temperature, and there was no significant statistical difference, demonstrating that this is a robust method for these factors (**Table 2**).

Range and linearity

The range between the lowest and highest scale values should be determined with precision, accuracy and linearity. Linearity is the method's ability to offer directly proportional results between the analyte concentration and measurement apparatus response. It is calculated from three authentic calibration curves. ICH specifies a minimum of five concentration levels.

Establishing an analytical curve is one of the first steps to be completed, and in many experiments, there is still the need for small adjustments in already advanced processes. Therefore, this step has to be carefully studied to avoid losses. For the acceptance of a calibration curve, it has to present a variation range from 80 to 120 percent of the test concentration. In studies with plants, the concentrations of the analyzed metabolite can vary largely and, as this infor-

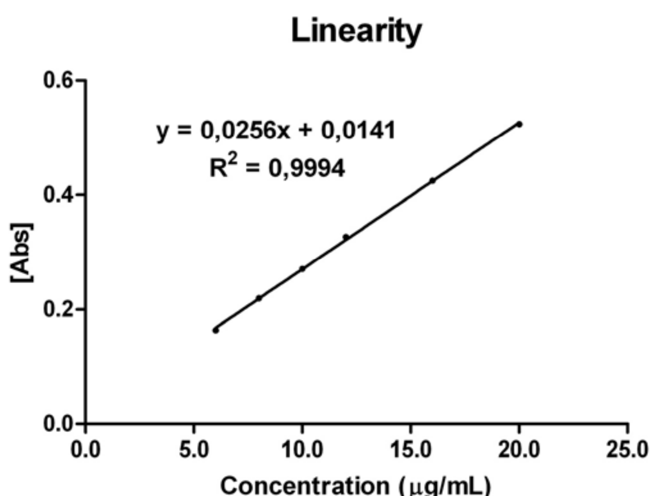

Fig. 5 Linearity using a Rutin standard (6.0-20.0 μg/mL), demonstrating the line equation and the linear correlation coefficient (R^2). From: **Peixoto Sobrinho TJS, Silva CHTP, Nascimento JE, Monteiro JM, Albuquerque UP, Amorim ELC** (2008) Validação de metodologia espectrofotométrica para quantificação dos flavonóides de *Bauhinia cheilantha* (Bongard) Steudel. *Revista Brasileira de Ciências Farmacêuticas*, in press, ©2008, with kind permission from Revista Brasileira de Ciências Farmacêuticas, São Paulo, Brasil.

Table 2 Results of the robustness test with the variations in analytical parameters in the submitted controls (μg/mL).

	Extraction time			Stability			Solvent mark	
	20 min	**30 min**	**40 min**	**20 min**	**30 min**	**40 min**	**Mark 1**	**Mark 2**
Average	16.527	16.446	15.484	15.741	16.446	16.107	16.446	16.175
SD	0.652	0.772	0.066382	0.282	0.772	0.0507	0.772	0.144
P%	3.95%	4.70%	0.43%	1.79%	4.70%	0.31%	4.70%	0.89%

SD = Standard deviation; P = Precision

From: **Peixoto Sobrinho TJS, Silva CHTP, Nascimento JE, Monteiro JM, Albuquerque UP, Amorim ELC** (2008) Validação de metodologia espectrofotométrica para quantificação dos flavonóides de *Bauhinia chei-lantha* (Bongard) Steudel. *Revista Brasileira de Ciências Farmacêuticas*, in press, ©2008, with kind permission from Revista Brasileira de Ciências Farmacêuticas, São Paulo, Brasil.

Table 3 Results of repeatability, intermediate precision and reproducibility of the analytical method with a spectrophotometer to quantify flavonoids in *Bauhinia cheilantha* (Bongard) Steudel.

Assay	Theoretical concentration (µg/mL)	N	Obtained concentration (µg/mL)	P (%)	A (%)
Repeatability	18.0	6	18.069	1.12	100.38
Intermediate precision	18.0	6	18.133	2.67	100.74
Reproducibility	18.0	6	18.990	0.44	105.50
	9.0	3	9.303	1.03	103.37
Accuracy	18.0	3	17.465	0.45	97.03
	27.0	3	25.843	0.31	95.71

N = Number of samples; P = Precision; A = Accuracy

From: **Peixoto Sobrinho TJS, Silva CHTP, Nascimento JE, Monteiro JM, Albuquerque UP, Amorim ELC** (2008) Validação de metodologia espectrofotométrica para quantificação dos flavonóides de *Bauhinia chei-lantha* (Bongard) Steudel. *Revista Brasileira de Ciências Farmacêuticas*, in press, ©2008, with kind permission from Revista Brasileira de Ciências Farmacêuticas, São Paulo, Brasil.

mation cannot be foreseen, it forces us to preliminarily measure the analyte concentration for the curve to be adjusted.

