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OPTIMIZATION AND VALIDATION OF A METHOD FOR THE QUANTIFICATION OF POLYSACCHARIDES OF *THUJA OCCIDENTALIS* LINN.

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ABSTRACT: The anthrone method has been employed for many years in the spectrophotometric determination of polysaccharides. However, taking into account that its use usually involves a series of steps and requires a long period of analysis, this study aimed to conduct a more detailed assessment of its application in the determination of total polysaccharides from the aerial parts of *Thuja occidentalis* Linn. Thus, this study attempted to optimize and validate a viable method for this species, whose antimetastatic and immunostimulant properties have been attributed to the metabolites analyzed in this study. The optimized and validated method showed satisfactory results, and this method lasts about 6 hours, in contrast to the diverse methodologies reported in the literature with an estimated duration of 41 hours. Thus, the presented method demonstrated to be a viable tool for the quality control of the plant drug and products derived from the aerial parts of *T. occidentalis*.

INTRODUCTION: Polysaccharides extracted from botanical sources have emerged as an important class of bioactive natural products, especially for their activities on the immune system and antitumor action^{1, 2}. Many polysaccharides isolated from plants have immunostimulatory action evidenced by their ability to regulate the function of macrophages^{3, 4}, to activate the complement system, to induce leukocyte chemotaxis³ and stimulate the humoral and cell-mediated immunity⁵.

Moreover, many studies also indicate that polysaccharides can effectively inhibit the growth of various tumors in experimental animals and raise their survival rate⁵. Some researchers have shown the association between the immunostimulant activity and the antitumor action for the polysaccharide fractions as reported to the polysaccharide fractions isolated from the roots of *Sophora flavescens*⁶, the alga *Enteromorpha intestinalis*⁴, the leaves of *Schisandra chinensis*⁵ and the aerial parts of *Thuja occidentalis*^{7, 8}.

Because of these actions, polysaccharides became a strong target of research in the field of natural medicines⁹, although they can also be widely used in other health products, functional foods and materials, and; therefore, they have a large market perspective¹⁰.

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In view of the therapeutic potential of these compounds and the regulatory needs for regularization of herbal medicines, there is the importance of accurate, fast and reliable analytical methods that can be used routinely in the quality control of plant inputs whose bioactive compounds are polysaccharides¹¹.

According to Martens and Frankenberger¹², the analysis to detect polysaccharide has an inherent complexity of their chemical nature and they can be determined by gas chromatography (GC) after derivatization, by spectrophotometry after treatment with chromogenic reagents and also by titrimetric and gravimetric method.

However, the colorimetric methods can be highlighted due to their simplicity, speed and because they do not require expensive instrumentation¹².

Among the colorimetric methods, the anthrone method is one of the most used and efficient quantification techniques¹¹. It has been successfully applied in the determination of soluble sugars from different plant samples, despite the big number of stages and its wide variability of the parameters^{11, 13, 14, 15}.

Thus, the focus of this study was to evaluate in detail the parameters of the anthrone method, aiming to optimize and validate a Spectrophotometric method capable of quantifying the total polysaccharides present in the aerial parts of *Thuja occidentalis* Linn. This species is popularly known as the tree of life or white cedar, and whose antimetastatic and immunostimulant properties have been attributed to its polysaccharides^{7, 8}.

MATERIAL E METHODS:

Material: Samples of the aerial parts of *T. occidentalis* were collected in the city of Cabo de Santo Augustinho, in Pernambuco (Brasil) on January 2013. The identification was carried out by the researcher and curator Dr. Rita Araújo Pereira and the voucher specimen (n^o. 87,752) was deposited at Herbarium of Agronomic Institute of Pernambuco, Brazil.

Fresh plant material was washed with purified water and sprayed with 70% alcohol (v/v). It was then placed in a circulating air oven (Fabbe-Primar[®]) for 84 hours at 40°C. After drying, the material was powdered in a knife mill (Model 340 Adamo[®]), passed through a 20-mesh sieve (0.84 mm) and placed in a glass container, which was sealed and properly maintained in the absence of light.

All reagents used were analytical grade: ethanol (Quimex[®] and Alphatec[®]), trichloroacetic acid - TCA (Vetec[®]), sulfuric acid - H₂SO₄ (Proquímicos[®] and Vetec[®]), anthrone (Sigma Aldrich[®]) and glucose (Sigma Aldrich[®]).

