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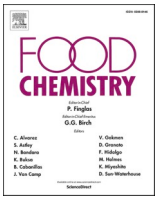

Analytical Methods

An accurate, cost-effective and simple colorimetric method for the quantification of total triterpenoid and steroidal saponins from plant materials

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ABSTRACT

Saponins are heterosides widely distributed in the plant kingdom. Their properties are used in many industrial sectors, such as food, cosmetics, agriculture, and pharmaceuticals, and their use is increasing due to the market trend to use natural ingredients. Although many techniques exist to quantify saponins (e.g., gravimetric, foaming, spectrophotometric or chromatographic), none of these allow simultaneous accurate, rapid and inexpensive analysis of both triterpenoid and steroidal saponins. A new colorimetric method constituted of *p*-anisaldehyde and sulfuric acid was developed and avoids all of the above disadvantages. Parameters used in this method allow a similar molar absorptivity for steroidal and triterpenoid saponins with high specificity in complex matrices reducing the sample preparation step and allowing quantification of saponins blends.

1. Introduction

Saponins are heterosides widely distributed in the plant kingdom (Vincken et al., 2007) also found in some marine organisms (Marston & Hostettmann, 1995). They are complex molecules constituted of a sapogenin associated with one or more osidic chains. Saponins possess several properties such as sweetness and bitterness (Heng et al., 2006; Schmid et al., 2021), foaming and emulsifying (Martín & Briones, 1999), and pharmacological as well as insecticide, molluscicide and antimicrobial activities (Sparg et al., 2004). These different physicochemical and biological properties are used in many industrial sectors such as in food, cosmetics, agriculture and pharmaceuticals (Güçlü-Üstündağ & Mazza, 2007). There is also an increase in demand for saponins due to the current market tendency to use natural ingredients (Güçlü-Üstündağ & Mazza, 2007). There are two main families of saponins; the steroidal saponins form of 27 carbon atoms and the triterpenoid saponins made up of 30 carbon atoms, which are the most abundant in plants. To ensure the quality of the extracts and raw materials used in the industry, it is necessary to develop rapid, accurate analytical methods, making it possible to quantify the total saponins content in complex matrices. Because structural diversity exists within these two groups of saponins, a method to assay both triterpenoid and steroidal saponins would be a major advantage to quantifying these compounds.

Many methods have been developed for detecting and measuring total saponins present in plants; usual methods use gravimetry and foam index (Tenon et al., 2017). However, these methods are not efficient enough to accurately quantify saponins. In the case of gravimetric method, to extract saponins with butanol tends to overestimate their amount due to the affinity of other compounds to this solvent. Moreover, the foaming method that measures the foam height is mainly used as a characterization element rather than a quantification element.

Individual quantification of saponins in vegetal samples has been reported in a large number of studies by chromatographic methods. There are quantitative methods using chromatography such as HPLC which coupled with different ultra-violet (UV), mass spectrometry (MS) or evaporative light scattering (ELSD) detectors allow for rapid, precise and robust measurements of the saponins content (Cheok et al., 2014; Man et al., 2010; Oleszek, 2002; Oleszek & Bialy, 2006). These devices are however expensive and it is necessary to optimize the chromatographic conditions to separate the various constituents present in each matrix plant which is time-consuming. In addition to this, quantification with chromatographic devices require standards of saponins. However, for this class of natural compounds, only a few representatives are commercially available as a standard, making it a very restrictive

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situation.

Other quantitative methods use thin-layer chromatography coupled with a densitometer. The advantage of this method is the speed of the analysis coupled with a separation of the compounds present in the matrix making possible the obtaining of accurate and robust measurements (Avula et al., 2011; Coran & Mulas, 2012). However, these methods must be automated to ensure the robustness of the quantification; it is then necessary to be equipped with relatively expensive modules. To this disadvantage is added the adaptation of the chromatographic conditions to analyse each plant matrices.

Quantification of total saponins by spectrophotometry has become popular due to its simplicity and better value compared to chromatographic methods. It consists in revealing the saponin pool using a chromogenic agent with acid and measuring the chromophore formed by a UV–vis spectrophotometer. The use of a standard calibration allows the quantification of saponins in the sample. The method usually used is that presented by Hiai and their collaborators (Hiai, Oura, Hamanaka, & Odaka, 1975; Hiai, Oura, Odaka, & Nakajima, 1975). The method uses a sulfuric vanillin solution to quantify triterpenoid saponins at a wavelength of 544 nm. Several standards of steroidal and triterpenoid sapogenins were tested, triterpenoid sapogenins had a maximum absorbance close to 540 nm while for steroidal sapogenins the maximum absorbance was around 460 nm with different molar absorption coefficients. In addition, the amount of sulfuric acid presents over 60% (v/v) in the reactional volume and causes the formation of interference close to the wavelengths of quantification, for example, rhamnose absorbs at 470 nm and sorbose at 520 nm (Hiai et al., 1976). These interferences can come from the plant matrix or the glycosides present on the saponins themselves, it is then necessary to carry out a pre-treatment before carrying out the quantification. There is another spectrophotometric method usually used and developed initially by Baccou and their collaborators (Baccou et al., 1977). They used sulfuric anisaldehyde to specifically reveal steroidal sapogenins at 430 nm. The specificity of the method has the advantage of directly quantifying steroidal saponins in a complex matrix without carrying out a pre-treatment. However, the conditions used do not allow the triterpenoid saponins to be revealed, reducing considerably the scope of this method.

