Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(9):4-10

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Optimization of the Folin-Ciocalteu Method for the Determination of Total Tannins from Guinea Grass (Panicum Maximum Grass)

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ABSTRACT

Folin-ciocalteu method was optimised and tested on panicum maximum grass samples and results showed that the method is reliable and consistent. Optimization was done in terms of volume of sample aliquot, volume of folins reagent, sodium carbonate, wavelength, extractant and ratio of volume of water to acetone in extractant. The volume of sample aliquot was found to be 0.1, while the volume of folins reagent was found to be 0.5 ml and the volume of sodium carbonate was found to be 2.5. 760 nm was found to be the best wavelength with 60% acetone being better to 50% methanol as an extractant. The quantity of tannins in panicum maximum grass was found to range from 0.63-0.67 µg/mL with an average of 0.65 µg/mL and the limit of detection was found to be 0.32 µg/mL while the limit of quantification was found to be 0.96 µg/mL. Statistical Analysis System *(SAS) was used to analyze data and the coefficient of variation was 12.6 with a standard error of 0.009369 and a standard deviation of 0.09554.*

Keywords: Optimised; Reliable; Consistent; Limit of detection; Limit of quantification

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INTRODUCTION

In arid and less developed countries, there is little animal feed supplementation as most farmers are subsistence and use 'free' range system where animals depend on native pasture for feeding during both the wet and dry seasons [1]. Natural pasture consists of various plant species and one of the most common as animal feed is browse plants. However, natural browse plants have higher levels of soluble tannin/phenolic compounds [2, 3]. These soluble tannin/phenolic compounds form complexes with proteins and carbohydrates [4, 5]. From the level of 2-4% tannins are beneficial as they bind proteins in the animal rumen making it not to be available for microbes which will otherwise use it for themselves [6]. These proteins will later become available to the animal down the tract after the pH has changed to around 8 [7]. At levels 5-9% the tannins are antinutritional as they completely bind the proteins such that even if the pH changes, the proteins won't be available. At these levels, tannins also reduce digestibility of fibre in the rumen [6]. At levels >9%, tannins are toxic and lethal to most animals [6]. Therefore it is important to know the levels of tannins in browse plants.

Currently, there is no official AOAC method of analysis for total tannins in grass which is the most common animal feed in Botswana and several methods have been developed for quantification of different types of tannins. Among these methods, the folin-ciocalteu method is the most highly reproducible and the most sensitive [5]. The folin-ciocalteu method is the only method that is used for quantification of total tannins; other methods like butanol-HCl method and vanillian-HCl method in methanol are used to measure condensed tannins while rhodanine method analyses gallotannins and wilson and hagerman method is employed for the determination of ellagitannins [5,8]. The Association of Official Agricultural Chemists onced listed folin-ciocalteu method for use on alcoholic beverages [9]. Hence it can be said that the folin-ciocalteu method is the only recommended method for total tannins.

Tannin's phenolic group is an excellent hydrogen donor that forms strong hydrogen bonds with the protein's carboxyl group. In the folin-ciocalteu method, polyphenols in plant extracts react with folin-ciocalteu reagent to form a blue complex that can be quantified by visible-light spectrophotometry [10]. folin-ciocalteu reagent is a mixture of phosphomolybdic and phosphotungstic acids and they are reduced to molybidenum and tungsten

oxides by -OH groups in the phenols [11]. The intensity of the colour is dependent upon the concentration of phenols and alkaline environment provided by the addition of sodium carbonate.

Previously, the Department of Agricultural Research Plants Laboratory (DAR-PL) was using the vanillin-HCl method, however, the method has several disadvantages [4, 12]. The disadvantages include; the method is not sensitive to low concentrations of tannin [13]. This is because low molecular weight flavanols overreact and large polymers underreact. In this method, catechin is used as standard but this monomer gives the maximum optical density leading to underestimation of large polymers [7]. It was also found that the method measures not only tannin but also flavan-3-ols and dihydrochalcones which are not tannins [4]. Also monomeric flavans gives higher color yield compared to tannins. Acetone which is the best extractor for tannins forms chromogen with acidified vanillin at peak range similar to that of tannins which interferes in the determination of tannins [4].