- 1. Preparation of the extractive solution:** The extractive solution was obtained by aqueous extraction under heating, based on the extraction methods and parameters described by Jiang *et al*¹⁶, Tang *et al*¹⁴, Tian *et al*¹⁵; Ye and Jiang¹⁷; Jin *et al*¹⁸ and Luo¹⁹. The extraction was performed in a water bath, using 3.0 g of plant material and 30 mL of distilled water for 2.5 hours. The extractive solution was cooled to room temperature (25°C) and filtered through cotton.
- 2. General method for obtaining the aqueous solution of polysaccharides (test solution):** Test solutions were obtained according to Tang *et al*¹⁴, with modification of the following parameters: it was used 2.0 mL (0.2 g/mL) of the extractive solution and the volume was adjusted with 10% trichloroacetic acid - TCA (v/v) to 0.24 mL. After being stored in a refrigerator for 24 hours at 4°C, the samples were centrifuged in a centrifuge (Excelsa 2 Fanen[®]) at 4000 rpm, for 15 minutes. This was made in order to remove proteins and to treat the supernatant with 80% ethanol (v/v). The proportion of supernatant: ethanol used was 1:5. Then the samples were stored in the refrigerator for 12 hours at 4°C and centrifuged at 4000 rpm for 15 minutes to make polysaccharides precipitate. Subsequently, the supernatant was discarded, and the polysaccharides were dissolved in distilled water (10 mL).

3. **Anthrone reagent:** Anthrone reagent was prepared based on the parameters described by Yemm and Willis¹³, using a concentration of 0.2% of anthrone (w/v) and a mixture of H₂SO₄ and water with the proportion modified from 5:2 to 6:1.

4. **General method for the quantification of total polysaccharides (anthrone method):**

The quantification of total polysaccharides was performed on a Ultraviolet-visible (UV-Vis) spectrophotometer (mini-1240, Shimadzu®), using the test solutions obtained from the extractive solution of *T. occidentalis* and the anthrone method as described by Yemm and Willis¹³. We also used distilled water to obtain the blank solution and the diluted samples from a glucose solution (100 µg/mL) to construct the standard curve, in the range of 20-100 µg/mL. In detail, the preparation of the samples was as follows: an aliquot of 5.0 mL of the anthrone reagent was added to the test tubes placed in a bath containing ice. The test tubes already had 1.0 mL of distilled water to obtain the blank solution, or 1.0 mL of diluted samples from the glucose solution in order to obtain the standard curve or 1 mL of the diluted solutions of polysaccharides of *T. occidentalis* (0.4 mL of test solution and 0.6 mL of water). After agitation using a vortex (MA 162, Marconi®) and heating for 10 minutes in a water bath (Novaética®) at 100°C, the absorbance of the sample was determined at a wavelength of 625 nm. All analyzes were performed in triplicate.

5. **Evaluation and optimization of the general method to obtain the test solution:**

a. **Removal of Interfering Proteins:** First, we analyzed the need for treatment of the extractive solution with 10% TCA (v/v) to remove the proteins. For this, samples were prepared without the addition of 10% TCA (v/v), with and without (after chilling) centrifugation. We also evaluated the samples treated with different amounts of TCA (0.0, 0.12, 0.24, 0.36 and 0.48 mL), which were centrifuged after spending 24 hours in the refrigerator, similar to the evaluation performed by Tang *et al*¹⁴. In order to verify the interference of protein

compounds, we determined the total polysaccharide content and protein dry weight of all samples, according to the loss on drying method as described in Brazilian Pharmacopoeia 5th Edition, 2013. After the establishment of the aliquot of 0.24 mL of 10% TCA (v/v), we evaluated the need to keep the samples in a refrigerator for 24 hours, subjecting them to analysis during periods of 0, 3, 6, 12 and 24 hours. Then the centrifugation time was analyzed (5, 10 and 15 minutes), maintaining a constant speed of 4000 rpm. For these analyzes, only the total polysaccharide content of the samples was verified.

b. **Precipitation of polysaccharides:** We initially analyzed the concentration of the ethanolic solution that would be used to precipitate the polysaccharides (60, 65, 70, 75, 80, 85, 90, 95 and 99.5%), similar to the evaluation performed by Tang *et al*¹⁴ (2011). After the establishment of absolute ethanol as the precipitating reagent, we evaluated the need to keep the samples in a refrigerator for 12 hours, subjecting them to analysis during periods of 0, 3, 6 and 12 hours. Next, we analyzed the ratio of the ethanol solution used (1:2, 1:3, 1:4 and 1:5) and the centrifugation time (5, 10 and 15 minutes) maintaining a constant speed of 4000 rpm.

Anthrone method: We evaluated several parameters of the anthrone method, such as the need for cooling in an ice bath during the preparation of the samples, prior to the addition of the anthrone reagent to the test tubes containing the test solution and the necessity of agitation of the sample by vortex. Other parameters assessed include the need for heating the samples in a water bath at 100°C and the interference of loss of water during the heating of the samples, using test tubes with and without caps. Additionally, the effect of different heating times was analyzed (2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 minutes).