Despite the different technologies and inventions developed over the last decades, there is no simple, fast and inexpensive method allowing accurate and total quantification of saponins in several complex matrices. In this paper, we present a rapid, specific, inexpensive and universal spectrophotometric method, which accurately measures total saponins of both types in several plant matrices. The method can also be used to quantify total saponins content in a mixture constituted of steroidal and triterpenoid saponins.

2. Material and methods

2.1. Chemical and materials

Four saponin plants: *Camellia oleifera* (defatted seed meal), *Chenopodium quinoa* (seed), *Trigonella foenum-graecum* (cotyledon) and *Yucca schidigera* (juice extract) were supplied by Nor-Feed (Beaucouzé, France) and named respectively Camellia, Quinoa, Fenugreek and Yucca. The standard of escin IB (≥97%, CAS: 26339-90-2) was purchased from Phytolab (Vestenbergsgreuth, Germany). Standards of protodioscin (≥98%, CAS: 55056-80-9) and escin (≥95%, CAS: 6805-41-0) were purchased from Merck KGaA (Darmstadt, Germany). Stock solutions of each standard were prepared at 5 $mg.mL^{-1}$ in methanol. Deionized water was obtained using an Elix advantage 15 system (Merck-Millipore, USA). Methanol, ethanol, ethyl acetate and dichloromethane (technical grade) were purchased from Carlo Erba (Milan, Italia). Sulfuric acid (>95%) and *p*-anisaldehyde (>99%) were purchased from Fisher Scientific (MA, USA).

2.2. Sample preparation

2.2.1. Comparison of extraction methods

To compare the effectiveness of the sample preparation, different extraction methods were trialled. Each sample of Camellia and Fenugreek plants was extracted in triplicate with a pressurized liquid extractor (PLE) Speed Extractor E-914 (Büchi, Switzerland); an ultrasonic extractor (US) Elmasonic S30(H) (Elma Schmidbauer, Germany) and a micro-wave extractor (MAE) Monowave 300 (AntonPaar, France). The solvent used during the extraction was filtered and evaporated under reduced pressure with a rotary evaporator Hei-Vap (Heidolph, Germany) for dryness. Different solvents, sample/solvent ratios (m/v), times, temperatures of incubation and number of cycles were tested. Optimal conditions for each method were selected. Methods were compared by following the yield of extraction (%m/m) and the total area (AU) in HPLC-ELSD corresponding to the saponins using the method explained in 2.4.

2.2.2. Extraction method for the quantification for analysis of saponins

Sample preparation was carried out in triplicate to obtain Camellia, Quinoa, Fenugreek and Yucca extracts. A weight of 500 mg of dried and ground saponin plant was solubilized in 50 mL of dichloromethane. The solution was extracted with an ultrasonic bath set at 50 °C for 5 min then filtered, the residue was extracted twice with dichloromethane. The residue is then solubilized with 50 mL of methanol and extracted with an ultrasonic bath set at 50 °C for 5 min and again filtrated, the residue is extracted twice with methanol. Filtrates were recovered and evaporated under reduced pressure with a rotary evaporator for dryness. The extraction yields were 18, 45, 22 and 28% (m/m) for Camellia, Quinoa, Fenugreek and Yucca samples respectively. Camellia and Fenugreek extracts were blended till obtaining mixtures corresponding to 20/80, 50/50 and 80/20 (m/m). Extract solutions were prepared in methanol at 10 $mg.mL^{-1}$. After centrifugation at 10 000 g for 10 min, supernatants were used for analysis.

2.3. HPLC- MS^n characterization

Camellia and Fenugreek extracts were analysed with an LC Waters 2795 with a UV detector (Waters, USA) coupled with a mass spectrometer Esquire 3000 plus composed of electrospray ionization and an ion trap analyser (Bruker, USA). Mass data was recorded with the following ionization conditions: a capillary voltage 3500 V and a nebulizer temperature of 340 °C together with a nitrogen sheath gas (207 kPa). The full scan mass acquisitions in the positive and negative mode were performed by scanning from 400 up to 1600 *m/z* range with a target value of 1100 *m/z*. Mass data were compared with databases and literature to tentatively identify compounds and saponins present in Camellia and Fenugreek extract.

2.4. HPLC-ELSD method

The total saponins content in Camellia, Quinoa, Fenugreek and Yucca extracts was determined by HPLC-ELSD according to the following method: an LC-2030C 3D with diode array detector (Shimadzu, Japan) was coupled with an Evaporative Light Scattering Detector LT-ELSD Model 90LT (Sedere, France). A Capcell Pak C_{18} AQ (4.6 × 150 mm, 3 μm) (Osaka Soda, Japan) was used. The mobile phase was composed of two solvents (A and B), where A corresponds to 0.1% formic acid (v/v) in water and B to acetonitrile. The gradient used was: 0–20 min from 0 % to 100 % B and held for 5 min. The flow rate was set at 1 $mL.min^{-1}$, and the temperature of the column was set at 25 °C. The nebulizer temperature of the ELSD was set at 50 °C with a gas pressure of 350 kPa. A volume of 20 μL of the supernatant obtained during sample preparation (2.2) was injected. Areas corresponding to saponins were integrated with the ELSD and the total saponins content in the extract was determined by a standard calibration curve.

