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Research Article

Micro-methods to determine the composition of selected secondary metabolites in Colombian forages

Ana Maria Calvo Salamanca[1*], **Erika Natalia Duran**[2], **Andrea Milena Sierra**[1], **Ronnal Esneyder Ortiz**[1], **Olga Lucia Mayorga**[1], **Claudia Ariza-Nieto**[1]

[1]Corporación Colombiana de Investigación Agropecuaria - AGROSAVIA. Tibaitata Research Center Mosquera, Colombia
[2]Universidad Nacional de Colombia. Department of Chemistry. Bogotá Colombia

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Abstract: Plants used as forage in livestock production are a source of secondary metabolites (SM) that are involved in important interaction in life, health, nutrition and animal performance. This study aimed to adapt and validate six spectrophotometric micro-methods for quantifying SM such as total phenols (TP), total tannins (TT), condensed tannins (CT), total saponins (TS), total alkaloids (TA), and total sterols (TE), in more than 700 Colombian tropical forage samples. The sample and reagent quantities used in the assays were reduced, preparation conditions were modified in some steps, and the colorimetric reactions were performed in 96-well microplates. The micro methods were validated for linearity, limits of detection and quantification, accuracy, precision, and percent recovery in materials such as grasses, legumes, and tree forages from six Colombian ecoregions. The coefficients of variation (CV), repeatability and reproducibility for all methods were less than 0.4 and 5.6%, respectively. The recoveries ranged from 84.8 to 88.4% for TP, 65.7 to 92.3 for TT, 59.8 to 80.2 for CT, 49.9 to 69.5 for TA, and 61.9 to 78.8% for TS. Micro assays promote minimal waste generation in the environment and allow the processing of a larger number of samples, thereby reducing reagent consumption. This is particularly useful in the analysis of forage materials when making decisions about the effects and functionality of these components in animal diets and their performance.

1. INTRODUCTION

Livestock production of ruminants in tropical countries, such as Colombia, is mostly based on native, locally adapted, and naturalized forage, including diverse grass forages (GF), legume forages (LF), tree forages (TF) and other forages (OF), which are characterized by intermediate forage production, high tolerance to abiotic stress, persistence, and considerable forage quality (Bush & Burton, 2015; (Pensiero *et al*., 2020). Likewise, in this ruminant production system, conserved forage (hay and silage), crop residues, and agro-industrial by-products are locally produced with low or no feed supplementation (Arowolo & He., 2018; Munidasa *et al*., 2021).

*CONTACT: Ana Maria Calvo SALAMANCA acalvo@agrosavia.co Corporación Colombiana de Investigación Agropecuaria –AGROSAVIA. Tibaitata Research Center Mosquera, Colombia

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This negatively affects the full production potential of animals (Arowolo and He, 2018). There is growing interest in improving the digestibility of these feed resources (Koura *et al*., 2023). In recent years, several methods have been explored to enhance ruminal microflora function, improve digestion and fermentation processes, and increase nutrient availability and utilization through supplementation with botanical extracts (Holman & Malau-Aduli, 2012). These extracts have mainly focused on studying the specificity of condensed tannin content against certain microbial groups, which can be used for selective modulation of the rumen microbiome (Busquet *et al*., 2006). However, it should be noted that forage materials may contain other secondary metabolites (SM) such as total saponins, phenolic compounds (total phenols and total tannins), total alkaloids, and total sterols, which, depending on their concentration, may have negative effects at the physiological and/or nutritional level or, in contrast , positively modulate the rumen ecosystem of animals (Al-Khayri *et al*., 2023).

Phenolic compounds (total phenols and total tannins): The Folin-Ciocalteu method, based on the oxidation-reduction principle, is suitable for the determination of phenolic compounds. Tannins are distinguished from non-tannins using a solid matrix, polyvinylpolypyrrolidone (PVPP), which can be used to determination of concentrations ranging from 5 to 50 µg/mL. The phenolic content in the extract was measured before and after treatment with PVPP, and the difference between these values was the tannin content (Makkar *et al*., 2007).

Total Alkaloids: The method allows the determination of precipitable alkaloids using Dragendorff's reagent and can be applied to simple alkaloids or plants containing alkaloids. However, this method is not applicable to plant materials containing purine alkaloids. The alkaloid precipitates were (BiI3) (Alk - HI) or DR (KBiI4). Bismuth forms a yellow bismuth complex {Bi [CS [CS (NH2)3]} (NO3)3 in nitric acid medium with thiourea.

Total Saponins: In method for the determination of total saponins, steroidal sapogenins with or without double bonds at C-5, triterpenoid and sterol sapogenins, and bile acids with an OH group at the C-3 position react with vanillin in an acidic medium to form chromogens with absorbance maxima at 455-460 nm, 460-480 nm, or 544 nm, depending on the nature of the saponins (Kuppusamy *et al*., 2018).

Several analytical methods have been reported for quantifying total phenols, tannins, condensed tannins, alkaloids, saponins, and sterols in plant materials, primarily using liquid chromatography (HPLC) (coupled with a UV and/or MS detector) and gas chromatography (GC) (coupled with an FID and/or MS detector). Although these methods offer high selectivity and sensitivity, they also present certain limitations. HPLC and GC quantification can be constrained by detector sensitivity (UV/DAD) (IUPAC, 1997; Kuppusamy *et al*., 2018), high operational costs (MS detector), and the need for multiple sample preparation or derivatization steps.

Moreover, the quantification of isolated compounds in biological matrices such as feed materials remains controversial in terms of analysis and quality control. Research suggests that the bioactivity of plant materials results from the combined effects of multiple compounds rather than from isolated synthetic additives (Vaou *et al*. 2022).

In contrast, UV/VIS spectrophotometry is widely used because of its simplicity and low implementation cost. Various colorimetric assays have been proposed for identifying and quantifying plant derivatives (Marques *et al*., 2013). To address these challenges, this study adapted and validated six spectrophotometric micro-methods for quantifying secondary metabolites (SM) in a variety of tropical forage resources used in livestock production in Colombia. These methods aim to reduce reagent consumption and minimize environmental waste while ensuring accurate and cost-effective analysis.

