A new method for quantification of total polyphenol content in medicinal plants based on the reduction of Fe(III)/1,10-phenanthroline complexes

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ABSTRACT

This paper proposes an alternative analytical spectrophotometric method for the total polyphenol quantification in the aqueous extract of plants. When these extracts are added to solutions of Fe(III), in presence of 1,10-phenanthroline, the absorbance values at 551 nm (A_{511nm}) due to the $Fe(phen)_3^{2+}$ complexes formed are proportional to the total polyphenol concentration expressed as pyrogallic acid (PA). A typical calibration graph of A_{511nm} values vs. PA is linear from 0.16 to 0.64 mg L^{-1} (r = 0.994, n = 6) with a limit of detection 0.041 mg L^{-1}. The results of the polyphenol content of the aqueous extracts of twenty medicinal Brazilian plants obtained with the proposed method were compared with values using the Folin-Ciocalteu reagent. A paired t-test revealed no statistically significant difference for the species analyzed. The total antioxidant capacity of the same twenty extracts was determined based on the scavenging of stable radical DPPH.

Keywords: Polyphenols; Medicinal Brazilian Plants; 1,10-Phenanthroline; Iron (III); Antioxidant Activity

1. INTRODUCTION

Since about 1930 studies have demonstrated that among all the metal ions which are complex by the uncharged ligand 1,10-phenantroline (1,10-phen), the iron(II) complexes formed, $Fe(phen)_3^{2+}$, is the species which produces more intense color [1]. This orange-red ferrous chelate is very stable (log β_3 = 21) [2] and has been used for the determination of iron ion in various types of samples after reduction of total iron to Fe(II) by the addition of a suitable reducing agent. For this purpose, several reducing compounds have been used like hydroxylamine hydrochloride, hydrazine, sodium sulphite (or hydrosulphite), stannous chloride, sodium formate, formaldehyde, sodium hypophosphite, ascorbic acid, hydroquinone and tannic acid [1].

Consequently, any reducing agent capable of converting Fe(III) to Fe(II) in a medium containing 1,10-phen could be indirectly determined by measuring the amount of $Fe(phen)_3^{2+}$ complexes formed. In spite of that, there are not many analytical methodologies available in literature for the quantification of reducing compounds using the $Fe(phen)_3^{2+}$ complexes. Some examples are presented in **Table 1**.

Ascorbic acid is the compound that has been more indirectly determined in complex matrices such as fruit juices, soft drinks and teas [3], fruits and vegetables [4], beers and soft drinks [5] and tablets [6]. The total concentration of tannins (or tannic acid) in teas and beers [7], hop [8] and tannery wastewater [9] was also determined. The majority of analytical methods available in the literature for these two analytes can probably be attributed to the fact that both are strong reducing agents in aqueous solution, which makes the procedure more easily performed.

α-Tocopherol, an oil-soluble vitamin, required the use of surfactants for its determination in oil facial [10] and milk powder [11]. Hydrogen sulfide was quantified in various samples (wastewater, lake water, coconut, sugarcane juice and egg) in acidic media waiting some minutes for the total reduction of Fe(III) [12].

Formaldehyde was determined in wastewater after reaction with an excess of a stronger reducing agent,

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Table 1. Selected spectrophotometric analytical methods for indirect determination of reducing compounds exploiting the $Fe(phen)_3^{2+}$ complex formation.

LR (g/mL)	LD (g/mL)	Analyte	Remarks	Ref.
0 - 8	NA	Ascorbic acid	Detn. of AA in canned fruit juices, cordials, tea and soft drinks. Background correction was needed and interference was noted in presence of TA, PA and sulphite.	3
0 - 2.8	NA	Ascorbic acid	Detn. of AA in fruits and vegetables based on the formation of the ionic pair between $Fe(phen)_3^{2+}$ and BPR in the presence of CTMAB (pH 6.0, HOAc-NaOAc). RSD 1.1 - 3.7%.	4
5 - 80 1 - 80	2.7 0.5	Ascorbic acid (NPC) Ascorbic acid (PC)	Two spectrophotometric FIA methods (NPHC and PHC) for the detn. of AA in soft drinks and beers. The NPHC is based on the reduction of Fe(III) forming the $Fe(phen)_3^{2+}$ complexes. The PHC uses the irradn. with visible light to achieve higher sensitivities. NFC has RSD 1.6% (n = 11) and 60 samples h^{-1}. PC has 1.0% (n = 11) and 40 samples h^{-1}.	5
0.50 - 4.00 1.00 - 8.00	NA NA	Ascorbic acid L-Cysteine	Simultaneous detn. of AA and L-Cys in tablet samples using the HPSAM based on the difference of the reaction rates between AA and L-Cys with Fe(III). RSD for 3.0 ppm (n = 6) for AA (2.3%) and L-Cys (2.7%).	6
0 - 30	NA	Tannins	FIA method for the detn. of tannins in tea and beer. GA as std. RSD for 10 mg L^{-1} GA 0.7% (n = 10); 60 samples h^{-1}.	7
0.10 - 5.0	0.05	Tannins	Detn. of tannins in tannery wastewater; RSD is 0.492 %.	8
0 - 300	0.84	Tannic acid	FIA kinetic method for detn. of TA in hops. TA is injected into a stream of Fe(III) and mixed with 1,10-phen (0.03 mol L^{-1} H_2SO_4 medium). 71 samples h^{-1} (RSD 1.1%).	9
5.17 - 400	0.86	α-Tocopherol	FIA method for the detn. Of -tocopherol in facial oil using SDS oil in water micro emulsion as single-phase extraction. 60 samples h^{-1} (RSD 0.03%).	10
0.21 - 43	0.05	α-Tocopherol	FIA method for detn. of α-tocopherol in milk powder. 30 samples h^{-1} (RSD 1.2%, n = 4).	11
035 - 1.40	NA	Hydrogen sulfide	Detn. of H_2S in lake water, industrial effluents, tender coconut, sugarcane juice and egg.	12
0.25 - 1.0	0.02	Formaldehyde	FIA method for detn. of HCHO in wastewater based on the conndensation of HCHO with $(NH_2OH)_2.H_2SO_4$ producing formaldoxime. Residual $(NH_2OH)_2.H_2SO_4$ reduces Fe(III) in presence of 1,10-phen. RSD was 2.03 % for 0.25 mg L-1. (n = 4). 15 samples h^{-1}.	13
0.18 - 180	0.09	Glucose	FIA method for detn. of glucose in wine and human blood plasma. The method is based on passing a glucose solution containing Q through a column immobilized with glucose oxidase. In the flow system Q is reduced to HQ glucose and the HQ formed reduces Fe(III) in a 1,10-phen medium. 24 samples h^{-1}. RSD was 0.78% (n = 5) for 5 M glucose.	14
1 - 120	0.5	Citric acid	FIA method for detn. of CA in beverages based on the photoreduction of Fe^{3+} to Fe^{2+} by CA under UV or visible radiation. RSD 2.8%. 40 samples h^{-1}.	15
0.16 - 0.64	0.066	Total polyphenols	Detn. of total polyphenol content in 20 medicinal Brazilian plants. PA as std.	this work