Therefore the aim of this work was to optimise and validate the folin-ciocalteu method for the determination of tannins in *panicum maximum* grass samples. This is because the folin-ciocalteu method can give inaccurate results therefore it is important to optimise this method for each type of forages [7, 14]. Inaccurate results come from the fact that interfering compounds such as ascorbic acid, tyrosine and possibly glucose are also measured [7,15,16]. Panicum maximum was chosen because it is one of the most productive native pastures in tropical areas and produces high yields of palatable fodder [17].

EXPERIMENTAL SECTION

Reagents

Acetone was obtained from SMM instruments Johannesburg, South Africa, folin-ciocalteu reagent was obtained from SMM instruments Johannesburg, South Africa, sodium carbonate was obtained from Rochelle, Johannesburg, South Africa, insoluble polyvinyl pyrrolidone (PVPP) was obtained from Sigma, Perth, Western Australia and Tannic acid was obtained from Merck, Galloping Hill Road Kenilworth, U.S.A.

Equipment

Drying oven was obtained from ELE International, New Delhi, India, Model 4 Laboratory hammer mill was obtained from Arthur H. Thomas Company, Philadelphia, USA. Weighing Balance was obtained from Adam Equipment in Danbury, Connecticut, USA, double-beam scanning spectrophotometer (PC model UVD 2950) was obtained from Labomed company, Los Angeles, United States, centrifuge (Model TJ-6) was obtained from Beckman Coulter, Brea, California, Pharmaceutical refrigerator (model MPR 311D(H)) was obtained from Sanyo Electrical Company, Japan and whirlimixer vortex was obtained from Fisons Ipswich, United Kingdom and water purification system was obtained from Millipore, Massachusetts, USA.

MATERIALS AND METHODS

Sampling and sample treatment

26 *Panicum maximum* grass samples were collected in the bush around Sebele Agricultural field, Sebele, Gaborone in a completely randomized method and were then dried at 70 °C for 48 h. Four replicates were made for each sample and were then powdered in laboratory harmer mill. 200 mg of each sample were then weighed into sample vials and 10 ml of 60% acetone was poured into each sample extract phenols. The samples were then kept for forty minutes with constant vortexing.

Standards

25 mg of tannic acid was weight into a beaker and diluted to 250 mL in a volumetric flask and diluting to the mark with deionized water. This solution contains 0.1 mg/mL of tannic acid. The following amount of this solution was pipetted into volumetric flasks; 0, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL. To these solutions; deionized water was added respectively in volumes of 2.0 mL, 1.9 mL, 1.8 mL, 1.7 mL, 1.6 mL and 1.5 mL then 0.5 mL of the folin-ciocalteu reagent and 2.5 mL of the sodium carbonate solution. These solutions were vortexed and kept for 40 min then absorbance 760 nm was recorded.

Before PVPP treatment

0.1, 0.2 mL and 0.5 of suitable aliquots of the tannin-containing extract will be taken in sample vial, then 1.9, 1.8 and 1.5 mL of deionized water was added followed by 0.1, 0.3, 0.5 and 1.5 mL of the folin-ciocalteu reagent and then 2.9, 2.7, 2.5, 2 and 1.5 mL of the sodium carbonate solution. The samples were vortexed, centrifuged at 3000 rpm for 10 min and absorbance at 760 nm after 40 min recorded.

After PVPP treatment

50, 100 and 150 mg of PVPP was weighed into sample vials, then 0.1, 0.2 mL and 0.5 of suitable aliquots of tannin-containing extract will be taken in sample vial, then 1.9, 1.8 and 1.5 mL of deionized water was added followed by 0.25, 0.5 and 1 mL of the folin-ciocalteu reagent and then 2.75, 2.5 and 2 mL of the sodium carbonate solution. The samples were vortexed, centrifuged at 3000 rpm for 10 min and absorbance at 760 nm after 40 min recorded.

Data treatment

Data were analyzed by the program SAS® Proprietary Software VERSION 9.4 Copyright (c) 2002-2012 by SAS Institute Inc., Cary, North Carolina, USA by one-way analysis of variance (ANOVA). The amount of total phenols as tannic acid equivalent was calculated from the above calibration curve. The total phenolic content was expressed on a dry matter basis.