2.5. Gravimetric method

The total saponins content in Camellia and Fenugreek extracts was determined with the gravimetric method according to (Budan et al., 2013). Extract (100 mg) was accurately weighed and dissolved in 50 mL of water. The solution was transferred into a separatory funnel and supplemented with 15 mL of ethyl acetate. The mixture was then shaken and the aqueous layer was recovered after the complete separation phase and partitioned twice again with ethyl acetate. The aqueous solution was then extracted with 10 mL of *n*-butanol and left to stand for complete separation. The aqueous phase was then extracted twice with 10 mL *n*-butanol and the combined organic phases were evaporated under reduced pressure to become dry and freeze-dried with an Alpha 2-4 LSCplus (Martin Christ, Germany) for 12 h. The saponin content was calculated as the mass of dried butanolic extract.

2.6. HPTLC analysis

Extracts of Camellia and Fenugreek were analysed by High-Performance Thin-Layer Chromatography (HPTLC). 50 μg of methanolic extracts and 0.4 to 8 μg of protodioscin and escin standards in methanol were spotted on a C_{18} plate Alugram® RP-18 TLC W/UV_{254} with an autosampler 4 (CAMAG, Switzerland). Migration was performed with a mixture of methanol/water/formic acid 65/35/1 (v/v/v). The plate was derivatized by spraying 0.5% (v/v) of *p*-anisaldehyde in a sulfuric methanol solution according to Jork (Jork et al., 1990) and heated at 150 °C with a TLC Plate Heater 3 (CAMAG, Switzerland) until visualization of the spots. Scans and UV spectra of the plate were recorded with a TLC Scanner 3 and TLC Visualizer (CAMAG, Switzerland). The spectra were processed with the software WinCats version 1.4.4.

2.7. Spectrophotometric method

UV spectra of protodioscin and escin IB standards were recorded in methanol respectively at 0.2 and 0.03 $mg.mL^{-1}$ with an Evolution 60 Spectrophotometer (Thermo Fisher Scientific, USA). Maximum wavelengths and molar absorption coefficients were determined.

2.7.1. Total saponins quantification (600 nm)

Total saponins contents were determined in Camellia, Quinoa, Fenugreek, Yucca and mixture extracts by spectrophotometric analysis. A volume of 100 μL of the supernatant obtained during sample preparation (2.2) was transferred into a capped test tube with 100 μL of a 50% *p*-anisaldehyde solution in methanol (v/v) and 2 mL of 50% aqueous sulfuric solution (v/v). This step was followed by the solution being immediately heated at 60 °C in a water bath for 20 min. Finally, the chromogenic reaction was stopped by transferring the capped test tube into an ice-water bath for 10 min. The reactional solution was transferred into a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, USA). The absorbance measurement was performed against a blank at 600 nm. The total saponins content in the extract was determined by using a standard calibration curve. To avoid drift of the absorbance between chromogenic reactions, standard solutions and samples were performed during the same chromogenic reaction batch.

2.7.2. Analytical method assessment

The analytical spectrophotometric method at 600 nm was evaluated. The analytical properties such as precision, selectivity, linearity, detection limit, quantification limit and accuracy were investigated in this context. The studies were conducted with standard solutions, samples and spiking experiments.

2.7.3. Total steroidal saponins quantification (425 nm)

The total steroidal saponins were determined in Fenugreek and mixture extracts with a method derived from the work of (Baccou et al., 1977). A volume of 100 μL of the supernatant obtained for sample preparation (2.2) was transferred into a capped test tube with 100 μL of a 10% *p*-anisaldehyde solution in ethanol (v/v) and 2 mL of 12.5% sulfuric solution in ethanol (v/v). The solution was then heated at 60 °C in a water bath for 20 min. Finally, the chromogenic reaction was stopped by transferring the capped test tube into an ice-water bath for 10 min. The reactional solution was transferred to the spectrophotometer. The absorbance measurement was performed against a blank at 425 nm. The total steroidal saponin content in the extract was determined by using a standard calibration curve with protodioscin as standard.

2.7.4. Total triterpenoid saponins quantification

The total triterpenoid saponin content was determined in mixture extracts using the values obtained during total saponins quantification (2.7.1) and total steroidal saponin quantification (2.7.3) according to the formula Eq. (1):

$$\textit{Total triterpenoid saponins content} = \textit{Total saponins content} - \textit{Total steroidal saponins content} \quad (1)$$

The standard of saponin used for mixture extracts was the protodioscin.

3. Results and discussion

Several methods have been developed in the past to determine the total saponins content. Unfortunately, none of these methods makes it possible to be at the same time accurate, fast, inexpensive and universal, which means allowing quantification of both steroidal and triterpenoid saponins in complex matrices. Therefore, we have developed a rapid and inexpensive spectrophotometric method that responds to the inconveniences and inadequacy of the existing techniques.