Statistical analysis: For the optimization of the method, the results were expressed as the mean of three determinations. Additionally, statistical analysis was performed by Student's t-test or One-

Way ANOVA or Two-Way ANOVA, when applicable, with a significance level of 95%.

a. **Validation of the analytical method:** For validation of the analytical method, the following parameters were evaluated: specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness. All analyzes were performed in triplicate and reliability of the parameters was checked by the coefficient of variation (CV) as a percentage, considering values that did not exceed 5%²⁰. Additionally, the results were statistically analyzed by analysis of variance (One-Way or Two-Way ANOVA, when applicable) with a significance level of 95%.

i. **Linearity, limits of detection and quantification:** Linearity was checked for the extractive solution and test solution from the analysis of three authentic curves. For the extractive solution, three curves were constructed using samples of the extractive solution at five concentration levels: 0.1, 0.15, 0.2, 0.25 and 0.3 g/mL, corresponding to a range of 50 to 150%. For the test solution, three curves were constructed with five aliquots of test solutions: 0.2, 0.3, 0.4, 0.5 and 0.6 ml, corresponding to a range of 50 to 150%. The curves were constructed by plotting the average values of absorbance versus concentration.

The results obtained were assessed by means of linear regression using the least squares method in order to determine the coefficient of determination (R^2). A coefficient of determination greater than 0.99 was required²⁰.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the equations $LOD = SD_a \times 3/S$ and $LOQ = SD_a \times 10/S$. In these equations, the standard deviation (SD) is the intercept with the Y axis of the three linearity curves obtained and the "S" is the average of the slopes of the respective curves²⁰.

Robustness: For the determination of the robustness of the method, various parameters were varied to determine their influence on the method. They include the following: manufacturer of absolute ethanol (Quimex[®] and Alphatec[®]), manufacturer of H_2SO_4 (Proquímicos[®] and Vetec[®]), centrifugation time for the deproteinization (4, 5 and 6 minutes) and for the precipitation of polysaccharides (14, 15 and 16 minutes) and brand of the centrifuge (Excelsa 2 centrifuge, FANEN[®] and 5810 R centrifuge, Eppendorf[®]).

The results were expressed as the mean of three determinations.

i. **Precision:** For the evaluation of the precision of the method, the repeatability and intermediate precision were assessed. The repeatability was observed for six individual determinations at 100% of the test concentration (0.2 g/mL). The intermediate precision or inter-assay precision was performed using the same repeatability parameters, but with the analysis being carried out by two different analysts on two different occasions.

ii. **Accuracy:** The accuracy of the method was determined by the recovery assay. For this, the standard glucose at a known concentration (10 μ g) was added to the samples at 75, 100 and 125% of the test concentration (0.15, 0.2 and 0.25 g/mL), covering the linearity range of the method. The recovery values, expressed as percentages, were determined by the ratio between the average concentrations determined experimentally and the corresponding theoretical concentrations²⁰.

Specificity: The specificity of the method was verified by overlapping the spectra of the following samples: test solution with the addition of the anthrone reagent, test solution without the addition of the anthrone reagent, glucose and quercetin (both with the addition of the reagent). The spectra were obtained at wavelengths ranging from 420 to 700 nm.

RESULTS E DISCUSSION

Evaluation and optimization of the method:

1. **Removal of Interfering Proteins:** The anthrone method has been successfully employed in the in the analysis of plant polysaccharides. In general, application of anthrone method for analyzing plant samples requires the isolation of polysaccharides by precipitation with ethanol and centrifugation^{11, 14, 15}. This step may, however, require a pretreatment which involves a deproteinization of the samples, once plant extracts generally have a large amount of pigments and proteins that may impede the isolation of polysaccharides. Furthermore, although anthrone reagent is specific, some proteins may be condensed with the reagent, forming products with various colors and this can affect the colorimetric determination of polysaccharides²¹.

Although deproteinization is considered as an essential step in the analysis of polysaccharides in plants, in some cases, protein removal is not required¹¹. Therefore, this condition was assessed in this study by employing the method of trichloroacetic acid (TCA). For the extractive solution obtained from the aerial parts of *T. occidentalis*, the deproteinization, with the addition of 10% TCA and centrifugation, demonstrated to be a necessary condition for the quantification of polysaccharides.

First, it was found that the test solutions obtained from the samples of the extractive solution that were not treated with 10% TCA (v/v) and were not centrifugated did not allow the precipitation of polysaccharides. The contrary happened to the samples that were centrifuged immediately or only after 24 hours in the refrigerator, and whose contents are described in **Table 1**.