2. MATERIAL and METHODS

2.1. Reagents and Analytical Solutions

Reagents: Sigma-Aldrich®, anhydrous sodium carbonate ($NaCO_3$) ammonium iron (III) sulphate dodecahydrate, thiourea 98% and sodium sulphide nonahydrate; Merck®; potassium hydroxide 85%, potassium iodide, and poly(vinylpolypyrrolidone) (PVPP) (Supelco®).

Methanol, formic acid, acetic anhydride, chloroform, sulfuric acid 97% (Merck®), nitric acid ethanol, n-butanol, petroleum ether, fuming hydrochloric acid, Acetone, Ascorbic acid (Supelco®); glacial acetic acid ≥ 99.8 % (Panreac).

Analytical solutions All solutions were prepared on the same day as the determinations, protected from direct sunlight, and stored at 4°C. Tannic acid [100 µg TAc⁄mL]. The tannic acid solution was prepared as reported in (Makkar *et al*., 1993) However, a mixture of methanol/water/formic acid (70:29.5:0.5) was used to maintain the same conditions in the sample extracts and then diluted 1:9 with the same mixture. Catechin [1000 $\mu g\ mL^{-1}$]. The stock solution was prepared by dissolving catechin (10 mg) in 10 mL of ultrapure water. Atropine [8.23 mg mL^{-1}]: The atropine solution was prepared by weighing 83.133 mg and dissolved in 10 mL of absolute ethanol, and a bismuth nitrate solution was prepared by dissolving 24.6331 mg in 4 mL of nitric acid and made up to 10 mL with ultrapure water. β-Sitosterol [1000 µg mL^{-1}]: The stock solution was prepared by dissolving 1.052 mg of β- Sitosterol in 1 mL of chloroform. Diosgenin [1000 µg mL^{-1}]: The stock solution was prepared by dissolving 15.5 mg of Diosgenin in 8 mL of absolute ethanol and diluting to 10 mL with ultrapure water. The solubility of diosgenin was improved by using ethanol instead of methanol.

The calibration curve was the same for the Total Phenols and Total Tannins method, which was prepared from a stock solution of tannic acid [100 µg mL^{-1}], and solutions within the concentration range of 3-50 µg TAc– mL^{-1} were obtained using the same extraction solution as the samples. For the condensed tannin method, Catechin solutions within the concentration range of [400 – 900 µg mL^{-1}] were used. The calibration curve for the total alkaloid method was prepared from a solution of [250 µg mL^{-1}] of bismuth nitrate, and solutions within the concentration range of 6-200 µg mL^{-1} were obtained by diluting with 40% nitric acid. For the total saponin method, a calibration curve was constructed within the concentration range of 10-100 µg mL^{-1} of Diosgenin. The calibration curve for the total sterol method was prepared from a solution of β- Sitosterol [1000 µg mL^{-1}] and solutions in the concentration range of 5 - 100 µgmL^{-1} were obtained. All dilutions were prepared and measured in triplicate.

2.2. Apparatus

For the measurement of total phenols and tannins, condensed tannins, total alkaloids, total saponins, and total sterols, a 96-well plate reader was used, with a wavelength range of 200 to 999 nm and a measurement range of 0.000 to 4.000 OD (Biotek, Synergy HT), a Merck/Millipore Milli- Q HX 7040 water purifier, a high-speed centrifuge, digital control, maximum speed 20000 rpm, temperature control: refrigerated (Hermle Z32 HK, manufactured by Benchmark), and a microcentrifuge maximum capacity x 1.5-2 mL, maximum speed 18000/31,514 rpm/RCF, temperature control -20 to +40°C (Hettich Micro 220R Zoor). A concentrator (Eppendorf, Concentrator plus/Vacufuge® plus) was Iused in the TS method. For the TE and TS methods, a fat extraction system (FOSS, SoxtecTM 2050, Denmark) and a spectrophotometre (Thermo Scientific GENESYSTM 30), with a single beam, wavelength range 325 – 1100 nm, spectral bandwidth 5 nm, accuracy ± 2 nm, and repeatability < ± 1nm were used.

2.3. Collection and Preparation of Plant Materials

A total of 719 materials of forages between Grass forages (GF), legume forages (LF), tree forages (TF), and other forages (OF) were collected between years 2017 to 2020 from six different regions in Colombia (Antioquia, Arauca, Cundinamarca, Boyacá, Caquetá and Caldas). Forage species were collected by means of unstructured sampling, for which a

homogenisation cut was made by manual pruning at a height between 0.5 and 1 m, depending on the architecture and growth points of each species (Hallé, 2010). For each plant , the total number of leaves and stems (diameter <1 cm) was harvested (Avendaño R. *et al*., 2003). The sample type was classified according to the plant parts as follows: 1) sample composed of leaves and stems, 2) leaves only, 3) stems only.

Samples were dried at 65 °C for 48 h until a constant weight was obtained to determine dry matter (DM), and then ground and sieved through a 1 mm sieve. For the percentage recovery test, 17 representative samples were selected, including GF (5), LF (5), and TF (7), as these materials contained all secondary metabolites of interest, as detailed in Table 1.

Table 1. Forage materials selected for adjustment and validation methods.

Family or Category	Plant material		Part
	Common name	Scientific name	
Grass forages	Bermuda grass	*Cynodon dactylon*	L-S
	Guinea grass	*Panicum máximum cv*	L-S
	Star	*Cynodon nlemfuensis*	L-S
	King grass purple	*Pennisetum purpureum x P. typhoides cv. morado*	L-S
	Sweet grass	*Paspalum notatum*	L-S
Legume forages	Matarratón	*Gliricidia sepium*	L-S
	Trupillo	*Prosopis juliflora*	L
	Leucaena	*Leucaena leucocephala*	L-S
	Clitoria	*Clitoria ternatea cv Tehuana*	L-S
	Bejuco de Chivo	*Centrosema plumieri*	L
Tree forages	Oregano	*Lippia origanoides Kunth*	L
	Olive tree	*Capparis odoratissima*	L
	Puy	*Tabebuia billbergii*	L
	Moringa	*Moringa oleifera*	L-S
	Sajarito	*Pisonia aculeata*	L-S
	Espino	*Pithecellobium lanceolatum*	L-S
	Uvita	*Cordia alba*	L-S

L: Leaf, S: Stem.