3.1. Sample preparation

Preliminary extraction experiments have been carried out to ensure the complete extraction of triterpenoid and steroidal saponins from plant materials. *Camellia oleifera* (Camellia) was used as a triterpenoid saponin plant and *Trigonella foenum-graecum* (Fenugreek) was used as a steroidal saponin plant. The experiments consisted in comparing several extraction procedures with different extraction modes: pressure liquid extraction (PLE), microwaves assisted extraction (MAE), ultrasonic-assisted extraction (UAE). The extraction performance was evaluated with the extraction yield (%) obtained as well as the integrated area in HPLC-ELSD (AU) corresponding to the saponins. The results showed that utilization of methanol at 50 °C gave the best results in terms of extraction yield as well as the integrated saponins area for both plants. Moreover, PLE, MAE and UAE gave similar results in terms of extraction yields and integrated area which ranged from [31.7–32.7%] and [6.93–6.94 AU] respectively for Fenugreek and from [19.5–21.7%] and [6.95–6.98 AU] respectively for Camellia. These similar results testify to the exhaustiveness of saponins extraction in these matrices. Therefore, ultrasonic-assisted extraction was chosen for its “easy to use” and

relatively better value aspects. The chosen sample preparation for this study consisted in performing three extraction cycles with methanol and evaporating to dry the filtrate after filtration to obtain an extract usable for the determination of total saponins content. During the development of the spectrophotometric method and as a precaution, a delipidation step with dichloromethane was added to eliminate the compounds which may cause interferences on the measurement such as triterpenoid and steroidal compounds. It should be noted that if these compounds are not present or have no impact on the spectrophotometric measure, the extraction step can be drastically simplified by using a single 10 min extraction cycle with methanol followed by centrifugation. The quantification of the saponins is then carried out with an aliquot of the supernatant.

3.2. Usual methods

3.2.1. HPLC-ELSD method

A liquid chromatography method coupled with an Evaporative Light Scattering Detector (HPLC-ELSD) was developed in-house to quantify saponins present in Camellia, *Chenopodium quinoa* (Quinoa), Fenugreek and *Yucca schidigera* (Yucca) extracts. This method is very reliable and was used as a reference to compare total saponins content obtained by the gravimetric and the spectrophotometric methods. Saponins are molecules with weak chromophores, their quantification with UV–vis detector remains difficult because of the maxima wavelengths close to the cutoff of the solvent and their various intensities. Indeed, escin IB and protodioscin, two of the standards used in this study, have only one maximum at 205 nm with a respective molar absorption coefficient of 22,172 and 3693 $L.mol^{-1}.cm^{-1}$. The use of an ELSD detector is well suitable for the quantification of these molecules because neither the optical properties of the compounds nor their capacity to form charged species has an impact on the ELSD response. The extracts obtained during the sample preparation are constituted of various metabolites. Because the ELSD detector gives no spectral information about molecules, preliminary work is, therefore, necessary to identify saponins from other constituents by using detectors suitable for their identification. Previous works in HPLC-MS^n were therefore carried out on extracts, the Fig. 1 shows ELSD chromatograms for Camellia and Fenugreek. Extracts are complex matrices mainly constituted of sugars, flavonoids and saponins. The area corresponding to saponins was integrated and the total saponins content was determined by external calibration. Quantification by ELSD is almost independent of the structure and therefore any saponin standard could be used with the result given as equivalent. For this study, similar standards to saponins found in studied plants were used. For Camellia and Quinoa, escin IB, a triterpenoid saponin was chosen. Whereas for Fenugreek and Yucca, protodioscin, a steroidal saponin was chosen. The total saponins content determination performed in triplicate by specifically calculating the sum of the areas of saponins for the four extracts is given in Table 1. The HPLC-ELSD method developed here permitted quantifying saponins in several extracts with a relative standard deviation (RSD%) which does not exceed 5% corresponding to a good level of precision for a method based on ELSD detector (Tenon et al., 2017). This method meets all of the criteria for its use in industry. However, the device is expensive and upstream work must be done to identify the saponins present in the samples, which can be time-consuming in the case of different matrices' analyses.

Table 1
Analysis results and comparison between HPLC-ELSD method and gravimetric method.

Extracts	HPLC-ESLD method		Gravimetric method	
	Found (%m/m)	RSD% (n = 3)	Found (%m/m)	RSD% (n = 3)
Camellia[a]	30.1	2.6	58.2	11.2
Quinoa[a]	9.6	4.7	–	–
Fenugreek[b]	28.7	2.8	50.9	9.0
Yucca[b]	29.9	4.1	–	–

[a] Values given as escin IB equivalent for HPLC-ELSD method.
[b] Values given as protodioscin equivalent for HPLC-ELSD method.

3.2.2. Gravimetric method

The saponins content in Camellia and Fenugreek extracts was estimated by the gravimetric method. This method widely used is based on the affinity of saponins with the butanol to specifically extract these latter. The results for the method presented in Table 1, show a saponin