TABLE 1: POLYSACCHARIDE CONTENT AND DRY WEIGHT OF THE PROTEINS THAT WERE REMOVED FROM THE SAMPLES SUBJECTED TO DIFFERENT CONDITIONS OF DEPROTEINIZATION

10% TCA (v/v) (mL)	Time in the refrigerator (h)	Polysaccharide content ($\mu\text{g/mL}$) \pm SD	Proteins* (%)	CV (%)
0.0	0	3.39 \pm 0.08	---	2.26
0.0	24	3.34 \pm 0.09	0.08	2.79
0.12	24	3.18 \pm 0.06	0.10	1.93
0.24	24	2.82 \pm 0.05	0.15	1.84
0.36	24	---	0.09	---
0.48	24	---	0.07	---

SD – Standard Deviation; CV – Coefficient of variation; *Dry weight of the proteins that were removed from the samples

There was no statistical significant difference in the polysaccharide content for the samples that were not treated with 10% TCA (v/v) as shown by Student's t-test, with the calculated t-value (0.74) lower than the tabulated t-value (2.78). However, after evaluating the treatment of the extractive solution with different volumes of TCA (0.0, 0.12, 0.24, 0.36, 0.48 mL), a statistical significant decrease in the polysaccharide content could be observed for the analyses with 0.0, 0.12 and 0.24 mL of the reagent as shown by ANOVA with the calculated F-value (41.64) larger than the tabulated F-value (5.14). In addition, the samples containing 0.36 and 0.48 ml of TCA were not adequate for the method, given that they did not enable the precipitation of the polysaccharides at later stages,

possibly due to the hydrolysis conferred by the higher content of acid.

Additionally, when we compared the polysaccharide content of the test solution with the dry weight of protein that was removed from the samples (Table 1), we found that a statistical significant reduction of the polysaccharide content was caused by increasing the deproteinization. This showed the interference of these compounds on the analysis and that their presence may cause an overestimation of the polysaccharide content in *T. occidentalis*. It is also worth noting that the samples that were not treated with 10% TCA (v/v) showed greater difficulty in solubilizing, in water, the polysaccharides that precipitated, confirming

that the removal of proteins is a necessary step for the method. Given these results, we selected the volume of 0.24 mL for the treatment of the samples. These results were similar to those obtained by Tang et al¹⁴ (2011).

After the establishment of the volume of 10% TCA (v/v), we evaluated if the samples would need to remain in the refrigerator. The data analyzed in the period of 0 [2.77 ± 0.13 (CV 4.71%)], 3 [2.83 ± 0.14 (CV 5.08%)], 6 [2.78 ± 0.17 (CV 6.05%)], 12 [2.65 ± 0.20 (CV 7.50%)] and 24 [2.68 ± 0.11 (CV 4.24%)] hours were not statistically different as shown by ANOVA, with the calculated F-value (0.68) lower than the tabulated F-value (3.48). Besides that, it was demonstrated by Student's t-test that there was a concordance between the results of 0 and 24 hours, with the calculated t-value (0.92) lower than the tabulated t-value (2.78). Therefore, the selected time was 0 h (immediate centrifugation).

Finally, we evaluated the centrifugation time (5, 10 and 15 minutes), maintaining a constant speed. The results to 5 [2.74 ± 0.10 $\mu\text{g/mL}$ (CV 3.67%)], 10 [2.66 ± 0.09 $\mu\text{g/mL}$ (CV 3.41%)] and 15 [2.78 ± 0.14 $\mu\text{g/mL}$ (CV 2.78%)] minutes showed no statistical significant differences as shown by ANOVA, with the calculated F-value (0.86) lower than the tabulated F-value (5.14). This result was consistent with the Student's t-test, with the calculated t-value (0.46) lower than the tabulated t-value (2.78) for each centrifugation time analyzed. Thus, we selected the shortest time for the method.

Precipitation of polysaccharides: After setting the parameters for the deproteinization of the extractive solution of *T. occidentalis* (0.24 mL of 10% TCA and immediate centrifugation at 4000 rpm for 5 minutes), the parameters for the precipitation of polysaccharides were analyzed. First, we verified the influence of the concentration of the ethanolic solution [60, 65, 70, 75, 80, 85, 90, 95 and 99.5% (absolute ethanol)].

The precipitation of polysaccharides was only observed for the test solutions treated with 70% or more of ethanol and a wide uniformity was only verified for the test solutions treated with 85% or more of ethanol. These macroscopic observations were then confronted with the polysaccharide

content determined by the anthrone method: 85 [2.85 ± 0.09 (CV 3.04%)], 90 [2.85 ± 0.06 (CV 2.18%)], 95 [2.99 ± 1.37 (CV 1.37%)] and absolute ethanol [3.07 ± 0.04 (CV 1.31%)].

Although a great uniformity of the precipitation has been demonstrated for the test solutions with 85% or more of ethanol, it was found by ANOVA a statistical significant variation among the results for the concentrations from 85 to 99.5% [calculated F-value (9.18) > tabulated F-value (4.07)] and from 90 to 99.5% [calculated F-value (15.22) > tabulated F-value (5.14)]. This reinforced the strong influence of ethanol as a precipitating agent. In what concerns the range from 95 to 99.5% of ethanol, there was no significant variation [calculated t-value (2.51) < tabulated t-value (2.78)], according to Student's t-test.

However, we chose to select the absolute ethanol as the precipitating reagent in order to reduce possible experimental errors and for reasons of practicality.