LR = linear range; LD = limit of detection; AA = ascorbic acid; TA = tannic acid; PA = pyrogallic acid; GA = gallic acid; CA = citric acid; RSD = relative standard deviation, NA = not available; CTMAB = cetyl trimethylammonium bromide; FIA = flow-injection analysis; SDS = sodium dodecyl sulfate; BPR = bromopyrogallol red; NPHC = nonphotochemical; PHC = photochemical; Q = quinone; HQ = hydroquinone; L-Cys = L-cysteine; HPSAM = H-point standard addition method; NB = nitrobenzene; CTMAB = hexadecyl(trimethyl)azanium bromide;

hydroxylamine sulfate, in which residue was then able to reduce Fe(III) in a medium containing 1,10-phen [13]. Glucose was analyzed in human blood plasma and wine after its redox reaction with quinone in an immobilized glucose oxidase column yielding hydroquinone, which then reduced Fe(III) [14]. Fe(III) was photochemically reduced by citric acid upon irradiation of visible and UV light and the Fe(II) formed was determined with 1,10-phen [15]. Despite these interesting strategies, the indirect determination of reducing agents weaker than ascorbic acid and tannins (such as formaldehyde, glucose and citric acid) in complex samples seems to demand more elaborate analytical procedures or consume more chemical reagents.

Lau *et al.* developed an accurate spectrophotometric method for the determination of tannins in tea and beer samples [16]. In that study, the reduction of Fe(III) to Fe(II) by tannins was carried out at 80°C for 20 min and then the Fe(II) formed was reacted with 1,10-phen (at pH = 4.4) to form the orange-red $Fe(phen)_3^{2+}$ complex. However, in order to reduce the interference from other reducing compounds in these samples, it was necessary to obtain a "tannin free" solution. For this purpose, a background correction was carried out by precipitating tannins twice (with gelatine and kaolin) which rendered the method tedious and time consuming [16].

In the present work, the analytical calibration graph obtained by using pyrogallic acid as reducing agent of Fe(III), in the presence of 1,10 phen, was used to determine the total polyphenol content in aqueous extracts of twenty Brazilian medicinal plants. No background correction was needed and the absorbance values obtained (at 511 nm) due to the $Fe(phen)_3^{2+}$ complexes formation, after the addition of 1,10-phen, were found to be

proportional to the polyphenols' concentration.

All results obtained by this reaction were compared with the values obtained with the Folin-Ciocalteu reagent which was recommended by the Brazilian Pharmacopoeia for determining the total content of polyphenol in plant species [17].

Some of the plants investigated in this study have not been extensively studied but have widespread use in folk medicine in the treatment of several diseases. For these species the value of the total polyphenol content is not yet standardized, so they are not available in the Brazilian Pharmacopoeia.

The values of the antioxidant capacity of the same twenty plant species were determined using the well-known method of the scavenging of the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and compared with their values of total polyphenol content.

2. MATERIAL AND METHODS

2.1. Apparatus

All spectrophotometric measurements were made in a HPUV 8453 (Agilent) spectrophotometer using a 1.0 cm optical path length glass cell.

2.2. Reagents

Reverse osmosis water (Quimis, Q842-210, Brazil) was used to prepare the solutions of analytical-grade chemicals (except when another solvent is indicated).

A 192 g L^{-1} $Fe(ClO_4)_3$, stock solution was synthesized and standardized as described in previous studies [18]. A diluted 3.54 g L^{-1} working solution was prepared by accurate dilution.

A 0.101 g L^{-1} pyrogallic acid ($C_6H_6O_3$, FW 126.11 g·mol^{-1}, 99%, Labsynth, Brazil,) stock solution was prepared by dissolving 0.0504 g in 500.0 mL. A 12.6 and 5.04 mg L^{-1} working solutions were obtained by dilution in water just before the use.