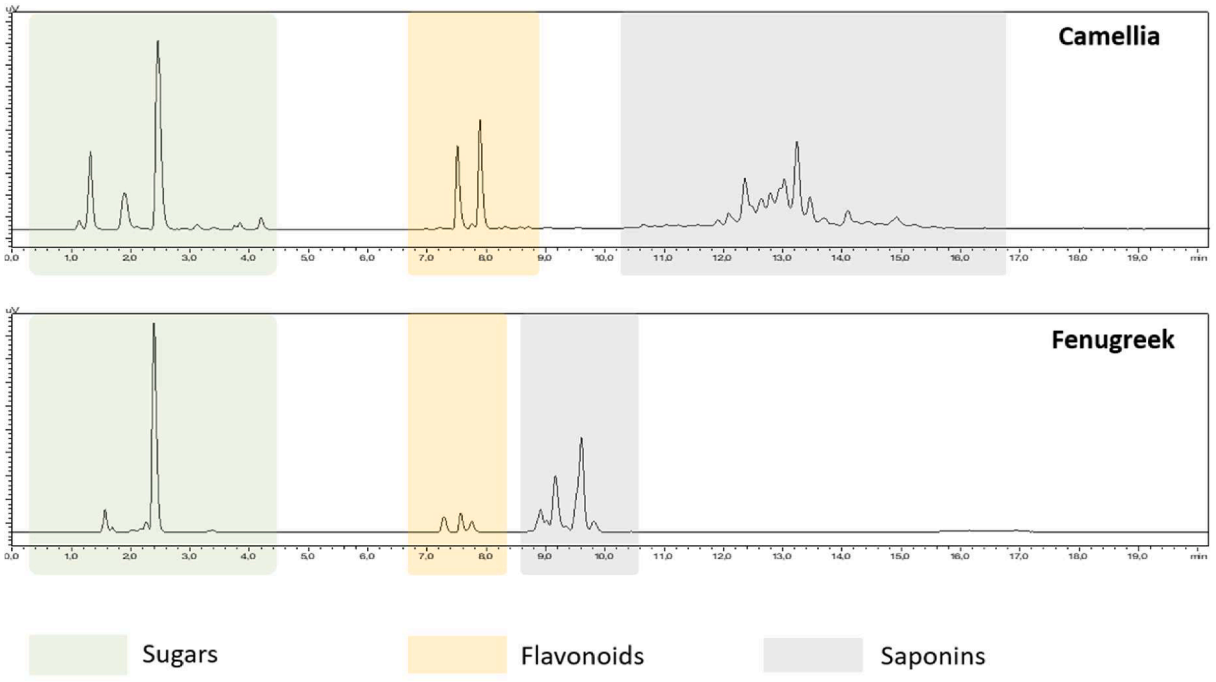

Fig. 1. HPLC-ELSD chromatograms of methanolic extracts for Camellia and Fenugreek.

content for Camellia and Fenugreek of 58 and 51% respectively (m/m). These values compared to those obtained with the HPLC-ELSD method lead to overestimations of up to 93%. These overestimations of the saponin content are due to other compounds still present in the butalonic extracts resulting from the low specificity and affinity of the saponins to butanol. To this lack of accuracy, is added a lack of precision, resulting in relatively high RSD% as well as an excessively long process compared to a method such as HPLC-ELSD.

3.3. Spectrophotometric method

In this study, we present a simple and cost-effective spectrophotometric method to quantify total saponins content in plants and their extracts. Usual spectrophotometric methods have many drawbacks: different molar absorption coefficients depending on saponin type, no-specific absorbance in complex matrices resulting in a biased measurement that requires specific sample preparation.

Preliminary tests were carried out using High-Performance Thin-Layer Chromatography (HPTLC) to determine the most suitable chromogenic reaction to quantify both steroidal and triterpenoid saponins in solution. The technique allows the separation of the different constituents of an extract on a plate, the use of a suitable staining reagent then permits the observation of the different chromophores of the extract. Coupled with a TLC scanner, the UV–vis spectra of each chromophore can also be obtained. Different staining reagents were tested on Camellia and Fenugreek extracts as well as on saponin standards: escin and protodioscin. The UV–vis spectra of each chromophore were then compared to determine a specific wavelength for the steroidal and triterpenoid saponins concerning the other constituents. The results showed that the use of *p*-anisaldehyde and sulfuric acid in controlled conditions led to the same λ_{max} for steroidal and triterpenoid saponin chromophores and a specific wavelength with regard to the other compounds in the extract as presented in Fig. 2.

There are few studies on the formation of chromophores in presence of sulfuric *p*-anisaldehyde for natural products proposed. A formation mechanism of the chromophore was recently proposed for the stigmasterol, a structure related to the sapogenins. After dehydration of alcohol function, the non-aromatic double bond then formed underwent condensation by the electrophilic *p*-anisaldehyde leading to a blue chromophore (Xu & Liu, 2021). These results are consistent with the absorbance observed with saponins at 600 nm in Fig. 2. With our conditions, the double bond involved in the condensation step could come from the dehydration of the hydroxyl present in C3 due to the acidic and heating conditions. To our knowledge, all the discovered saponins possess a free hydroxyl or a saccharide chain attached by an ether linkage in C3 (Vincken et al., 2007). This oxygenated position shared by all saponins seems to be a major key for the formation of an identical chromophore at 600 nm whether steroidal or triterpenoid saponins. Further studies are however necessary to confirm the mechanism and the regioselectivity associated with saponins.

Other constituents like polyphenols and polysaccharides are also present in the methanolic extract of saponin plants. The electron-rich aromatic rings of polyphenols like flavonoids react with *p*-anisaldehyde to form reddish chromophores (Xu & Liu, 2021). Absorbance is therefore around 300–500 nm and does not interfere with saponins as observed in Fig. 2. Sugars could react by a double bond via hydroxyl dehydration leading to a blue chromophore as stated for saponins. However, dehydration of sugar is more demanding in energy than saponins. Controlled conditions such as percentage of sulfuric acid and heating temperature, could avoid the development of these potential interfered chromophores.