2.4. Methods Adapted to Micro Essays

Total Phenols and Tannins Method: Sample preparation for both was based on a previous report (Makkar *et al*., 2007), with some modifications. Quantities and volumes were adjusted to perform the preparation in 15 mL conical tubes, 100 mg of forage material was weighed, and the volume of methanol/water/formic acid extraction solution (70.0:29.5:0.5) was reduced four times from 20 mL to 5 mL. The extraction process was performed in duplicate, with centrifugation at 6000 rpm for 8 minutes at 4°C. After centrifugation, the supernatants were combined and stored in a 15 mL conical tube to ensure the complete extraction of total phenols and tannins.

The precipitation of total tannins was based on the method described by (Makkar *et al*., 2007) with some modifications. The amount of polyvinylpolypyrrolidone (PVPP) was reduced 4-times and weighed into 2 mL microtubes from 100 mg to 25 mg of PVPP, and 250 µL of the concentrated extract was added and diluted with ultrapure water (1:1). Shaking was performed and the mixture was allowed to stand to enable matrix swelling and tannin addition. It was then centrifuged at 6000 rpm for 5 minutes at 4°C.

The total phenol content was quantified in a 96-well microplate. A dilution of the concentrated extract (1:19), was prepared, and 75 µL of the diluted extract or standard was added to each well. Then, 38 µL of 1N Folin-Ciocalteu's reagent and 187 µL of 20% sodium carbonate were added. The plate was incubated at room temperature for 40 minutes in the dark, and absorbance was measured at 755 nm using a microplate reader.

For total tannin quantification, 300 µL of the supernatant was transferred to another microtube (tube 2), and the PVPP precipitate was discarded. In Tube 2, 152 µL of 1N Folin-Ciocalteu's reagent and 748 µL of 20% sodium carbonate were added. The mixture was vortexed for 10 s, incubated in the dark for 35 minutes, and centrifuged at 6000 rpm for 5 minutes at 4°C. Finally, 300 µL of the supernatant was transferred to a well of a microplate, and the absorbance was measured using a microplate reader.

Condensed tannin method: The sample preparation was based on previous reports (Makkar *et al*., 2007) with some modifications. The extraction was carried out in conical tubes of 15 mL, 100 mg of forage was weighed, and the extraction solution was modified using acetone/water/formic acid/ascorbic acid (70.0:24.0:0.5:0.1) and a petroleum ether/acetic acid solution in proportion (99:1), of which 5 and 3 mL were added, respectively. The mixture was subjected to ultrasonication, and the organic phase was discarded. The tubes were centrifuged at 3500 rpm/10 min at4 °C, the supernatant was collected in another 15 mL conical tube, and the extraction was performed once more. The remaining acetone-ether was evaporated in a cabinet for 40 min and brought to 5 mL using ultrapure water. The quantification reaction was performed in 96-well microplate, a 37.5 µL aliquot of the condensed tannins extract or standard solution was added, diluted (1:1) with 70% acetone, 217.5 µL of butanol-HCl solution (95:5) and 7.5 µL of ferric reagent (2% ferric ammonium sulfate in HCl 2N), stirred in a plate shaker and incubated in a water bath at 90 °C/60 min covered with aluminum foil, then the plate was placed in an ice bath until room temperature. The absorbance was read at 550 nm using a microplate reader.

Total alkaloid method: The preparation and quantification of samples were based on the alkaloid precipitation method with Dragendorff's Rrwas reduced 100 fold, from 10 to 0.1 g. Consequently, the volume of the solvent used for extraction, the final volume of concentration, the amount of 10% ethanolic KOH, and the final volume of the extract were proportionally reduced 10 times, passing from 10 mL to 1 mL of the concentrated extract, and 1 mL of 10% alcoholic KOH was added. Reflux extraction was performed in a thermostatted bath at 80°C in screw-capped tubes with a stopper, and the extraction time was increased twofold. The extract was transferred to a 15 mL conical tube, vortexed for 10 s, and centrifuged at 2000 rpm/5 min/4°C to separate the plant material from the liquid phase. The alkaloid precipitation step was adjusted for a 2 mL microtube, and 700 µL of supernatant and 330 µL of 2% sulfuric acid were added until a pH between 2 and 2.5 was reached. Then, 300 µL of Dragendorff's reagent was added, vortexed for 30 s, allowed to stand for 5 minutes and centrifuged at 8000 rpm/5 minutes/4°C. The procedure was repeated with the addition of the same amount of Dragendorff's reagent, after which the supernatant was discarded and 200 µL of 1% Sodium Sulfide was added to the precipitate, vortexed for 30 s, and centrifuged at 8000 rpm/5 minutes/4°C. This procedure was repeated once more with the addition of sodium sulfide, and the supernatant was discarded. The precipitate was dissolved in 200 µL of concentrated nitric acid, allowed to stand for 5 minutes and then 300 µL of ultrapure water was added. The staining reaction was performed in a 96-well microplate; 40 µL of the acid extract or standard solution was added, 200 µL of 3% thiourea was added, and the absorbance was determinated using a microplate reader (Sreevidya & Mehrotra, 2003).

Total saponins method: Determination was based on previous reports (Makkar *et al*., 2007) (Hiai *et al*., 1976) and modifications were made, such as the reduction of the sample amount and the extraction volume, which were reduced 200 and 100 times from 10 g to 50 mg and 100 mL to 1 ml respectively, to adjust it to a micro method. Fifty milligrams of previously defatted plant material was weighed into a 2 mL microtube, and 1 mL of 50% aqueous methanol was added. The mixture was stirred on an orbital shaker for 12 h at room temperature. Then, the mixture was centrifuged at 6000 rpm/10 min and the supernatant was collected in another microtube, the extraction process was repeated, and the supernatants were mixed. Because of the volume of the extract, no rotary evaporator was used, but a Speed Vacuum was used at 42 °C until a 75% reduction in the initial volume was obtained. The tubes were centrifuged, and

the supernatant was transferred to another microtube and an equivalent volume of chloroform was added to eliminate pigments. The tubes were vortexedfor 30 s, centrifuged at 6000 rpm/5 minutes, the aqueous phase was transferred to another microtube (upper), and the organic phase was discarded (lower). The procedure was repeated two more times. An equal volume of n-butanol was added to the concentrated extract, vortexed for 15 s, centrifuged at 6000 rpm for 5 minutes and the upper phase was transferred to another 2 mL microtube. Extraction with n-butanol was repeated, and the supernatants were collected. Then, n-butanol was completely evaporated under vacuum at 45 °C and the residue was resuspended in 1.30 mL of ethanol 80 % (v/v). Total saponins were quatified using a a 96-well microplate, and 25 µL of sample or standard, 25 µL of 8% Vanillin and 250 µL of 72% (v/v) sulfuric acid were added slowly from the inner side of the wall. The plate was gently shaken for 5 minutes on a plate shaker and then placed in a water bath at 60°C for 10 minutes. Subsequently, the plate was cooled in ice water for 5 minutes and the absorbance was recorded on a microplate reader.