A 3.28 g·L^{-1} 1,10-phenanthroline (1,10-phen) stock solution was prepared by dissolving 1.64 g of 1,10-phen monohydrate ($C_{12}H_8N_2.H_2O$, FW 198.26 g·mol^{-1}, 99.5%, Merck, Brazil,) in 10.0 mL ethanol (CH_3CH_2OH, FW 46.06 g·mol^{-1}, 99.5%, Synth, Brazil) and then diluted in water in a 500.0 mL volumetric flask.

A 3.72 g·L^{-1} ethylenediaminetetraacetic acid (EDTA) solution was prepared by dissolving 1.86 g of disodium ethylenediaminetetraacetate dihydrate ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$, 372.24 g·mol^{-1}, 99%, Synth, Brazil) in a 500.0 mL volumetric flask.

Acetate buffer solution (HAc/KAc) pH 4.6 prepared by dissolving 14.3 mL of glacial acetic acid (CH_3COOH, FW 60.05 g·mol^{-1}, 99.8%, Merck, Brazil) and 20 g of potassium acetate (CH_3COOK, FW 98.15 g·mol^{-1}, 99%, Merck, Brazil) in water in a 1.0 L volumetric flask.

The Folin-Ciocalteu (FC) reagent was prepared as described in Brazilian Pharmacopoeia [17]. 20 g of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$, FW 329.86 g·mol^{-1}, 99%, Vetec, Brazil), 4.0 g of phosphomolybdic acid ($H_3Mo_{12}O_{40}P \cdot H_2O$, FW 1825.55 g mol^{-1}, 99%, Sigma, Brazil), and 10 mL of phosphoric acid (H_3PO_4, FW 98.00 g·mol^{-1}, 85%, d = 1.88 g·mol^{-1}, Merck, Brazil) were dissolved in 150 mL of water. This solution was heated under reflux for 2 hours and after cooling at room temperature, the solution was diluted to 200 mL with water. When the FC reagent is not being used it is kept in a refrigerator at 8°C.

A 10 % (m/v) sodium carbonate (Na_2CO_3, FW 105.99 g·mol^{-1}, 99%, Vetec, Brazil) solution was prepared in water.

A 50% (v/v) methyl alcohol (CH_3COH, FW 32.04 g mol^{-1}, d = 0.792 g cm^{-3}, Merck, Brazil) and a 70% (v/v) acetone (CH_3OCH_3, FW 58.08 g·mol^{-1}, d = 0.79 g cm^{-3}, Merck, Brazil) solutions (both 1.0 L) were prepared in water.

A 23.7 mg·L^{-1} DPPH (2,2-diphenyl-1-picrylhydrazyl, $C_{18}H_{12}N_5O_6$, FW 394.32 g·mol^{-1}, Sigma-Aldrich) solution was prepared daily just before the use by dissolving 2.40 mg in methyl alcohol in a 100.0 mL volumetric flask.

2.3. Procedures

2.3.1. Sample Preparation for Polyphenol Determination

The procedure described in the Brazilian Pharmacopoeia was used to extract the soluble polyphenol content of plant samples [17]. 0.75 g of dry material of each plant was transferred to a 250 mL erlenmeyer containing 150 mL of water which was maintained on a water-bath for 30 min at 80°C - 90°C. After cooling, the mixture was transferred to a 250.0 mL volumetric flask and made up with water and left the plant material being decanted. This solution was filtered and then discarding the first 50 mL of the filtrate. Then 5.0 mL of the filtrate above were transferred to a 25.0 mL volumetric flask which was completed with water.

2.3.2. Sample Preparation for Antioxidant Capacity Determination

1.00 g of the dry material was transferred to a 100 mL beaker in which was added 40 mL of a 50% (v/v) methyl alcohol aqueous solution. After homogenization, the mixture was allowed to stand for 1 hour at room temperature. This mixture was centrifuged (15,000 rpm, 15 min, diameter 12 cm) and the supernatant was transferred to a 100.0 mL volumetric flask. Next, 40 mL of a 70% (v/v) acetone aqueous solution material were added in the centrifuged. After homogenization, the mixture was also allowed to stand for 1 hour at room temperature.

Then, this mixture was centrifuged (15,000 rpm, 15 min, diameter 12 cm) and the supernatant obtained was transferred to the same 100.0 mL volumetric flask. Water was used to fill the volume of this flask [19].

2.3.3. Reference Procedure for Polyphenols Quantification (FC Reagent)

The procedure was carried out as described in the Brazilian Pharmacopoeia for total polyphenol quantification (with a 10-fold reduction in the volume of all reagents used) [17]. A calibration graph was obtained by mixing aliquots of 100 to 700 μL from a 12.6 mg·L^{-1} pyrogallic acid, PA, standard solution, with 200 μL of FC reagent and completed with a 10% Na_2CO_3 solution in a 5.0 mL volumetric flask.

Five mL of the aqueous extracts of plants (item 2.3.1) were transferred to a 25.0 mL volumetric flask and completed with water.

The multiple standard addition method was utilized for analysis of all aqueous extracts, as follows: 100 - 300 μL (depending on the species) of the aqueous extract were transferred into five flasks (5.0 mL) followed by addition of 200 μL of FC reagent. In four out of five 5.0 mL volumetric flasks, aliquots of 100, 300, 500 and 700 μL of a 12.6 mg·L^{-1} of PA standard solution were added and then the flasks were completed with 10% Na_2CO_3 solution.