In the case of *B. cheilantha* flavonoids, the average of three intervals with authentic repetitions was used, and they comprised six concentrations of rutin solution 0.5 mg/mL (6.0-20.0 µg/mL). After visual linear reaction, the results were statistically analyzed to define the correlation coefficient (minimum accepted $R^2 = 0.99$), and the regression equation, the linear adjustment and the relative standard deviation. **Fig. 5** shows the linearity graph obtained in this study.

Detection and quantification limits

The detection limit (DL), is defined as the minimum analyte concentration present in the sample that can be detected by the apparatus without being quantified. The quantification limit is defined as the minimum analyte concentration present in the sample that can be determined with precision and accuracy, under operational conditions.

The limits reflect the apparatus capacity and not the limitation of the applied methodology or the extraction technique. They are calculated from the extrapolation of linearity, by means of Equations 2 and 3, or through successive dilutions of the standard by observing their precisions.

$$DL = \frac{3.3\, xSDa}{CI} \quad \text{(Equation 2)}$$

$$QL = \frac{10xSDa}{CI} \quad \text{(Equation 3)}$$

where SDa is the relative standard deviation of the first curve concentration level, and CI is the curve inclination (which is the a value in the curve, $y = ax + b$).

In our study, the estimated Detection (DL), and Quantification (QL), limits were 0.75 and 2.50 µg/mL, respectively (Peixoto Sobrinho *et al*. 2008). With these results, we verified that the method presents high sensibility for the detection and quantification of the standard, without suffering from an alteration of equipment intrinsic factors.

Precision and accuracy

Precision is the evaluation of the proximity of the obtained results in a series of measurements for the same sample, at three concentration levels (low, medium, high, in triplicate), or six determinations with 100% of the test concentration. Precision is calculated through three tests: repeatability (agreement in results for the same analyst), intermediate precision (agreement in results for different analysts), and reproducibility (agreement in results for different laboratories). It can be calculated according to Equation 4. Accuracy indicates the proximity between the measured value and, the real concentration value, and it should be calculated from three concentration levels (low, medium and high).

$$P_{(\%)} = \frac{SD}{ACD} x100 \quad \text{(Equation 4)}$$

$$A_{(\%)} = \frac{AEC}{TC} x100 \quad \text{(Equation 5)}$$

where P(%) is the precision, SD is the standard deviation, ACD is the average concentration determined, A(%) is the accuracy, AEC is the average experimental concentration and TC is the theoretical concentration.

With our example for *B. cheilantha*, repeatability and intermediate precision assays were determined by six samples with the same concentration (18 µg/mL), executed on the same day (intraday), and on two consecutive days by different analysts (interday).

The results were expressed as relative standard deviation. Reproducibility tests were performed in another laboratory and involved the measurement of six samples with the same concentration (18 µg/mL). The accuracy was evaluated by three controls (in triplicate) in low (9.0 µg/mL), medium (18 µg/mL), and high (27 µg/mL), concentrations, and it was individually calculated for each control (**Table 3**).

In all precision assays (**Table 3**), the results found did not exceed 5%; values were between 95.71 and 105.50% (Peixoto Sobrinho *et al*. 2008). These data confirm that the proposed quantification method by spectrophotometry (in the visible), is precise and exact according to the norms established in ICH.

FINAL CONSIDERATIONS

We have presented techniques for determining tannin and flavonoid dosage, which are easy to execute and help synthesize the contributions previously presented in the literature. We also summarize the precise relationship between equipment and reagents as well as protocols for preparing solutions. Our contribution facilitates the implementation and optimization of this type of analysis for different plant species.

The furnished data is aimed at illustrating each stage of the analysis and the way in which the results should be expressed. All analyses must be performed in triplicate in order to minimize possible weighing and pipetting errors, which would directly interfere with solution concentrations and consequently, with the final result.

In the context of ethnobotanical and ethnopharmacological studies, such analysis could help with the selection of interesting plants for pharmacological studies, and, under the conservationist point of view, it could aid in the elaboration of management plans of highly demanded species due to their phytochemical attributes.

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