The next step was the evaluation of the need for the test solutions remain 12 hours in the refrigerator. The data analyzed in the period of 0 [2.90 ± 0.10 (CV 3.43%)], 3 [3.03 ± 0.03 (CV 5.08%)], 6 [2.91 ± 0.12 (CV 4.15%)] and 12 [2.95 ± 0.14 (CV 4.62%)] hours were not statistically different, as shown by ANOVA, with the calculated F-value (0.95) lower than the tabulated F-value (4.07). Furthermore, it was demonstrated by Student's t-test that there was a concordance between the results of 0 and 24 hours, with the calculated t-value (0.50) lower than the tabulated t-value (2.78). Then, the selected time was 0 h.

Next, we analyzed the proportion of ethanolic solution used to precipitate the polysaccharides in the supernatant, obtaining the following results: 1:2 [2.91 ± 0.20 $\mu\text{g/mL}$ (CV 6.96%)], 1:3 [3.02 ± 0.11 $\mu\text{g/mL}$ (CV 3.58%)], 1:4 [3.08 ± 0.11 $\mu\text{g/mL}$ (CV 3.56%)] and 1:5 [3.04 ± 0.12 $\mu\text{g/mL}$ (CV 4.14%)] supernatant: ethanol. As assessed by ANOVA for all the proportions (1:2, 1:3, 1:4 and 1:5) [calculated F-value (0.74) < tabulated F-value (4.07)], and by Student's t-test for the proportions of 1:2 and 1:5 [calculated t-value (0.90) < tabulated t-value (2.78)]; the polysaccharide content was statistically equivalent.

However, once the amount of ethanolic solution is a critical point for the precipitation of polysaccharides and once the ration 1:2 resulted in the highest CV (%), the ratio 1:3 was selected for the method. Besides that, the ratio 1:3 is a more suitable ratio from the economic point of view in relation to the ratio 1:5 used in the general method (2.5.).

Finally, we analyzed the centrifugation time (5, 10 and 15 minutes) of the test solutions, obtaining the following results: 5 [$2.78 \pm 0.20 \mu\text{g/mL}$ (CV 7.39%)], 10 [$3.03 \pm 0.19 \mu\text{g/mL}$ (CV 6.14%)] and 15 [$2.98 \pm 0.09 \mu\text{g/mL}$ (CV 2.95%)] minutes. A significant variation could not be observed among these times using ANOVA [calculated F-value (1.86) < tabulated F-value (5.14)] and Student's t-test for 5 and 15 minutes [calculated t-value (1.54) < tabulated t-value (2.78)], and for 10 and 15 minutes [calculated t-value (0.44) < tabulated t-value (2.78)]. However, the polysaccharides that precipitated after 5 and 10 minutes of centrifugation were looser, making difficult the complete removal of the supernatant.

Therefore, since it is a critical step and taking also into consideration the CV (%), the duration of 15 minutes was chosen for this stage. This time was the same as the one described in the general method (2.5.).

To sum up, the following parameters for the precipitation of polysaccharides of the extractive solution of *T. occidentalis* were set: absolute ethanol, ratio 1:3 (extractive solution: ethanol) and immediate centrifugation at 4000 rpm for 15 minutes. Thus, it can be observed as shown in **Figure 1**, that the optimized method for the removal of interfering proteins and the precipitation of polysaccharides was significantly simpler and more practical in relation to the general method, given that the number of extensive steps that were eliminated, reducing the test duration from 41 hours to about 6 hours.

Anthrone method: Employing the optimized method, the parameters and possible sources of error of anthrone method were separately evaluated as shown in **Table 2 and 3**.