In both graphs (calibration and multiple standard additions with the samples) the absorbance was measured at 715 nm (A_{715nm}) after 30 min. using water as reference solution.

2.3.4. Proposed Procedure for Polyphenols Determination $Fe(phen)_3^{2+}$ Complexes)

The calibration graph was obtained by transferring different volumes (0.4 to 2.5 mL) of 5.04 mg·L^{-1} PA standard solution to testing tubes (12 mm × 120 mm) containing 2.5 mL of 3.54 g·L^{-1} $Fe(ClO_4)_3$ solution. The tubes were then placed in a water bath and maintained at 80°C for 20 min. After, the solutions contained in these tubes were transferred to 25.0 mL volumetric flasks and then were adding 2.5 mL of acetate buffer solution (pH 4.6), 5.0 mL of 3.28 g·L^{-1} 1,10-phen and 2.5 mL of 3.72 g L^{-1} EDTA. The volume was completed with water and after cooling the absorbance measurements were made at 511 nm.

The multiple standard addition method was used in all extracts samples analysis as follows: 100 μL of the samples of aqueous extracts (item 2.3.1) were transferred to five testing tubes. In four out of five tubes, aliquots of 0.4, 0.8, 1.2 and 1.6 mL of a 5.04 mg·L^{-1} of PA standard solution were added. Next, the procedure for obtaining the calibration graph was repeated.

In both graphs (calibration and multiple standard additions with the samples) the absorbance values, after cooling, were measured at 511 nm.

A mixture containing 354 mg·L^{-1} $Fe(ClO_4)_3$, 656 mg·L^{-1} 1,10-phen, 372 mg·L^{-1} EDTA in acetate buffer (pH = 4.6) medium, after undergoing the above procedure, was used as reference solution.

2.3.5. Determination of the Total Antioxidant Capacity Based on the Scavenging of the Stable Radical DPPH

The DPPH scavenging assay for the total antioxidant capacity determination was carried out based on the procedure described by Sánchez-Moreno *et al*. [20]. The DPPH• is a stable free radical in solution and its concentration can be followed by the absorbance measurements at 515 nm (A_{515nm}). The decrease in absorbance value by adding aliquots of plant extract is related to the total antioxidant capacity.

Firstly, the absorbance values at 515 nm (A_{515nm}) of five DPPH diluted solutions (2.37 - 18.9 mg L^{-1}) obtained from the 23.7 mg L^{-1} DPPH stock solution were taken using methyl alcohol for zeroing the spectrophotometer. The DPPH calibration graph determined by linear regression was achieved by plotting the A_{515nm} values as a function of DPPH concentration.

$$A_{515nm} = a + b \times DPPH \qquad (1)$$

In order to evaluate the total antioxidant capacity of plant extracts the following procedure was performed. A control solution was prepared adding 0.125 mL of an aqueous solution containing methyl alcohol and acetone (40 mL of 50% (v/v) methyl alcohol + 40 mL of 70% (v/v) acetone in 100 mL) in a 5.00 mL volumetric flask and the volume completed with the 23.7 mg·L^{-1} DPPH solution. The value corresponding to half of the A_{515nm} of the control solution is substituted in Equation (1) to obtain the corresponding DPPH value (in mg·L^{-1}), and the respective value in g of DPPH is calculated.

Another calibration curve is obtained in the presence of plant extracts. For each plant extract (item 2.3.2) four different diluted solutions varying from 0.05 - 0.5 mg·L^{-1} of dry material were prepared in the 50% methyl alcohol aqueous solution. A 0.125 mL of these plant extract dilution were transfer to a 5.00 mL volumetric flask and the volume completed with the 23.7 mg·L^{-1} DPPH solution. The A_{515nm} values were taken after 15 minutes using methyl alcohol to zeroing the spectrophotometer. The extract calibration graph was obtained by plotting these A_{515nm} values as a function of the dry material solutions where C_{DM} is in mg·L^{-1} as in Equation (2). It shows the decrease of the absorbance values due to the scavenging of the DPPH by the polyphenols.

$$A_{515nm} = a - b \times C_{DM} \qquad (2)$$

In order to express the total antioxidant capacity, the

half value of A_{515nm} of the control solution (obtained with DPPH solution without addition of plant extracts) is substituted in Equation (2). The value found corresponds to the dry material sample required to reduce by 50% the initial concentration of DPPH (EC_{50}). The EC_{50} results were expressed as g DPPH/g dry material (**Table 2**).