Total sterols method: Sample preparation was based on previous reports (Araújo *et al*., 2013), with modifications. Briefly, sterols were extracted with 80 mL of petroleum benzene using Soxhlet for 1.5 h from 2 g of previously dried and ground feed materials. The extract was dried to complete dryness in an extraction oven, and the residue was resuspended with 5 mL of chloroform and made up to 10 mL with the same solvent. An aliquot of 100 µL for GF and FT extracts and 50 µL for LF was taken, 500 µL of Lieberman-Buchard reagent (LBR) was added and the volume was adjusted to 1.5 mL with chloroform. Absorbance was measured in a spectrophotometer with a quartz cell after the addition of LBR prepared according to the method described by (Adu *et al*., 2019; Awad *et al*., 2000; Kenny, 1952).

2.5. Method Validation

The micro methods used for the determination of secondary metabolites in forages were validated, according to the (AOAC, 2024) guidelines in terms of linearity, limits of detection (LOD) and quantification (LOQ), precision and accuracy (Santo *et al*., 2005).

2.5.1. *Linearity*

The linearity of the spectrophotometric system was evaluated using the dilutions of the standards prepared as described in the Calibration section. The relative responses of the analytes, measured as mean absorbances, were plotted against the respective concentrations of the curves constructed using the method of least squares linear regression.

2.5.2. *Limits of detection (LOD) and quantification (LOQ)*

The LOD and LOQ for each method were quantified by analysis of ten independently prepared blanks. Based on the guidelines of the International Union of Pure and Applied Chemistry (IUPAC, 1997), the LOD values correspond to three times the standard deviation of ten blank solutions, each measured once. The LOQ values were the concentrations equal to ten times the standard deviation of the peak area of each element in the blank solutions divided by the slope of their calibration curves.

2.5.3. *Precision*

The repeatability was calculated by testing two different concentrations of the pure standard five times on the same day. The experiment was repeated with the same concentrations on different days to determine the intermediate precision and the intra-day and inter-day relative standard deviation of the concentrations evaluated.

2.5.4. *Accuracy*

Recovery was determined by the added and recovered method by adding a known amount of standard to the diet. Each sample was fortified with 0.4, 0.8, 1.2 and 5.0 mg of Tannic Acid, Catechin, Atropine, and Diosgenin respectively. Recovery values were expressed as percentages to express the ratio between the experimentally determined total secondary metabolite contents and their theoretical concentrations. Each sample was measured six times, and the amount recovered was calculated.

2.6. Statistical Analysis

To analyze the variability in the concentration of secondary metabolites in the samples considered, a descriptive analysis was performed. The statistical parameters included the number of data points (n), maximum (Max) and minimum (Min) values, mean (Mean), and standard deviation. The analysis considered different levels of general grouping, including category and plant part. All analyses were conducted using software SAS Enterprise 7.2

3. RESULTS

3.1. Reaction Time

Table 2 shows the reaction stability time for all, standards and extracts evaluated. The data confirm that instability of the reaction product occurs after 1 hour, resulting in a decrease in the absorbance read after this time. On the other hand, the reproducibility of the method ensures that the response of the method would not be significantly affected if the determination is performed within this time interval.

Table 2. Stability for each of the metabolites evaluated.

Standard	Total phenols	Total tannins	Condensed Tannis	Total Alkaloids	Total Saponins	Total Sterols
Stability (min)	60	60	Stable	Stable	Stable	15 - 20

3.2. Method Validation

3.2.1. *Linearity, LOD and LOQ*

The calibration curves were evaluated after regression analysis, and linearity was estimated from the coefficients of determination (R2) for concentrations between 80 to 120% of the working concentration. The data presented in Table 3 indicate that the standard curves were linear; the R values indicate that more than 99% of the experimental variability could be explained by the linear models, confirming the satisfactory relationship between analyte concentrations and spectrophotometric responses. In terms of LOD and LOQ the spectrophotometric procedure was sensitive for the detection and quantification of secondary metabolites in the samples of forage extracts, without presenting significant interferences in the instrumental technique. Regression analyses showed that the linear correlation between concentration and signal intensity was significant ($p < 0.05$).

Table 3. Linearity and limits of detection and quantification for secondary metabolites.

Metabolite	Total Phenols	Total Tannins	Condensed Tannins	Total Alkaloids	Total Saponins	Total Sterols
Standard	Tannic acid	Tannic acid	Catechin	Bismuth nitrate	Diosgenin	β-sitosterol
Calibration range (ug-mL^{-1})	30 a 50	30 a 50	100 a 1000	10-200	6-230	10- 110
LOD (ug-mL^{-1})	2.7	2.6	5.9	3,9	7,4	3
LOQ (ug-mL^{-1})	3.3	3.3	11.2	10.2	14.7	3
Regressionequation	y = mx + b	y = mx + b	y = mx + b	y = mx + b	y = mx + b	y = mx + b
Correlation coefficient (r)	0.9964	0.9964	0.9998	0.9996	0.9759	0.9999
Slope (m)	0.0184	0.0184	0.00009	0.0033	0.0114	0.5075
Intercept (c)	0.0873	0.0873	0.0675	0.0453	0.1723	0.0996

3.2.2. *Precision*

Table 4 shows the precision of each method, evaluated as the relative standard deviation (RSD) and the repeatability of the method. The RSD values are considered acceptable since they do

not exceed 5% of the target value (Santo *et al.*, 2005). This indicates that it is an accurate method and shows that the adaptation to micro methods is appropriate for the quantification of DM in forage resources.