A colorimetric method in solution was thus developed and the reaction conditions were optimized: concentration of *p*-anisaldehyde (2.3% v/v), sulfuric acid (45.5% v/v), reactional solvent (water), temperature (60 °C) and time of incubation (20 min). This colorimetric method allows the development of a sapogenin chromophore with a maximum absorbance at 600 nm. The molar absorptivity obtained at this wavelength is similar for steroidal and triterpenoid saponins making it possible to quantify them in a mixture and with any standard of saponin. Moreover, absorption at 600 nm is only specific for sapogenins, making it possible to quantify directly in the presence of other compounds such as sugars and polyphenols, and so considerably reduces the stages of sample preparation. Validation studies for the proposed method were carried out and described in this paper, comprehensive results from the method validation were given in Table 2.

3.3.1. Selectivity

Selectivity describes the influence of other components on the

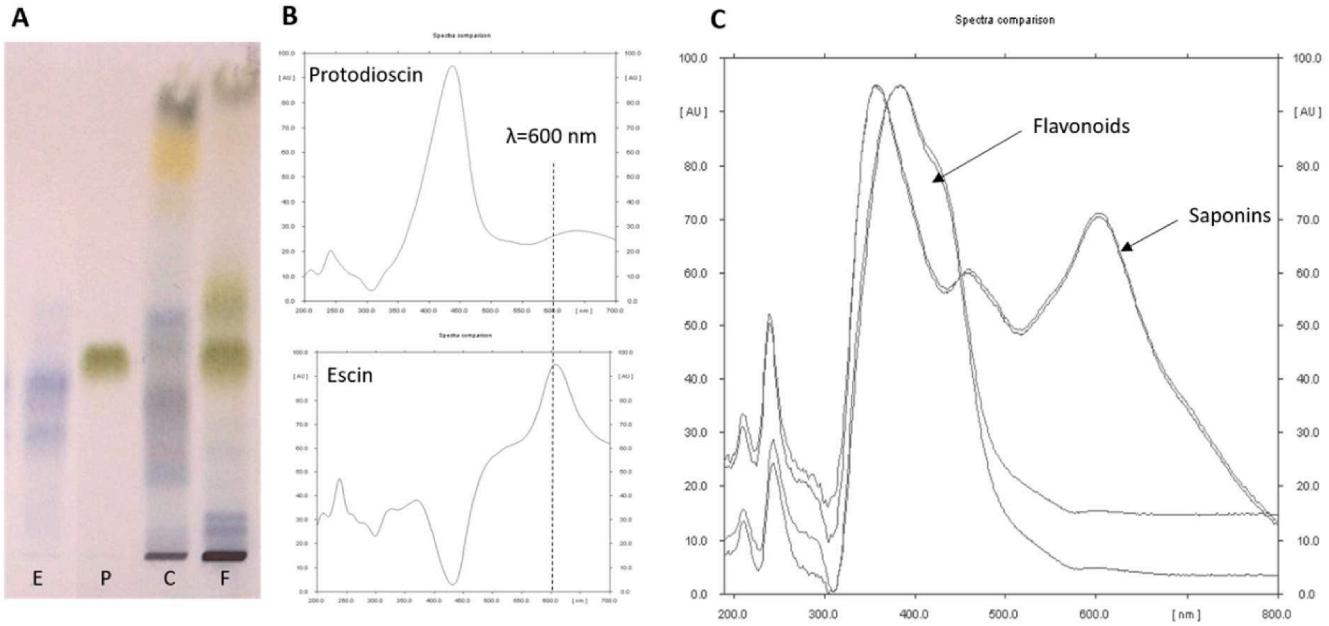

Fig. 2. HPTLC with *p*-anisaldehyde as reagent (A) with E: Escin, P: Protodioscin, C: Camellia, F: Fenugreek; (B) UV–vis spectra for chromophores of protodioscin and escin; (C) UV–vis spectra for chromophores of saponins and flavonoids from Camellia extract (see 2.6 for experimental conditions).

Table 2
Analytical characteristics of the spectrophotometric method.

Parameters	Triterpenoid saponins		Steroidal saponins
	Standard escin IB	Standard escin	Standard protodioscin
Regression equation	Abs. = (5.95 ± 0.24) × 10^{-3} (µg.mL^{-1}) + (22.5 ± 1.2) × 10^{-3}	Abs. = (5.08 ± 0.07) × 10^{-3} (µg.mL^{-1}) + (55.4 ± 0.7) × 10^{-3}	Abs. = (7.30 ± 0.31) × 10^{-3} (µg.mL^{-1}) + (46.1 ± 1.2) × 10^{-3}
Correlation coefficient (r^2)	0.9934	0.9973	0.9918
Linear working range (µg.mL^{-1})	19–230	20–230	18–230
LOD (n = 6, µg.mL^{-1})	6	6	5
LOQ (n = 6, µg.mL^{-1})	19	20	18
ε_{600nm} (L.mol^{-1}.cm^{-1})	2744 ± 99	2630 ± 159	2745 ± 84
Repeatability (n = 6, RSD%)	2.0–1.4 (Camellia extract)		0.7–1.7 (Fenugreek extract)
Intermediate precision (n = 12, RSD%)	2.6 (Camellia extract)		2.3 (Fenugreek extract)
Recovery (R%)	(Camellia extract)		(Fenugreek extract)
Spiking 50% (n = 3, %)	99.2–102.8		102.2–106.7
Spiking 100% (n = 3, %)	96.3–99.0		99.4–103.1

measured signal. If the generated signal is selective for the analyte, the method gives more sensitive and accurate results. The characterization of camellia and fenugreek extracts showed that the main compounds were sugars, flavonoids and saponins. To assess their impact, solutions of galactose and camelliaside A (the main sugar and glycosylated flavonol found in Camellia), were subjected to the chromogenic reaction. The solutions analysed in the proportions found in the extract gave no absorbance at 600 nm. Two calibration graphs were drawn up, one with a standard solution of escin in pure solvent and the other with the addition of standard escin in Camellia extract in the range of 100 to 200% of nominal saponin content. Then, the curves for both experiments were compared. The difference between the curves showed a matrix effect enhancement of only 4.1% for the standard addition method, indicating no enhancement or suppression of the signal by potential interferences present in the sample. These results clearly support the selectivity of the method.