Table 2. Total polyphenols quantification using the Folin-Ciocalteu reagent and the $Fe(phen)_3^{2+}$ complexes, expressed as pyrogallic acid.
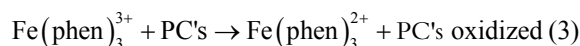
Plant	Common Brazilian name	Part used	Use in folk medicine [30]	Total polyphenols content[a]		Antioxidant capacity
				Folin-Ciocalteu [17]	$Fe(phen)_3^{2+}$	EC_{50}[b] [19, 20]
Annona muricata L.	Graviola	Leaves	Anti-diarrhea; against spasms	1.90 ± 0.06	1.06 ± 0.80	9.09
Baccharis trimera (Less.) DC.	Carqueja	Leaves and stalk	Female infertility and male impotence; liver problems; stomach dysfunctions	0.83 ± 0.09	0.27 ± 0.05	2.44
Bauhinia splendens Kunth	Escada de Jabuti	Barks	Hemorrhoids and anti-rheumatic	1.42 ± 0.14	1.33 ± 0.03	25.0
Casearia sylvestris Sw.	Guaçatonga	Barks	Burns injuries; herpes; skin injuries; gastritis	1.90 ± 0.14	1.40 ± 0.30	0.182
Carapa guianensis Aubl.	Andiroba	Barks	Ulcers; bronchitis; cicatrizant	2.55 ± 0.19	0.59 ± 0.05	25.0
Cordia ecalyculata Vell.	Porangaba	Leaves	Diuretic	1.30 ± 0.08	2.05 ± 0.66	8.33
Costus spicatus (Jacq.) Sw.	Canarana	Leaves	Pain and swelling	0.47 ± 0.10	0.49 ± 0.20	14.3
Dipteryx odorata (Aubl.) Willd.	Cumaru	Seeds	Spasm; headache;	1.40 ± 0.12	0.75 ± 0.21	14.2
Geissospermum laeve (Vell.) Miers	Pau Pereira	Barks	Fever; stomachache; dizziness	1.53 ± 0.06	0.16 ± 0.03	4.55
Hamamelis virginiana L.	Hamamelis	Leaves	Hemorrhoids, anal eczema, bleeding, angina	3.12 ± 0.08	3.11 ± 0.38	0.48
Himatanthus drasticus (Mart.) Plumel	Sucuúba	Barks	Inflammation; gastritis	0.57 ± 0.06	0.46 ± 0.04	0.97
Hymenaea courbaril L.	Jatobá	Barks	Cough; bronchitis	1.41 ± 0.19	1.43 ± 0.50	14.2
Lippia grandis Shauer	Salva de Marajó	Leaves	Headache; stomachache	2.40 ± 0.10	2.71 ± 0.20	9.09
Maytenus ilicifolia Mart. ex Reissek	Espinheira Santa	Leaves	Skin cancer; ulcers; indigestion; chronic gastritis; dyspepsia	0.70 ± 0.04	0.71 ± 0.10	7.69
Salacia impressifolia (Miers) A.C. Sm.	Miraruira	Stalks	Diabetes	3.52 ± 0.39	1.70 ± 0.48	33.3
Schinus terebinthifolia Raddi	Aroeira	Leaves	Washing wounds; ulcers	5.57 ± 0.52	3.52 ± 0.60	100
Stachytarpheta cayennensis (Rich.) Vahl	Gervão	Leaves	Stomach tonic and stimulant of gastric functions; anthelmintic	1.92 ± 0.05	1.72 ± 0.04	9.97
Stryphnodendron adstringens (Mart.) Coville	Barbatimão	Barks	Leucorrhoea; hemorrhoids; bleeding; diarrhea	11.8 ± 0.3	11.9 ± 1.9	1.59
Plantago major L.	Tanchagem	Leaves	Diuretic; anti-rheumatic, bronchitis	1.20 ± 0.08	0.90 ± 0.13	0.29
Vismia duckei Maguire	Lacre	Leaves	Anti-rheumatic; dermatosis	0.40 ± 0.06	0.12 ± 0.02	2.78

[a] average and standard deviation values expressed as g pyrogallic acid/100 g dry material. [b] EC_{50} expressed as g DPPH/g dry material.

3. RESULTS AND DISCUSSION

3.1. Chemical Features

The spectrophotometric method proposed to measure the total content of polyphenols in aqueous vegetable extracts is based on the reduction of Fe(III) by polyphenolic compounds (PC's) in a water bath at 80°C for 20 min. The Fe(II) formed reacts with the 1,10-phen ligand yielding the reddish-orange $Fe(phen)_3^{2+}$ complexes according to Equation (3):

$$Fe(phen)_3^{3+} + PC's \rightarrow Fe(phen)_3^{2+} + PC's \text{ oxidized} \quad (3)$$

In the proposed procedure the ratio "total metalic ion: ligand" = 1:3 was kept, with 10 % excess of the latter. Thus, in all working solutions the concentrations of metal ion and ligand were 354 mg·L^{-1} Fe(III) and 656 mg·L^{-1} 1,10-phen.

Most studies involving the formation of $Fe(phen)_3^{2+}$ kept the pH of working solution between 3 to 6, so in the present study acetate buffer was used to maintain the pH at 4.6. Addition of a suitable masking agent, like EDTA, is needed for the complexion of the unreacted residual Fe(III) in order to avoid the absorbance interference of $Fe(phen)_3^{3+}$ [3,16].

The order of addition of reagents (Fe(III) + PA + buffer solution + EDTA + 1,10-phen) is required to obtain consistent and reproducible results. It was noted that the development of a color solution is very slow at room temperature, so the final solution was kept in a water bath (80°C for 20 min.) as done in previous works [16].

The absorption spectra of the Fe(III) solution in presence of 1,10-phen before and after addition of PA standard solution are represented in the **Figure 1**. The absorbance value at 511 nm, A$_{511nm}$, due to the $Fe(phen)_3^{2+}$ complexes is linear with PA concentration from 0.16 to 0.64 mg·L^{-1}, described by the equation A$_{511nm}$ = 0.0929 + 1.172 × PA (r = 0.994, n = 6). The limit of detection, LD, calculated as three times the standard deviation of the intercept, divided by the slope of the regression equation, obtained by the least-squares method for a calibration graph, was 0.041 mg·L^{-1}. The relative standard deviation, RSD, for 10 measurements of 0.24 mg·L^{-1} PA standard solution was 1.3%. The angular coefficient, defined as apparent absorptivity for PA (at 511 nm) was (1.18 ± 0.11) L·cm^{-1} mg^{-1} for 12 calibration graphs (RSD 8.6%) which makes the proposed method very sensitive to polyphenol-rich complex samples such as plant extracts [18,21].