Table 4. Accuracy of methods for secondary metabolites evaluated.

Metabolite	Total Phenols	Total Tannins	Condensed Tannins	Total Alkaloids	Total Saponins	Total Sterols
Standard	Tannic acid	Tannic acid	Catechin	Bismuth nitrate	Diosgenin	β-sitosterol
Repeatability (RSD, %)[a]	2.31	2.31	1.02	0.90	2.86	1.10
Intermediate accuracy (RSD, %) [a]	2.35	2.35	1.07	1.89	4.03	2.52

[a] The inter-day relative standard deviation of the concentrations evaluated.

3.2.3. *Accuracy*

The forage samples with added standard showed an average relative recovery for total phenols ranging from 84.8 to 88.4 %, for total tannins between 65.7 and 92.3 %, 59.8 and 80.2 %,49.9 and 69.5 %, and 61.9 and 78.8 % for condensed tannins, total alkaloids and total saponins, respectively. The values obtained in terms of recovery are shown in Table 5. The recovery percentage recoveries were close to 90%. These recovery percentages are within the limits established by the (AOAC, 2005) for the validation of spectrophotometric methods.

Table 5. Accuracy in terms of recovery evaluated through standard addition.

Metabolite	Compound	N	Forage type	Recovery, %,		
				Media	Max	Min
Total phenols	Tannic Acid	30	GF	87.48±20.51	118.4	63.32
		30	LF	88.46±17.15	118.82	69.83
		42	TF	84.89±12.90	99.96	55.42
Total tannins	Tannic Acid	30	GF	92.39±22.40	139.26	48.61
		30	LF	74.11±26.19	122.6	38.31
		42	TF	65.78±36.94	140.52	14.47
Condensed tannins	Catechin	30	GF	80.20±35.95	156.01	41.09
		30	LF	60.97±38.56	144.68	19.23
		42	TF	59.82±53.79	182,7	6.15
Total alkaloids	Atropine	30	GF	69.59±30.34	132.46	12.8
		30	LF	66.88±26.09	117.21	30.89
		42	AF	49.96±23.74	103.4	15.45

3.3. Application

To validate the quantification method of secondary metabolites (SM), 719 forage samples were collected from different regions in Colombia. Various parts of the forage were analyzed, including 469 leaf-stem samples, 131 leaf samples, 89 stem samples, 16 leaf-stem-panicle samples, 11 grain-leaf-stem-panicle samples, 2 fruit samples, and 1 flower-leaf-stem sample. Table 6 shows the average concentrations of total phenols, total tannins, condensed tannins, total alkaloids, total saponins, and total sterols in different forage species. Additionally, the association between plant parts and the average, secondary metabolite (SM) content is illustrated in Figure 1 for the parts of the plant with the highest number of samples: leaf, leaf-stem, and stem.

Table 6. Descriptive statistics of composition of SM expressed in g Kg^{-1} DM, distributed in: Grass forages (GF) (n=143); legume forages (LF) (n=204); tree forages (TF) (n=325), and other forages (OF) (n=47).

Metabolite	Forage type	Min	Mean	Max	Stdv
Total Phenols	GF	3.311	12.938	36.230	6.324
	LF	2.452	26.136	181.920	22.215
	TF	1.286	27.060	199.180	31.341
	OF	3.748	15.363	72.590	14.919
Total Tannins	GF	0.408	8.048	29.120	6.158
	LF	0.085	16.775	96.450	15.394
	TF	0.186	16.992	137.110	20.033
	OF	0.158	8.604	40.680	9.189
Condensed Tannins	GF	1.011	1.325	8.970	1.469
	LF	0.9..39	3.023	45.100	5.719
	TF	1.015	3.638	62.740	8.100
	OF	1.023	2.205	19.250	3.600
Total Alkaloids	GF	0.754	5.350	15.060	4.620
	LF	0.769	3.805	13.370	3.585
	TF	0.474	3.831	16.000	3.295
	OF	0.610	4.652	13.230	3.387
Total Saponins	GF	0.700	4.005	22.380	5.571
	LF	0.348	9.035	80.940	12.313
	TF	1.463	10.616	84.380	16.675
	OF	0.696	7.539	27.950	8.488
Total Sterols	GF	0	0.566	8.470	1.661
	LF	1.650	1.801	13.850	2.696
	TF	0.187	1.910	11.360	2.645
	OF	0.586	2.218	8.210	2.599

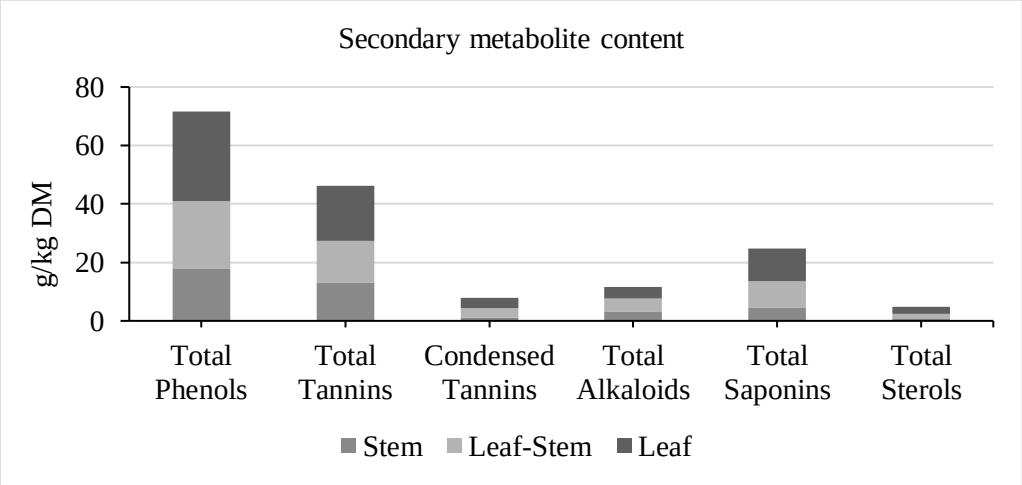

Figure 1. Average secondary metabolite content and plant part relationship. Source: Own source.