RESULTS AND DISCUSSIONS

Adjustment of the mass of tannic acid

12.5, 25, and 50 mg of tannic acid was weight into a beaker and diluted to 250 mL in a volumetric flask and diluting to the mark with deionized water. These solutions respectively contain 0.05, 0.1 and 2 mg/mL of tannic acid. The following amount of this solution was pipetted into volumetric flasks; 0, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL. To these solutions; deionized water was added respectively in volumes of 2.0 mL, 1.9 mL, 1.8 mL, 1.7 mL, 1.6 mL and 1.5 mL then 0.5 mL of the folin-ciocalteu reagent and 2.5 mL of the sodium carbonate solution. These solutions were vortexed and kept for 40 min then absorbance at 760 nm was recorded. 0.1 mg/ml tannic acid was found to be better in terms of the intensity of the color of the standards as 0.05 mg/ml was too light and R^2 flactuated and with 0.2 mg/ml the color was so intense especially for the highest standard. The optimization of mass of tannic acid was very important as tannic acid was found to vary in composition [18].

Adjustment of the acetone:water ratio

Percentage of water to acetone was tested from 40 to 90% and it was found that 60% was best (figure 1). This same ratio was found by Karimi et al, 2015 when determining total phenols in oregano leaves [19]. Chavan and Amarowicz did and extraction experiment by varying the strength of acetone in water and find that 80:20 was the best for beach pea leaves [20].

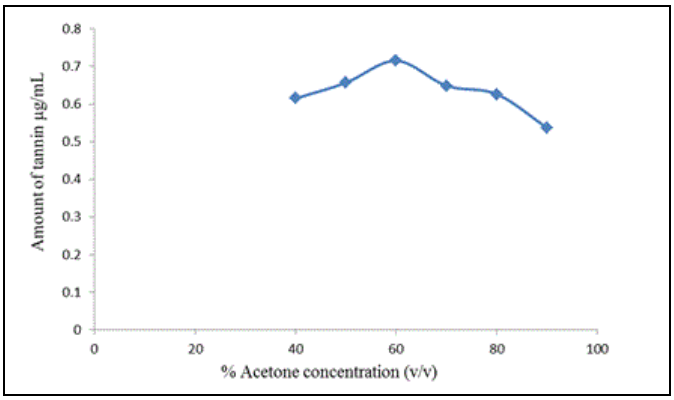

Figure 1: Showing amount of tannins got under different concentrations of acetone

Adjustment of the volume of Folin and sodium carbonate

25 mg of tannic acid was weight into a beaker and diluted to 250 mL in a volumetric flask and diluting to the mark with deionized water. These solutions respectively contain 0.05, 0.1 and 2 mg/mL of tannic acid. The following amount of this solution was pipetted into volumetric flasks; 0, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL. To these solutions; deionized water was added respectively in volumes of 2.0 mL, 1.9 mL, 1.8 mL, 1.7 mL, 1.6 mL and 1.5 mL then 0.25, 0.5 and 1 mL of the folin-ciocalteu reagent and 2.75, 2.5 and 2 mL of the sodium carbonate solution.

These solutions were vortexed and kept for 40 min then absorbance at 760 nm was recorded. 0.5 ml of folins reagent was found to be most optimum figure 2 while 2.5 ml was found to be the most optimum for sodium carbonate Figure 3.