3.3.2. Linearity

Linearity describes the range for which the concentration of analyte shows a linear relationship with the measured signal. Linearity was investigated in a solution for different standards: escin IB, protodioscin and escin. The studies showed that the absorbance for each standard was highly linear in the range 20–230 µg.mL^{-1}. The regression equations obtained from five points calibration in triplicate were as indicated below Eq. (2):

$$\textbf{\textit{Escin IB}}: Abs. = (5.95 \pm 0.24) \times 10^{-3} C(\mu g.ml^{-1}) + (22.5 \pm 1.2) \times 10^{-3} \quad (2a)$$

With regression coefficient of r^2: 0.9934

$$\textbf{Protodioscin}: Abs. = (7.30 \pm 0.31) \times 10^{-3} C(\mu g.ml^{-1}) + (46.1 \pm 1.2) \times 10^{-3} \quad (2b)$$

With regression coefficient of r^2: 0.9918

$$\textbf{Escin}: Abs. = (5.08 \pm 0.07) \times 10^{-3} C(\mu g.ml^{-1}) + (55.4 \pm 0.7) \times 10^{-3} \quad (2c)$$

With regression coefficient of r^2: 0.9973

Statistical analysis of the results according to the lack-of-fit test for escin IB, protodioscin and escin gave respectively for the range 50–230 µg.mL^{-1} a $F_{calculated}$ of 0.047, 0.029 and 0.189 which are lower than the Snedecor table value (3.71 for $p = 0.05$).

3.3.3. Limit of detection

Limit of detection (LOD) was defined as the lowest analyte concentration that can be determined by the analytical process. LOD was determined with procedural blanks consisting of repeating six times a blank solution without analyte over the whole procedure under repeatability conditions. The quantity of saponins in blanks is determined from the calibration curve with each standard: escin IB, protodioscin and escin. LOD was calculated with the following equation Eq. (3):

$$LOD = 3 \times s_{blank} \quad (3)$$

where s_{blank} was the standard deviation obtained for the blank solutions, the number 3 is a constant with a confidence level of 95% according to the IUPAC. The LOD for escin IB, protodioscin and escin were calculated respectively as 6, 5 and 6 µg.mL^{-1}.

3.3.4. Limit of quantification

Limit of quantification (LOQ) was defined as the lowest analyte concentration that can be quantified by the analytical process. It was based on a standard deviation of 6 replicates of procedural blanks in repeatability conditions and calculated with the following equation Eq. (4):

$$LOQ = 10 \times s_{blank} \quad (4)$$

According to the IUPAC, the LOQ was expressed in terms of relative standard deviation, which is the maximum tolerated value traditionally equal to 10%. The LOQ was determined to be 19, 18 and 20 µg.mL^{-1} for escin IB, protodioscin and escin.

3.3.5. Molar absorption coefficient

From the linearity range obtained, the molar absorption coefficient was determined for the three standards. Solutions of the three standards were subjected to the same chromogenic reaction in the range 50–230 µg.mL^{-1}. The molar absorption coefficients were determined, corresponding to the slope of the calibration curves Eq. (5):

$$\textbf{EscinIB}: \varepsilon_{600nm} = 2744 \pm 99 L.mol^{-1}.cm^{-1} \quad (5a)$$

$$\textbf{Protodioscin}: \varepsilon_{600nm} = 2745 \pm 84 L.mol^{-1}.cm^{-1} \quad (5b)$$

$$\textbf{Escin}: \varepsilon_{600nm} = 2630 \pm 159 L.mol^{-1}.cm^{-1} \quad (5c)$$

This method allows to obtain a chromophore that is sensitively the same for the three different saponins and whose global molar absorption coefficient is 2707 ± 109 L.mol^{-1}.cm^{-1} at 600 nm.

The major advantage of this method is that any saponin standard can be used to quantify the saponins in a sample, whether it consists of triterpenoid, steroidal or both saponins. In addition, purified saponins are quite expensive like escin IB and protodioscin, the use of a mixture of saponins like escin standard could be an inexpensive alternative to quantify saponins in samples. Because the chromogenic reaction can cause a drift of the absorbance between runs, it is preferable to quantify saponins in samples with a standard performed on the same chromogenic reaction.

3.3.6. Precision

The precision of an analytical method describes the variation in the results under stipulated conditions. In this study, precision was defined under repeatability conditions and intermediate precision. The study was performed on Camellia and Fenugreek extracts with escin IB and

protodioscin respectively as standard. Repeatability was determined by measuring each sample six times under the same operating conditions according to the analytical method. Intermediate precision was determined by repeating the repeatability over two days. Precision was expressed as relative standard deviation RSD%. Values obtained under repeatability conditions were 1.7 and 1.2% for Camellia and Fenugreek extracts. Under intermediate precision conditions, the RSD% were 2.6 and 2.3% respectively for Camellia and Fenugreek extracts, which were acceptable results for a spectrophotometric method.