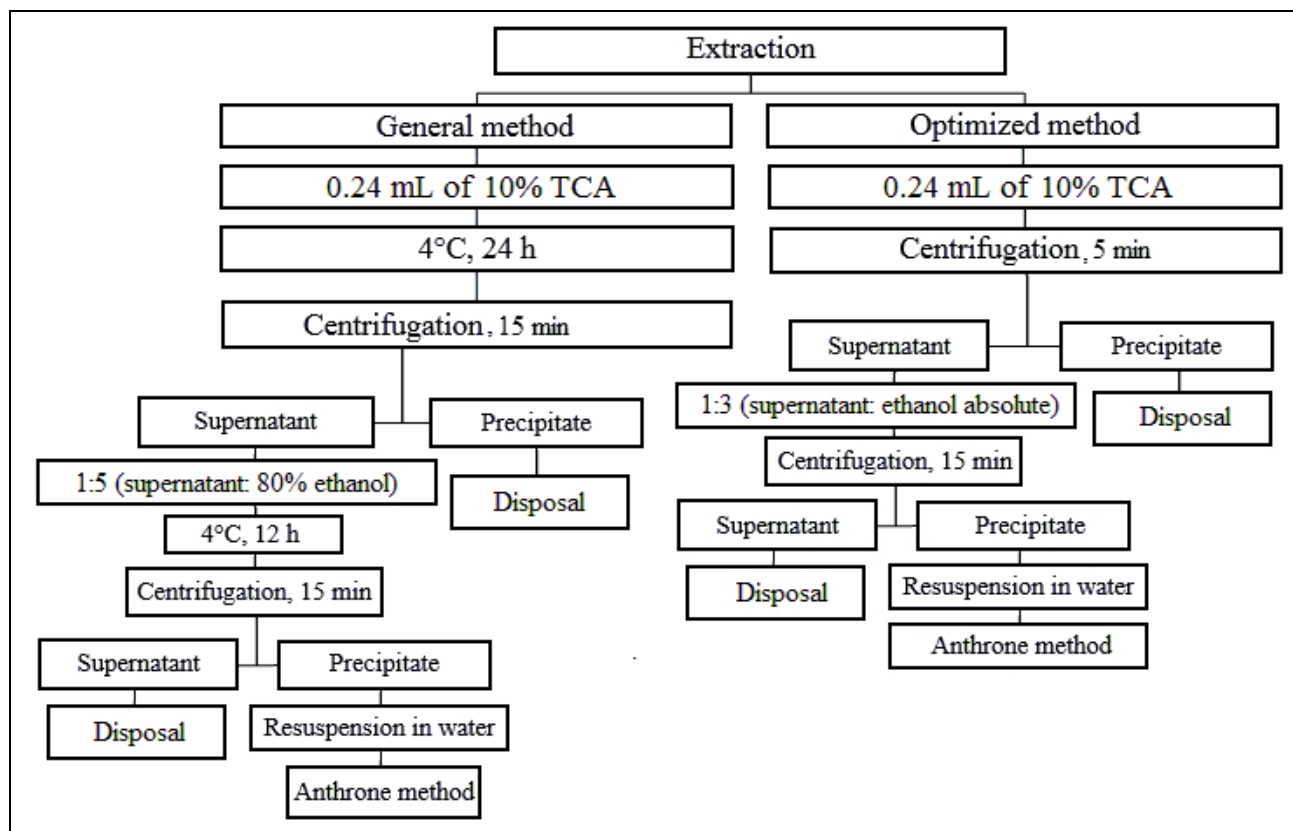


FIGURE 1: FLOWCHART OF THE GENERAL AND OPTIMIZED METHOD FOR THE REMOVAL OF INTERFERING PROTEIN AND THE PRECIPITATION OF POLYSACCHARIDES

TABLE 2: POLYSACCHARIDE CONTENT DETERMINED BY THE ANTHRONE METHOD, VARYING SOME DIFFERENT PARAMETERS

Parameter	Polysaccharide Content ($\mu\text{g/mL}$) \pm SD	CV (%)
Standard* - General Method	2.99 \pm 0.09	2.97
Absence of ice bath	2.93 \pm 0.05	1.59
Absence of agitation using vortex	0.36 \pm 0.06	16.60
Absence of bath with heating	0.25 \pm 0.04	14.59
Presence of test tube caps	2.98 \pm 0.05	1.84

SD – Standard Deviation; CV – Coefficient of variation. *Preparation of the samples that will be read, on UV/Vis spectrophotometer, in an ice bath with agitation using vortex, heating in a water bath at 100°C, and absence of the test tube caps.

TABLE 3: POLYSACCHARIDE CONTENT DETERMINED BY THE ANTHRONE METHOD, VARYING THE TIME OF HEATING IN A WATER BATH AT 100°C

Time of heating (min)	Polysaccharide Content ($\mu\text{g/mL}$) \pm SD	CV (%)	Calculated t-value
2.5	2.46 \pm 0.07	2.87	8.60
5.0	2.81 \pm 0.01	0.48	3.51
7.5	3.00 \pm 0.004	0.12	0.91
10.0	2.96 \pm 0.07	2.39	---
12.5	3.09 \pm 0.06	2.13	2.27
15.0	2.82 \pm 0.06	2.23	2.51

SD – Standard Deviation; CV – Coefficient of variation. * tabulated t-value = 2.78

Anthrone reagent was prepared as described by Yemm and Willis¹³, but the ratio of H₂SO₄ and water was adjusted to 6:1, instead of 5:2. This happened because although the reagent with a ratio of 5:2 has an appropriate percentage of acid (71.42%), when we prepare the samples that will be read on UV/Vis spectrophotometer by adding 1.0 mL of diluted sample of polysaccharide (0.4 mL of test solution and 0.6 mL of water) in 5.0 mL of reagent; the acid concentration reaches 59.5%. This results in a low solubility of anthrone, whereas when we use the ratio of 6:1, the acid concentration is reduced from 85.71% to 71.3%²³.

Among the parameters evaluated by Student's t-test, it was found that the addition of a step of cooling in an ice bath before adding the anthrone reagent did not affect the color intensity of the final complex [calculated t-value (1.13) < tabulated t-value (2.78)] (Table 2), as also demonstrated by Olennikov and Tankhaeva²³. Therefore, in order to simplify the process, this step was removed from the optimized method.