4. DISCUSSION

The adaptation of micromethods to determine secondary metabolites in forages is crucial for reducing reagent and sample consumption, lowering costs, and minimizing environmental

impact. Additionally, micromethods offer increased sensitivity and precision, making them ideal for large-scale screening and research.

Results indicate that, for all secondary metabolites except total sterols, forage trees had the highest content compared to other forage categories. This is because the concentration of secondary metabolites is related to the age of the forage and other aspects such as the content of primary nutrients and the influence of soil and climate (Ramos *et al*., 1998; Verdecía, 2021). Forage trees were the longest-lived materials collected compared to other groups, such as grasses and legume forages. Also, the functional role of secondary metabolites derived from trees and shrubs in ruminants shows specificity against certain microbial groups, which can be utilized for the selective modulation of the rumen microbiome (Guerriero *et al*., 2018; Martin *et al*., 2009) consequently affecting animal productivity. Among the metabolites that have been shown to influence ruminant production are saponins, tannins, organosulfur compounds, essential oils, lignin's, alkaloids, antioxidants, etc. Currently, more than 200,000 defined structures of secondary compounds are reported, demonstrating the vast area that still needs to be researched (Patra & Saxena, 2011; Patra & Saxena, 2009). In this context, the variation in metabolite contents in forages can have positive functional roles or become toxic or detrimental to the digestion of the feed resource when consumed by the animal and reaching the rumen. Ideally, there should be a moderate content of secondary metabolites in the diet of ruminants, contributing to ruminal functionality without affecting the parameters of rumen dynamics, such as ration digestibility, fermentation profile, or passage rate (Ramírez-Restrepo & Barry, 2005).

Condensed tannins in plants reduce ruminal methanogenesis by decreasing hydrogen formation and inhibiting methanogens (Jenko *et al*., 2018; White & Lawler, 2002). The second most important group is saponins, whose antiprotozoal effect is attributed to the binding of saponins with the cholesterol in the protozoan cell membrane, causing its lysis. Since approximately 25% of methanogens are in symbiosis with ciliated protozoa, the inhibition of methanogenesis is mainly attributed to the adverse effect of saponins on hydrogen-producing ciliated protozoa and cellulolytic bacteria, reducing the availability of hydrogen for methanogenesis (Ellis, 1993).

Based on the information reported, compositional elements are provided that could be used to implement nutritional strategies using plants with a high presence of secondary metabolites without affecting animal performance in silvopastoral systems in Colombia (Santacoloma-Varón & Granados, 2010). The consumption of forages in grazing is influenced by the type of secondary metabolites consumed and the feeding sequence. For example, in cattle, a higher grazing frequency was observed when they were first allowed to graze on legumes containing tannins or saponins. In sheep, forage consumption with high alkaloid content was higher when supplemented with tannins or saponins. This study suggests that secondary compounds interact to influence grazing intake and that this feeding sequence can be manipulated. Jayanegara *et al*. (2009) found that combining high-quality plants with plants with high phenolic content (tannins) in the diet of ruminants reduces methane emissions without affecting rumen fermentation.

In this context, it's important to consider that many of these secondary compounds are employed by the plant for various functions and are associated with specific parts of the plant and the dynamics of each forage. Because the concentration of secondary metabolites (SM) is higher in leaves compared to other parts of the plant such as the leaf-stem mix and the stem. This is because leaves engage in competitive relationships with other plants, acting as allelopathic agents, and against fungal, bacterial, and viral invasions with substances known as phytoalexins (Harborne., 1992; Harborne., 1993; Sepúlveda-Jiménez, *et al* 2004; Wallace 2004); in mutualistic relationships by attracting pollinators and seed dispersers; as molecules carrying information related to possible defensive functions (Stafford, 1967); as protection against ultraviolet radiation and desiccation; and as nitrogen reserves (Ellis, 1993). Environmental and soil conditions where the plant is grown also influence this, as well as the cutting age, season, and ecotype (Ariza-Nieto *et al*., 2018).

5. CONCLUSIONS

Quantitative analysis is essential for evaluating forage quality. In this study, six analytical micro methods were successfully adapted and validated using UV-Vis spectrophotometry to quantify secondary metabolites in grasses, legumes, and tree forages commonly used in livestock feeds. The validated methods demonstrated strong linearity, precision, and accuracy under the studied conditions. Therefore, micro-assays are recommended for the routine analysis of secondary metabolites in various forage materials, providing a reliable tool for improving our understanding of their functional roles in animal nutrition.

A total of 719 forage samples were collected from six ecoregions in Colombia, representing different plant groups, including grasses, legumes, trees, and other forage types. The results revealed significant differences ($p<0.0001$) in secondary metabolite composition based on plant parts, highlighting the importance of considering plant structure when assessing forage nutritional value. This study contributes valuable insights into the variability of secondary metabolites in forages, paving the way for more targeted nutritional strategies in livestock production.

Furthermore, this study emphasizes the potential of secondary metabolites to influence livestock health and productivity. The presence of bioactive compounds in forage materials can contribute to improved digestion, enhanced immune responses, and methane emission reduction in ruminants. Understanding the variability in secondary metabolite content across different forage types and plant parts enables the development of more effective feeding strategies, optimizing both animal performance and environmental sustainability.

Overall, these findings provide valuable insights for future research on forage composition and its implications for animal nutrition. The use of validated micro-assays can support more precise dietary formulations, promoting sustainable livestock systems while maintaining forage quality and functionality.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Ana Maria Calvo Salamanca Methodology, formal analysis, writing/original draft preparation, writing/reviewing and final editing. **Erika Natalia Duran Cruz** Methodology, formal analysis, writing/original draft preparation, writing/reviewing and final editing. **Ronnal Esneyder Ortiz Cuadros,** Methodology, formal analysis, writing/original draft preparation, writing/reviewing and final editing. **Claudia Janeth Ariza-Nieto** Conceptualization, methodology, formal analysis, writing/original draft preparation, writing/reviewing and final editing, visualization, supervision. **Andrea Milena Sierra,** Data collection, methodology, formal analysis, writing/original draft preparation, writing/reviewing and final editing. **Olga Lucia Mayorga Mogollón** Contributed to conceptualization, methodology, formal analysis, writing/original draft preparation, writing/reviewing and final editing, visualization, supervision.