3.2. Interference Study

In a previous published article dealing with the determination of tannins in tea and beer samples using the
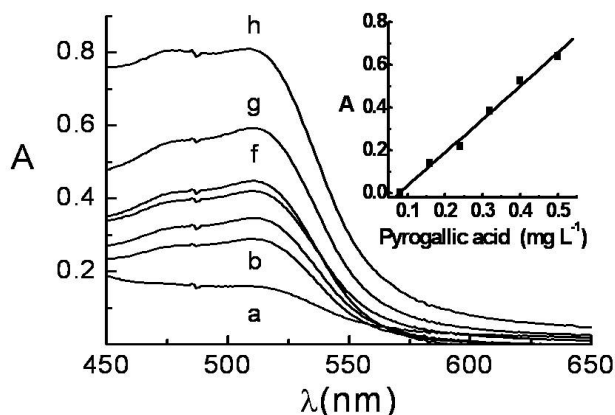

Figure 1. Absorption spectra of: (a) = Fe(III) 354 mg L^{-1} + acetate buffer solution pH = 4.6 (HAc 0,025 mol L^{-1}/KAc 0,020 mol L^{-1}) + 1,10-phen 656 mg L^{-1} + EDTA 372 mg L^{-1}, (b to g) = (a) + Pyrogallic acid (mg L^{-1}) 0.8, 0.16, 0.24, 0.32, 0.40 and 0.50, respectively. Absorbance measurements using water as reference solution. Inset figure: Calibration graph for pyrogallic acid using the absorbance at 510 nm of Fe(phen)$_3^{2+}$ complexes (b = 1.0 cm).

$Fe(phen)_3^{2+}$ complexes, it was pointed out that most of the compounds normally found in those samples showed no interference [16]. So in that study a background correction based on the precipitation of tannins with gelatin and kaolin was carried out to minimize interference due to other reducing substances (resulting in a "free tannin solution") before the absorbance measurements.

In the present work the influence of some non-phenolic compounds usually present in aqueous plants extracts was verified regarding to the reduction of Fe(III) to Fe(II) before the addition of 1,10-phen. Solutions containing one fixed concentration of PA (0.252 mg·L^{-1}) and three solutions containing this PA solution plus three known concentrations (0.252, 2.52 and 6.31 mg·L^{-1}) of the possible interfering species were analyzed following the proposed procedure (item 2.3.3.).

The interference criterion adopted was that the A$_{511nm}$ value of the mixture varied more than 10% from the A$_{511nm}$ value obtained for the 0.25 mg·L^{-1} PA solution alone.

Ascorbic acid (AA), a strong reducing agent, reduced Fe(III) to Fe(II) and a positive interference was observed when it was present in a PA:AA = 1:1 ratio (0.252/0.252 mg L^{-1}).

Citric acid (CA) also showed a positive interference when present in a CA:AA = 1:1 ratio (0.252/0.252 mg L^{-1}), which could be probably attributed to the photochemical reduction of Fe(III) to Fe(II) in the presence of CA [15].

Saponin has also shown a positive interference when present in 1:1 ratio.

Regarding sugars, interference was observed for fruc-

tose, glucose, lactose, sucrose and galactose from a 10-fold excess PA: sugars ratio = 0.252/2.50 mg·L^{-1}.

Nevertheless, the concentrations of those substances in aqueous extracts of plants species obtained from dry materials are usually lower than the polyphenols, then none significant interference of these substances is expected. Besides, for the polyphenols quantification by the present procedure, dilutions from 5 to 25-fold are necessary (item 2.3.1), which would probably prevent any possible interference that could arise from any reducing sugars, saponin, ascorbic and citric acids.

3.3. Statistical Treatment

Since we are dealing with samples of variable composition, the paired *t*-test [22] was performed for statistical comparison of the results for polyphenols quantification, represented by g PA/100 g of dry material, obtained with proposed method ($Fe(phen)_3^{2+}$ complexes) and accepted method (FC reagent). As can be seen in **Table 2**, the *t*-test value obtained (1.52) is less than the value tabulated (1.63) meaning that there is no statistically significant difference for the species analyzed.

3.4. Recovery Study

The recovery rate experiments were accomplished by spiking the sample with three different known amounts of [PA] standard solution, and the [PA] added was determined using the calibration graph "absorbance *vs*. [PA] standard solution".

The values obtained for the rates of recovery (**Table 3**) ranged from 61% - 121% (average 96%), which can be considered reasonable in complex samples as plant extracts.