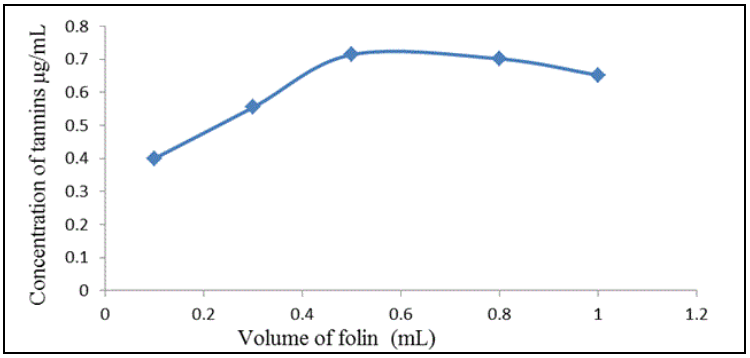

Figure 2: Showing amount of tannins got under varying volumes of folin

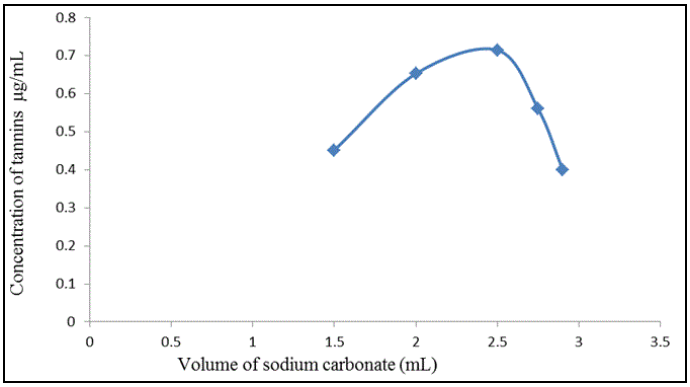

Figure 3: Showing amount of tannins got under varying volumes of sodium carbonate

Adjustment of the wavelength

25 mg of tannic acid was weight into a beaker and diluted to 250 mL in a volumetric flask and diluting to the mark with deionized water. This solution contains 0.1 mg/mL of tannic acid. The following amount of this solution was pipetted into volumetric flasks; 0, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL. To these solutions; deionized water was added respectively in volumes of 2.0 mL, 1.9 mL, 1.8 mL, 1.7 mL, 1.6 mL and 1.5 mL then 0.5 mL of the folin-ciocalteu reagent and 2.5 mL of the sodium carbonate solution. These solutions were vortexed and kept for 40 min then absorbances at 600, 650, 700, 720, 740, 760, 780, 800, 820 and 850 nm was recorded.

Wavelength of 760 nm was also found to be optimal figure 4. Wei, 2010 observed that the same wavelength was the best when performing an Online Determination of Trace Amounts of Tannic Acid in Coloured Tannery Wastewaters by Automatic Reference Flow Injection Analysis [21].

Standard solutions of tannic acid concentrations of 0, 5, 10, 15, 20 and 25 µg/mL were prepared and used for the calibration curve. The calibration equation was found to be y = 0.03238x - 0.00085 where y is the absorbance and x is the tannic acid concentration with a correlation coefficient of 0.9992.

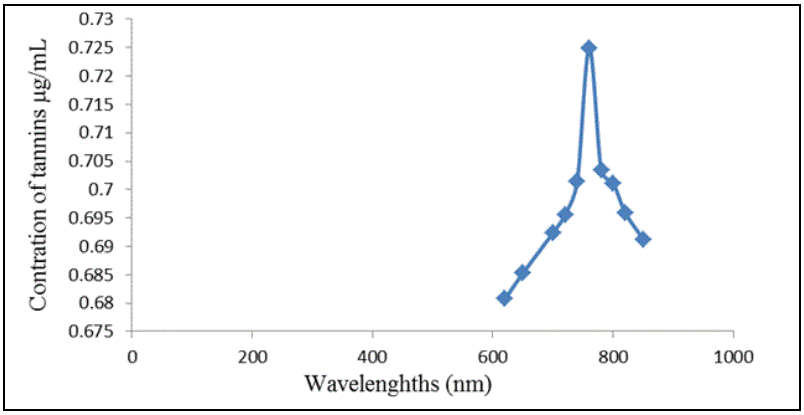

Figure 4: Showing amount of tannins got under varying wavelengths.

60% acetone was found to be the better extractant on fine material (<1 mm mesh) for tannins compared to 50% methanol Table 1. The extraction efficiency of fine material was found to be twice than that in coarse material. The coarse type of finess (<2 mm mesh) is the one that is used for most of the routine analysis currently carried out in most Plant Analysis Laboratory whereas the fine one was tested specifically for tannin analysis.

Table 1: Comparison of 50% methanol extraction and 60% acetone

PMX material	Methanol extraction (50%)	Acetone extraction (60%)
Fine	0.287474	0.607526
Coarse	0.155421	0.340947

Accuracy and Precision

The recovery for this method was found to range from 70 to 85% which is good as it range with some data by Wulasanri, Desmiaty and Purwitosari [22]. Statistical Analysis System (SAS) was used to analyze data and 104 readings were made and the mean was found to be 0.6533 µg/mL, coefficient of variation was 12.62 with a standard error of 0.009369 and a standard deviation of 0.09554. The method detection limit, limits of detection and quantification and signal to noise ratio were calculated by using the method developed by Shrivastava and Gupta, 2011 [23] and Ripp 1996 [24]. The precision was tested by analysing the same between days and weeks and the precision was good as shown by low values of coefficient of variation, standard error and standard deviation.