Orcid

Ana Maria Calvo Salamanca https://orcid.org/0000-0003-1345-5891

Erika Natalia Duran https://orcid.org/0000-0002-5741-1973
Andrea Milena Sierra https://orcid.org/0000-0001-6725-2526
Ronnal Esneyder Ortiz https://orcid.org/0000-0003-3977-1462
Olga Lucia Mayorga https://orcid.org/0000-0001-7872-6109
Claudia Ariza-Nieto https://orcid.org/0000-0001-9965-0625

REFERENCES

Adu, J.K., Amengor, C.D.K., Kabiri, N., Orman, E., Patamia, S.A.G., & Okrah, B.K. (2019). Validation of a simple and robust liebermann–burchard colorimetric method for the assay of cholesterol in selected milk products in Ghana. *International Journal of Food Science*, *2019*, 1–7. https://doi.org/10.1155/2019/9045938

Al-Khayri, J.M., Rashmi, R., Toppo, V., Chole, P.B., Banadka, A., Sudheer, W.N., Nagella, P., Shehata, W.F., Al-Mssallem, M.Q., Alessa, F.M., Almaghasla, M.I., & Rezk, A.A.-S. (2023). Plant secondary metabolites: The weapons for biotic stress management. *Metabolites*, *13*(6), 716. https://doi.org/10.3390/metabo13060716

AOAC. *Official Methods of Analysis*, (2005). Method 931-01 phosphorus in plants. In Official methods of analysis. Arlington: Association of official analitycal chemists.

AOAC. *Official Methods of Analysis* (2024). *AOAC International*. https://www.aoac.org/official-methods-of-analysis/

Araújo, L.B.D.C., Silva, S.L., Galvão, M.A.M., Ferreira, M.R.A., Araújo, E.L., Randau, K.P., & Soares, L.A.L. (2013). Total phytosterol content in drug materials and extracts from roots of Acanthospermum hispidum by UV-VIS spectrophotometry. *Revista Brasileira de Farmacognosia*, *23*(5), 736–742. https://doi.org/10.1590/s0102-695x2013000500004

Ariza-Nieto, C., Mayorga, O., Mojica, B., Parra, D., & Afanador-Tellez, G. (2018). Use of LOCAL algorithm with near infrared spectroscopy in forage resources for grazing systems in Colombia. *Journal of Near Infrared Spectroscopy*, *26*(1), 44-52. https://doi.org/10.1177/0967033517746900.

Avendaño R.J., Carlos, O.M., & Ramírez S.M. (2003). Caracterización de los componentes vegetales consumidos por ovinos y bovinos en plantas de tagasaste (Chamaecytisus proliferus ssp. palmensis). *Agricultura Técnica*, *63*(1). https://doi.org/10.4067/s0365-28072003000100006

Arowolo, M.A., & He, J. (2018). Use of probiotics and botanical extracts to improve ruminant production in the tropics: A review. *Animal Nutrition 4*(3), 241-249. https://doi.org/10.1016/j.aninu.2018.04.010

Awad, A.B., Chan, K.C., Downie, A.C., & Fink, C.S. (2009). Peanuts as a Source of β-Sitosterol, a Sterol with Anticance Properties. *Nutrition and Cancer, 36*(2), 238-241. https://doi.org/10.1207/S15327914NC3602_14.

Busquet, M., Calsamiglia, S., Ferret, A., & Kamel, C. (2006). Plant extracts affect in vitro rumen microbial fermentation. *Journal of Dairy Science*, *89*(2), 761-771. https://doi.org/10.3168/jds.s0022-0302(06)72137-3

Bush, L., & Burton, H. (2015). Intrinsic Chemical Factors in Forage Quality. *ASA, CSSA, and SSSA Books*, 367–405. https://doi.org/10.2134/1994.foragequality.c9

Ellis, B. (1993). *Phenolic Metabolism in Plants.*Helen A. Stafford , Ragai K. Ibrahim. *The Quarterly Review of Biology*, *68*(4), 596–597. https://doi.org/10.1086/418355

Guerriero, G., Berni, R., J. Armando Muñoz-Sanchez, Apone, F., Abdel-Salam, E. M., Qahtan, A.A., Alatar, A.A., Cantini, C., Cai, G., Hausman, J.-F., Siddiqui, K.S., Hernández-Sotomayor, T., & Faisal, M. (2018). Production of plant secondary metabolites: Examples, tips and suggestions for biotechnologists. *Genes*, *9*(6), 309-309. https://doi.org/10.3390/genes9060309

Hallé, F. (2010). *Arquitectura de los árboles*. *Bol. Soc. Argent. Bot. 45*(3-4), 405-418. https://www.uv.mx/personal/tcarmona/files/2016/08/Halle-2010.pdf

Harborne, J.B (1993). Introduction to Ecological Biochemistry. Academic Press England.

Harborne, J.B. (1992). *Chapter 20 Phenolic compounds. In E. Heftmann, Chromatography Fundamentals and Applications of Chromatographic and Electmphoretic Methods*. Elsevier.

Hiai, S., Oura, H., & Nakajima, T. (1976). Color reaction of some sapogenins and saponins with vanillin and sulfur1c acid. *Planta Medica, 29*(02), 116–122. https://doi.org/10.1055/s-0028-1097639

Holman, B.W.B., & Malau-Aduli, A.E.O. (2012). Spirulina as a livestock supplement and animal feed. *Journal of Animal Physiology and Animal Nutrition, 97*(4), 615–623. https://doi.org/10.1111/j.1439-0396.2012.01328.x

IUPAC. (1997). Compendium in chemical terminology. Oxford: Blackwell Scientific publications.