Regarding the agitation using vortex, it was found that the samples that were not subjected to this step remained with two phases and; therefore showed different values from the samples subjected to the general method [calculated t-value (42.67) > tabulated t-value (2.78)] and high coefficient of variation (Table 2). Because of these reasons, this step was kept in the method.

Likewise, the step involving the heating of the samples in a water bath at 100°C was essential, once the characteristic blue-green color did not appear for the samples that were not heated [calculated t-value (49.18) > tabulated t-value (2.78)] (Table 2). Although there are reports saying that the heating caused in the reaction mixture is sufficient as a heat source for the color development, Scott and Melvin²⁴ noted that it is necessary to maintain a high temperature during the reaction in order to obtain consistent results, placing the mixture in a bath with temperature control for some time.

Furthermore, the heating and the difference in the rate of reaction of anthrone with ketohexoses or aldohexoses have been used to differentiate the presence of glucose and fructose in mixtures. At room temperature, anthrone forms a blue color with fructose while a similar coloration is only obtained for glucose after heating^{22, 25}. Therefore, based on the results obtained in this study, it is possible to estimate that the samples of *T. occidentalis* does not have fructose in their constitution.

Additionally, it was shown that the possible loss of water in the process of heating is a negligible source of error once there was no statistical significant variation of the polysaccharide content for the samples heated in test tubes with and without caps [calculated t-value (0.14) < tabulated t-value (2.78)] (Table 2).

It is noteworthy that a similar result was also shown by Scott and Melvin²⁴, although the form of the authors' evaluation has been restricted to verifying the weight of the test tubes containing the samples before and after heating.

Finally, taking into account that the variation of the heating time is one of the error sources in the anthrone method²⁴ and that the maximum color development is achieved when the rate of formation of the chromogen, and its destruction in a heated acid reaches equilibrium²², the influence of the heating time was evaluated as provided in Table 3.

Although a significant variation in the polysaccharide content among all heating times could be verified using ANOVA [calculated F-value (46.88) > tabulated F-value (3.10)], it was verified by Student's t test the presence of different behavior among the time intervals, such as the significant increase in the polysaccharide content reached until 7.5 minutes, followed by increased stability in the range from 7.5 to 12.5 minutes, with a slight decrease after 12.5 minutes (Table 3).

Thus, in order to decrease sources of error relating to heating, the selected time for the method was 10.0 minutes, which is consistent with the data reported in the literature. This is because the heating time of 10 minutes at 100°C has been efficiently adopted to estimate soluble sugars in plant materials¹³.

It is known that some sources of error, such as the heating time, do not exert significant variation for the same group of samples once they will be constant for all samples. This will happen if the bath has an excellent circulation to heat uniformly all the test tubes²⁴, a prerequisite for obtaining reproducible results²⁵.

Validation of the analytical method:

- 1. Linearity, limits of detection (LOD) and quantification (LOQ):** To assess the linearity, linear regression was performed on the curves obtained by plotting five

concentrations of the extractive solution in the range from 0.1 to 0.25 g/mL and five aliquots of test solution in the range from 0.2 to 0.6 mL. The data obtained by means of linear regression using the least squares resulted in values of coefficients of determination (R^2) of 0.9987 and 0.9945 for the extractive solution and test solution, respectively. This indicates that more than 99% (99.87 and 99.45%, respectively) of experimental variability is explained satisfactorily by the equations ($y=1.5067x + 0.1661$, for the extractive solution, $y = 8.4345x - 0.0956$, for the test solution), confirming the linear relationship between the concentration of the analyte and the spectrophotometric response.

The data allowed us to affirm that the performance of the method meets the requirements recommended for the analysis of this parameter.

Considering the limits of detection (LOD) and quantification (LOQ), the results were 0.052 and 0.175 mg/g for the extractive solution, and 0.053 and 0.177 mg/g for the test solution. Based on these results, it was found that the procedures provide sensitive spectrophotometric detection and quantification of total polysaccharides *T. occidentalis* with the desired reliability.

- 2. Robustness, Precision and Accuracy:** The data (Table 4) showed that the method was robust for all the parameters, once all the calculated F-values were lower than the tabulated F-values (One-Way ANOVA).

The method provided accurate spectrophotometric response for the two analyzed levels: repeatability and intermediate precision. Regarding the repeatability, the results presented an average polysaccharide content of 3.14 mg/g and a CV of 4.78%, which is below the maximum of 5% recommended by RE n°899²⁰ and of 15% for plant materials²⁶.