MDL = (t-value) x (Standard deviation) = 2.326 x 0.09554 = 0.22 µg/mL

LOQ = 10 x (Standard deviation) = 10 x 0.09554 = 0.96 µg/mL.

LOD = 3.3 x (Standard deviation) = 3.3 x 0.09554 = 0.32 µg/mL.

S/N = mean/(Standard deviation) = 0.6533/0.09554= 6.8.

The limits of quantification and detection for this method were comparable to those found in literature, [11, 25, 26].

Effect of tannin level on animal nutrition

As mentioned in the early, for tannins to make any significant change in animal nutrition, the levels must at least be 2%, however, in this case the tannin level in percentage is 0.00006533% (or 0.6533 µg/mL). This means that the level of total tannins in these samples of *Panicum Maximum* grass were not enough to have any impact, whether it is good or in the worse scenario as mentioned in the introduction.

The spiking level was chosen in the range Calculated MDL < Spike Level < 10 x Calculated MDL as suggested by Jeffrey Ripp in Analytical Detection Guide of 1996 [24]. Hence the spike levels of 0.2 to 2 microgram/ml were used as shown Table 2.

Table 2: Recovery of the folin-ciocalteu method form aqueous samples

Samples and spike level (µg/mL)	Recovery
Water; 0.2, 0.6, 1, 1.4, 2	75, 70, 72, 71, 76
Sorghum; 0.2, 0.6, 1, 1.4, 2	76, 78, 83, 82, 85
Panicum Maximum grass; 0.2, 0.6, 1, 1.4, 2	81, 87, 83, 79, 84

Comparison with other methods

The folin-ciocalteu method was compared with other methods including the vanillian-HCl method for the determination of tannins. The LOD for the vanillian-HCl method was found to be 2.1 µg/mL compared with an improved value of 1.05 µg/mL obtained by combining this method with flow injection spectrophotometry [27]. On sorghum the value for the vanillian-HCl method was found to be in the range of 3000 µg/mL while that of the folin-ciocalteu method was found to be 0.2298 µg/mL. The Sorghum was used in this case as the vanillian-HCl method was initially developed for estimation of condensed tannins in sorghum. For the detection limits, *panicum maximum* grass was used and limit of detection was found to be 0.32 µg/mL while the limit of quantification was found to be 0.96 µg/mL. The value obtained is close to the one obtained by Pelozo et al, 2008 who did a Spectrophotometric determination of tannins and caffeine in preparations from *paullinia cupana* var. *sorbilis* and obtained a detection limit of 0.18 µg/ml [28]. Leamsomrong et al, 2009 proposed a flow injection analysis system for the determination of total phenolic compounds by using folin-ciocalteu Assay method and the LOD was found to be 0.0231 µg/mL [29]. Blainski et al 2013, developed the folin ciocalteu method for the determination of the total phenolic content from *Limonium Brasiliense L* and the LOD was found to be 1.0 µg/mL while the LOQ was determined to be 3.34 µg/mL [11]. Bobo-García et al, (2015) developed and

validated microplate methods for total phenolic content and antioxidant activity on polyphenolic extracts and LOD and LOQ were respectively found to be 0.74 and 2.24 µg/mL.

CONCLUSION

The folin-ciocalteu method was customized and successfully used for the determination of total tannins from *panicum maximum* grass samples. The level of total tannins in this grass samples were found to be too low to cause any harm or even a beneficial effect in animal nutrition. The method was optimised in terms of volume of sample aliquot, volume of folins reagent, sodium carbonate, wavelength, extractant and ratio of volume of water to acetone in extractant. SAS was used to analyze data for the coefficient of variation, standard error and standard deviation and the limits of detection and quantification were determined. The LOD and LOQ for the folin-ciocalteu method were found to be better than the vanillian-HCl method with good precision and accuracy.

ACKNOWLEDGEMENTS

I would like to acknowledge Prof Harinder Makkar at FAO for his valuable contribution in this work and also for giving our Laboratory the manual on tannins, Herrmann Baum Gartner at University of Hohenheim for helping with PVPP, Geoffrey Mmusi for helping with the vanillian-HCl method and my colleagues at DAR-PL for their assistance in this project.

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