Jayanegara, A., Togtokhbayar, N., Makkar, H., & Becker, K. (2009). Tannins determined by various methods as predictors of methane production reduction potential of plants by an in vitro rumen fermentation system. *Animal Feed Science and Technology, 150*(3) 230-237. https://doi.org/10.1016/j.anifeedsci.2008.10.011

Jenko, C., Bonato, P., Fabre, R., Perlo, F., Tisocco, O., & Teira, G. (2018). Adición de taninos a dietas de rumiantes y su efecto sobre la calidad y rendimiento de la carne. Ciencia, docencia y tecnología, 224-241. http://www.scielo.org.ar/scielo.php?script=sci_arttext&pid=S1851-17162018000100010&lng=es&tlng=es

Kenny, A. (1952). The determination of cholesterol by the Liebermann-Buchard reaction. *Biochemistry Journal, 52*(4), 611-619. https://doi.org/10.1042/bj0520611

Koura, B.I., Shipandeni, M., & Cutrignelli, M.I. (2023). Sustainable Feeds for Animal Nutrition in Tropical Areas. *Animals, 13*(8), 1379. https://doi.org/10.3390/ani13081379

Kuppusamy, P., Dong, K., Song, C.E., Ilavenil, S., Srigopalram, S., Valan, M., & Choon, K. (2018). Quantification of major phenolic and flavonoid markers in forage crop Lolium multiflorum using HPLC-DAD. *Revista Brasileira de Farmacognosia 28*(3), 282-288. https://doi.org/10.1016/j.bjp.2018.03.006

Makkar, H.P.S., Siddhuraju, P., & Becker, K. (2007). Trypsin inhibitor. *Methods in Molecular Biology™*, 1–6. https://doi.org/10.1007/978-1-59745-425-4_1

Makkar, H., Blummel, M., Borowy, N., & Becker, K. (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *Journal of the Science of Food and Agriculture, 61*(2), 161-165. https://doi.org/10.1002/jsfa.2740610205

Marques G.S, Leão, W.F., Lyra, M.A.M., Peixoto, M.S., Monteiro, R.P.M., Rolim, L.A., Xavier, H.S., Neto, P.J.R., Soares, L.A.L. (2013). Comparative evaluation of UV/VIS and HPLC analytical methodologies applied for quantifi cation of flavonoids from leaves of Bauhinia forfi cata. *Brazilian Journal of Pharmacognosy, 23*(1), 51-57. https://doi.org/10.1590/S0102-695X2012005000143

Martin, C., Morgavi, D.P., & Doreau, M. (2010). Methane mitigation in ruminants: from microbe to the farm scale. *Animal, 4*(3), 351-365. https://doi.org/10.1017/S1751731109990620

Munidasa, S., Eckard, R., Sun, X., Cullen, B., McGill, D., Chen, D., & Cheng, L. (2021). Challenges and opportunities for quantifying greenhouse gas emissions through dairy cattle research in developing countries. *Journal of Dairy Research, 88*(1), 3-7. https://doi.org/10.1017/S0022029921000182

Patra, A., & Saxena, J. (2011). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry Journal, 71*(11-12), 1198-1222. https://doi.org/10.1016/j.phytochem.2010.05.010

Patra, A., & Saxena, K. (2009). The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. *Nutrition Research Reviews, 22*(2) 204-219. https://doi.org/10.1017/S0954422409990163

Pensiero, J.F., Zabala, J.M., Lorena, & Richard, G.A. (2020). Native and Naturalized Forage Plant Genetic Resources for Saline Environments of the Southernmost Portion of the

American Chaco. Springer EBooks, 339–380. https://doi.org/10.1007/978-3-030-52592-7_18

Ramírez-Restrepo, C., & Barry, A. (2005). Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Animal Feed Science and Technology, 120*(3), 179-201. https://doi.org/10.1016/j.anifeedsci.2005.01.015

Ramos, G., Frutos, P., Giráldez, F.J., & Mantecón, A.R. (1998). Ramos, G.; Frutos, P.; Giráldez, F. J. & Mantecón, A. R. Secondary plant compounds in herbivore nutrition. *Arch. Zootecn. 47*, 597-620.

Santo, M., & Lecumberry, G. (2005). El proceso de medición: Análisis y comunicación de datos experimentales [The measurement process: Analysis and communication of experimental data]. Rio cuarto: Universidad Nacional de Rio Cuarto.Argentina.

Santocoloma-Varón, L.E. (2010). Evaluación del contenido de metabolitos secundarios en dos especies de plantas forrajeras encontradas en dos pisos térmicos de Colombia. *Revista de Investigación Agraria y Ambiental*, 31-35. https://doi.org/10.22490/21456453.890

Sepúlveda-Jiménez, G., Porta-Ducoing, H., & Rocha-Sosa, M. (2004). La participación de los metabolitos secundarios en la defensa de las plantas [The participation of secondary metabolites in plant defense]. *Revista Mexicana de Fitopatología*, 355-363. https://www.redalyc.org/pdf/612/61221317.pdf

Sreevidya, N., & Mehrotra, S. (2003). Spectrophotometric method for estimation of alkaloids precipitable with dragendorff reagent in plant materials. *Journal of AOAC International, 86*(6), 1124-1127.

Stafford, H.A. (1967). Biosynthesis of phenolic compounds in first internodes of Sorghum: Lignin and related products. *Plant Physiology*, 450-455. https://doi.org/10.1104/pp.42.3.450

Vaou, N., Stavropoulou, E., Voidarou, C.C., Tsakris, Z., Rozos, G., Tsigalou, C., Bezirtzoglou, E. (2022). Interactions between medical plant-derived bioactive compounds: Focus on antimicrobial combination effects. *Antibiotics (Basel), 11*(8), 1014. https://doi.org/10.3390/antibiotics11081014

Verdecía, D.H.-H.-M. (2021). Metabolitos primarios y secundario de seis especies de árboles, arbustos y leguminosas herbáceas. *Cuban Journal of Agricultural Science*, 77-93. http://scielo.sld.cu/scielo.php?script=sci_arttext&pid=S2079348020210001000008&lng=es&tlng=es

Wallace, R.J. (2004). Antimicrobial properties of plant secondary metabolites. *Proceedings of the Nutrition Society, 63*, 621-629. https://doi.org/10.1079/PNS2004393

White, R.G., & Lawler, J.P. (2002). Can methane suppression during digestion of woody and leafy browse compensate for energy costs of detoxification of plant secondary compounds? A test with muskoxen fed willows and birch. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology*, *133*, 849-859. https://doi.org/10.1016/S1095-6433(02)00152-6