TABLE 4: ROBUSTNESS OF THE OPTIMIZED METHOD FOR THE DETERMINATION OF POLYSACCHARIDES OF *T. OCCIDENTALIS*

Parameters	Variables	Time (min)	Mean (mg/g) ± SD	Statistical evaluation
Manufacturer of ethanol	Quimex® Alphatec®	---	3.02 ± 0.10	calculated t- value = 0.86 tabulated t-value = 2.78
		---	3.08 ± 0.07	
Manufacturer of H ₂ SO ₄	Proquímicos® Vetec®	---	3.02 ± 0.10	calculated t- value = 0.37 tabulated t-value = 2.78
		---	2.98 ± 0.09	
Centrifugation time	Deproteinization	4	2.91 ± 0.15	calculated F-value = 0.82 tabulated F-value = 5.14
		5	2.98 ± 0.03	
		6	3.03 ± 0.12	
	Precipitation of polysaccharides	14	2.95 ± 0.05	calculated F-value = 4.48 tabulated F-value = 5.14
		15	2.98 ± 0.03	
		16	2.90 ± 0.01	
Brand of the centrifuge	5810 R Eppendorf® Excelsa 2 Fanen®	---	2.79 ± 0.07	calculated t- value = 1.83 tabulated t-value = 2.78
		---	2.87 ± 0.01	

SD – Standard Deviation; CV – Coefficient of variation.

The intermediate precision was demonstrated through Two-Way ANOVA and according to this statistical evaluation the procedures were precise

for analyzes performed by different analysts on the same day and different days ($p < 0, 05$) (Table 5).

TABLE 5: RESULTS OF INTERMEDIATE PRECISION

Analysts	Day 1 (mg/g) ± SD	Day 2 (mg/g) ± SD	Statistical evaluation (Analyst)	Statistical evaluation (Day)
Analyst 1	3.16 ± 0.09	3.21 ± 0.05	calculated F-value = 0.71	calculated F-value = 5.32
Analyst 2	3.11 ± 0.09	3.20 ± 0.02	tabulated F-value = 2.84	tabulated F-value = 5.32

SD – Standard Deviation.

The accuracy of the method, calculated by the recovery test was evaluated from two aspects: the ability to extract the analyte and the repeatability of the responses (Table 6). According to the obtained data, there was no significant interference of the

method on the recovery of polysaccharides because the responses were remarkably accurate, with recovery rates within desirable limits (near 100%), which was confirmed by the lower CV at all levels tested²⁰.

TABLE 6: RESULTS OF THE RECOVERY TEST

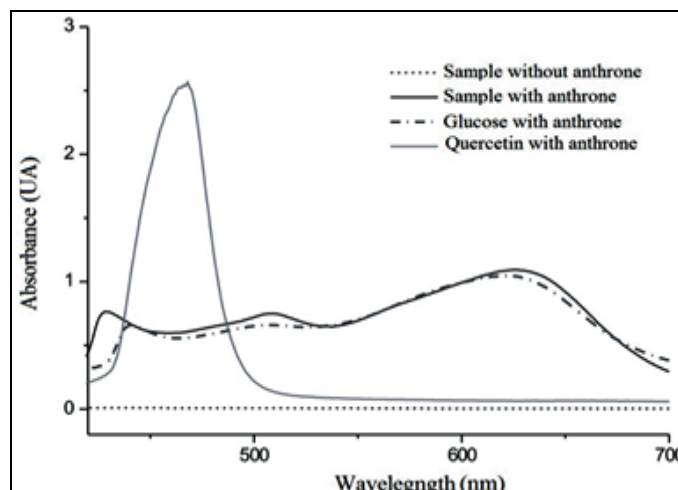
Extrative solution (g/mL)	Theoretical value (µg/mL)	Real value (µg/mL) ± SD (CV%)	Recovery (%)
0.15	3.00	2.99 ± 0.04 (1.46)	99.81
0.20	3.59	3.51 ± 0.02 (0.49)	97.85
0.25	4.41	4.31 ± 0.03 (0.78)	97.76

SD – Standard Deviation; CV – Coefficient of variation

Specificity: Figure 2 shows the superposition of spectra of the following samples: test solutions with and without the anthrone reagent, glucose with the reagent and quercetin also with the reagent.

When we compared the spectra of the test solutions and glucose with the one of quercetin, the data presented showed that there is no significant similarity between them.

This confirms that the wavelength of 625 nm for the method is satisfactory for the chosen marker of the species.

**FIGURE 2: SPECTRA OBTAINED FROM THE ANALYSIS OF SPECIFICITY**

CONCLUSION: The optimized and validated spectrophotometric analytical method for the quantification of total polysaccharides showed performance according to the recommended sanitary specifications for bioanalytical methods, proving to be a viable and suitable tool for the quality control of the plant drug and products derived from the aerial parts of *T. occidentalis*. Also, taking into account the variability and complexity in the plant kingdom, we suggest a similar evaluation of the parameters described in this work for other plant species.

In conclusion, this work demonstrated that the anthrone method was appropriate for *T. occidentalis*. Besides that, the satisfactory results were obtained by employing a smaller number of steps in a short time interval (about 6 hours) in contrast to the different methods reported in the scientific literature that last 41 hours.

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