3.5. Antioxidant Capacity

It can be noted from **Table 2** that the DPPH radical scavenging activities for the analyzed species (excluding *Stryphnodendron adstringens* (Mart.) Coville) are polyphenol content-dependent and decreased in the following order: *Schinus terebinthifolia* Raddi > *Salacia impressifolia* (Miers) A.C. Sm. > *Bauhinia splendens* Kunth ≈ *Carapa guianensis* Aubl> *Costus spicatus* (Jacq.) Sw. ≈ *Dipteryx odorata* (Aubl.) Willd. ≈ *Hymenaea coubaril* L. > *Stachytarpheta cayennensis* (Rich.) Vahl ≈ *Annona muricata* L. > *Lippia grandis* Shauer > *Cordia ecalyculata* Vell .≈ *Maytenus ilicifolia* Mart. ex Reissek > *Geissospermum laeve* (Vell.) Miers > *Vismia duckei* Maguire ≈ *Baccharis trimera* (Less.) DC. > *Stryphnodendron adstringens* (Mart.) Coville ≈ *Himatanthus drasticus* (Mart.) Plumel > *Hamamelis virginiana* L. > *Plantago major* L. ≈ *Casearia sylvestris* Sw.

A significant correlation (r = 0.785) was found between the antioxidant capacity values (g DPPH/g dry material/g) and the values of the total polyphenols content (g PA/100 g dry material) obtained with the FC reagent. Considering the values of the total polyphenols content obtained with the $Fe(phen)_3^{2+}$ complexes, this correlation is somewhat lower (r = 0.520) but still significant. Those results show that the $Fe(phen)_3^{2+}$ complexes could be also used to determine the antioxidant capacity of plant extracts.

3.6. Critical Considerations about the Use of the $Fe(phen)_3^{2+}$ Complexes and the FC Reagent in the Analysis of Total Polyphenol Content.

Herein are highlighted some relevant aspects of the use of $Fe(phen)_3^{2+}$ complexes and FC reagent in the analysis of the polyphenol content from the chemical point of view on which they are based.

a) In order to have quantitative oxidation of the phenolic compounds with the formation of the $Fe(phen)_3^{2+}$ complexes, the pH value should be between 2 - 9. In the majority of studies, the pH was kept between 3 - 6 using acetate or citrate buffer solutions. In the present study the pH was maintained at 4.6 with acetate buffer which is most commonly employed. Besides, citric acid was found to reduce Fe^{3+} to Fe^{2+} by the action of light [15]. In contrast, analyses with FC reagent must be conducted in an alkaline medium (pH = 10) which is easily achieved by the addition of sodium carbonate solution. At this pH the phenolic compounds are present as phenolates which are easier to oxidize. This may be the cause of obtaining results generally higher than those obtained with other methods [23-25]. Although the pH ranges in which these two reactants ($Fe(phen)_3^{2+}$ complexes and FC reagent) used are different, both methods have low selectivity in the analysis of polyphenols, for example, the specific identification of any polyphenol in a complex mixture is not possible.

b) It has been mentioned that the reduction of Fe(III) involves slow kinetic processes and that could hinder the quantification of reducing compounds in complex samples. In fact, it can be one of the reasons for the small number of analytical methods available in the literature using the $Fe(phen)_3^{2+}$ complexes for determining analytes that are potentially reducing agents (**Table 1**). The reduction of Fe(III) by cysteine in a medium containing 1,10-phen occurs slowly, but is accelerated in the presence of Cu(II) as a catalyst. This led to the development of a flow injection analytical method for spectrophotometric determination of copper [26]. The use of catalysts could be a good alternative for the development of analytical methods for other weak reducing agents. However, the procedure here presented for the determination of polyphenol content was carried out in a water bath at 80°C for 20 minutes and this incubation period

Table 3. Recovery rates from aqueous extracts of twenty Brazilian medicinal plants obtained with three different quantities added of pyrogallic acid (PA) using the $Fe(phen)_3^{2+}$ complexes.

Plants	Common Brazilian name	PA concentration (mg L^{-1})			
		Diluted aqueous extracts sample	Added	Found	Recovery (%)
Annona muricata L	Graviola	0.120	0.159	0.120	75
			0.240	0.183	76
			0.319	0.212	66
Baccharis trimera (Less.) DC.	Carqueja	0.252	0.0794	0.0820	103
			0.240	0.266	111
			0.319	0.330	104
Bauhinia splendens Kunth	Escada de Jabuti	0.0744	0.0794	0.0845	106
			0.240	0.221	92
			0.319	0.314	98
Casearia sylvestris Sw.	Guaçatonga	0.161	0.159	0.102	64
			0.240	0.146	61
			0.319	0.272	85
Carapa guianensis Aubl	Andiroba	0.248	0.159	0.140	88
			0.240	0.238	99
			0.319	0.335	105
Cordia ecalyculata Vell.	Porangaba	0.0668	0.159	0.129	81
			0.240	0.236	98
			0.319	0.235	74
Costus spicatus (Jacq.) Sw.	Canarana	0.0492	0.159	0.165	104
			0.240	0.232	97
			0.319	0.276	87
Dipteryx odorata (Aubl.) Willd.	Cumaru	0.163	0.159	0.130	82
			0.240	0.250	104
			0.319	0.348	109
Geissospermum laeve (Vell.) Miers	Pau Pereira	0.0706	0.0794	0.0858	108
			0.159	0.163	102
			0.319	0.351	110
Hamamelis virginiana L.	Hamamélis	0.0387	0.0794	0.0782	98
			0.159	0.140	88
			0.240	0.194	81
Himatanthus drasticus (Mart.) Plumel	Sucuúba	0.182	0.159	0.169	106
			0.240	0.256	107
			0.319	0.300	94
Hymenaea courbaril L.	Jatobá	0.161	0.159	0.139	87
			0.240	0.230	96
			0.319	0.288	90
Lippia grandis Shauer	Salva-de- Marajó	0.337	0.159	0.141	89
			0.240	0.264	109
			0.319	0.329	103
Maytenus ilicifolia Mart. ex Reissek	Espinheira-Santa	0.184	0.159	0.146	92
			0.240	0.256	107
			0.319	0.334	105
Salacia impressifolia (Miers) A.C. Sm.	Miraruira	0.214	0.159	0.125	79
			0.240	0.256	107
			0.319	0.375	117

Continued

Schinus terebinthifolia Raddi	Aroeira	0.0933	0.159	0.166	105
			0.240	0.253	106
			0.319	0.317	99
Stachytarpheta cayennensis (Rich.) Vahl	Gervão	0.206	0.159	0.144	90
			0.240	0.206	86
			0.319	0.289	91
Stryphnodendron adstringens (Mart.) Coville	Barbatimão	0.232	0.159	0.184	116
			0.240	0.237	99
			0.319	0.275	86
Plantago major L.	Tanchagem	0.185	0.159	0.146	92
			0.240	0.235	98
			0.319	0.320	100
Vismia duckei Maguire	Lacre	0.150	0.0794	0.0706	88
			0.240	0.286	119
			0.319	0.387	121

should allow all reducing compounds (even the weaker) to react completely with Fe(III). This could explain the reasonable agreement between the total polyphenol content values obtained with the $Fe(phen)_3^{2+}$ complexes and the FC reagent (**Table 2**). Moreover, such heating step makes the unnecessary use of surfactants (like cetyl-trimethylammonium bromide), as previously claimed in determining polyphenols in tea samples [27].

c) The color of the solution of the $Fe(phen)_3^{2+}$ complexes is very enduring. When stored in polyethylene bottles, the orange red solution remains unchanged for over a month outside the refrigerator (even in daylight). The reaction with the FC reagent is based on the reduction of phosphomolybdic-phosphotungstic acid forming a blue color complex which quickly fades.

d) The preparation of the FC reagent is very time-consuming. One of the ways of preparing it uses sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), phosphomolybdic acid ($H_3Mo_{12}O_{40}P \times H_2O$) and phosphoric acid ($H_3PO_4$) and it is necessary to keep the mixture of reactants boiling under reflux for 2 hours. Another way utilizes sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), hydrochloric acid (HCl) and lithium sulfate ($Li_2SO_4 \cdot 4H_2O$). In this case, it is necessary to boil under reflux for 10 hours. Alternatively, the ligand used in the proposed procedure (1,10-phen) is easily prepared by dissolving in a hydroethanolic solution (see item 2.2).

e) The reagents used in the proposed method (ferric salt, 1,10-phen, acetate buffer and EDTA) are less expensive and more available than those used to prepare the FC reagent.

f) If necessary, the $Fe(phen)_3^{2+}$ complexes can be extracted in organic solvents (chloroform, nitrobenzene, etc) with large anions (e.g., any long-chain alkyl sulphate or sulfonate) or even using ClO_4^- [28]. An analytical step

involving extraction can increase the sensitivity of the analyte under investigation.

g) When using the $Fe(phen)_3^{2+}$ complexes, the order of addition of reactants is mandatory. Previous studies have shown that complete reduction to Fe(III) is most easily accomplished by adding the reagents in the following order: Fe(III) + reducing agent + 1,10-phen + buffer [16]. In the present study the order was: Fe(III) + pyrogallic acid (or plant extract) + acetate buffer solution + EDTA + 1,10-phen, otherwise, discordant and non-reproducible results were obtained. For measurements using FC reagent, the order should be: pyrogallic acid (or plant extract) + FC reagent + sodium carbonate [25].

h) Analysis with FC reagent produces residue which is not generally recycled and needs to be properly disposed. Conversely, 1,10-phen can be recycled and even reused if the residue obtained after several analyzes is separately collected and concentrated. The residue is acidified with concentrated H_2SO_4 and the solution obtained is boiled in order to decompose the complex. Then, $CuSO_4$ solution is added until complete precipitation of $Cu(phen)SO_4 \cdot 2H_2O$. The precipitate is collected, washed with H_2SO_4 and with water, and then dissolved in a NH_4OH solution. A 1,10-phen liberated is extracted from this solution by shaking with $CHCl_3$ and being isolated by evaporation [1].

Given these observations, the spectrophotometric procedure proposed here to evaluate the total content of polyphenols in the aqueous extracts of plants is simple, easily operated and can be carried out with common laboratory equipment. The reagent used (*i.e.* 1,10-phen) is less expensive and easier to prepare than the FC reagent. Moreover, it is suitable for routine analysis and may be applied to aqueous plant extract samples containing low levels of polyphenols.

4. ACKNOWLEDGEMENTS

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APPENDIX
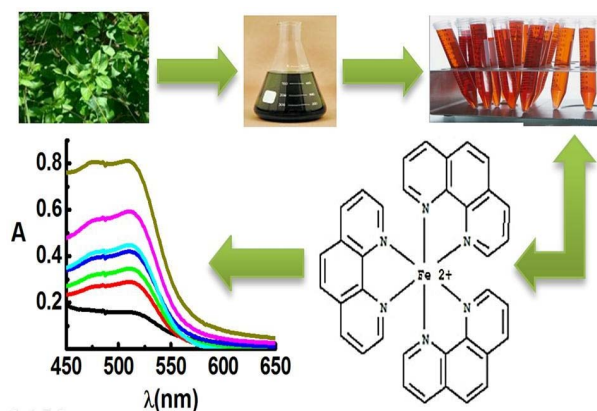
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