3.3.7. Accuracy

The accuracy of an analytical method describes the difference obtained experimentally with the reference value accepted is conventionally true. Because no certified reference material containing saponins was available, accuracy was performed by spiking samples with standard solutions. Two levels of saponin standard were added, corresponding to 50% and 100% of the nominal saponins content present in the sample. The study was performed with Camellia and Fenugreek extracts with respectively escin IB and protodioscin as standards. The accuracy was evaluated in triplicate by finding the recovery with the following equation Eq. (6):

$$R\% = \frac{C_{O+S} - C_O}{C_s} \times 100 \quad (6)$$

Where C_{0+S} is the saponin content found for the spiked sample, C_0 is the saponin content found in the initial sample and C_s is the saponin content added in the sample. Camellia and Fenugreek extracts with a known amount of standard have shown a recovery in the range of 96.3–106.7%. The recoveries were within acceptable levels indicating that the response provided by other compounds present within these two matrices has no impact on the overall result.

3.4. Applications of the spectrophotometric method

3.4.1. Determination of total saponins content in samples

Determination of total saponins content in four plant extracts: Camellia, Quinoa, Fenugreek and Yucca were performed to see the applicability of the proposed method. The standard used for the quantification corresponded to the saponins type present in the sample: escin IB for Camellia and Quinoa, protodioscin for Fenugreek and Yucca. Each determination was performed in triplicate and compared with the HPLC-ELSD method presented above. In addition to that, the accuracy by spiking experiment at 50% of the nominal saponins content (as R%) and precision under repeatability (as RSD%) were calculated. Results obtained for samples are displayed in Table 3, the spectrophotometric method gave good RSD% (<2.7%) and recoveries (100.9–106.1%) indicating a good precision and accuracy of the method for several matrices.

The total saponins content found for the four extracts were in the range of 9.5–30.5% (m/m), these values are quite similar to those found by the HPLC-ESLD method developed in-house (Table 1). These results show that the spectrophotometric method proposed here possesses all criteria to be used in total saponins measuring processes. Therefore, it is not necessary to carry out upstream work on the samples because of the specificity of the method for quantifying sapogenins in complex matrices.

The slight downside of this method is the limit of quantification (~20 μg.mL^{-1}) therefore, this method does not apply to very low contents in saponins. However, chromogenic reaction coupled with extraction step presented above permits quantifying saponins in plants with amounts lower than 1% (m/m) which are enough for most applications.

Table 3
Analysis results for total saponins content in four extracts by spectrophotometric method (600 nm).

Extracts	Found (%m/m)	RSD% (n = 3)	Recovery (%)
Camellia[a]	30.5	1.7	100.9
Quinoa[a]	9.5	1.2	106.1
Fenugreek[b]	28.7	1.2	104.9
Yucca[b]	28.1	2.7	102.7

[a] Values given in escin IB equivalent.
[b] Values given in protodioscin equivalent.

3.4.2. Determination of total saponins content in saponins mixtures

The spectrophotometric method presented here gives a sapogenin chromophore identical for steroidal and triterpenoid saponins at 600 nm (see above molar absorption coefficient). With this feature, it is possible to quantify the total content of saponins in a sample containing both steroidal and triterpenoid saponins. To see the applicability of the proposed method, mixtures of Camellia and Fenugreek extracts were performed in different ratios: 20/80, 50/50, 80/20 (m/m). Before quantifying saponins in these mixtures, the total saponins content was determined with protodioscin as standard in both extracts. The amount of saponins was 26.1% for Camellia (i.e., triterpenoid type) and 29.4% (m/m) for Fenugreek (i.e., steroidal type). From these results, theoretical content in saponins was calculated for prepared mixtures and displayed in Table 4. Total saponins contents were then determined experimentally in extract mixtures, values are compared against calculated values with relative bias (Bias%).

Results found experimentally were relatively close to the calculated values with a bias% which did not exceed 3.2%. These values clearly support the applicability of the method for the determination of total saponins in mixtures constituted of both saponin types.

To our knowledge, no spectrophotometric method can quantify simultaneously the total saponins content in a steroidal and triterpenoid mixture. Chromatographic methods could achieve similar results. However, as stated above, devices are expensive and require substantial work to separate and identify the saponin areas from the other constituents in the chromatogram.

3.4.3. Determination of steroidal and triterpenoid saponins contents in mixtures

Previous works carried out by (Baccou et al., 1977) have shown that soft conditions of *p*-anisaldehyde (0.13% v/v) and sulfuric acid (12.5% v/v) permit to react only with the spirostan and furan structures present in steroidal saponins regardless of the C3 hydroxyl function. Their spectrophotometric method made it possible to selectively quantify steroidal saponins from complex matrices at 425 nm.

Our first studies have shown that the method was not selective enough regarding complex samples. In a Fenugreek/Camellia mixture with a ratio of 50/50 (m/m), interferences could lead to an overestimation of the steroidal saponins content by >30%. To reduce this overestimation, several modifications were evaluated in Bacou's initial method. The results showed that exchanging ethyl acetate by ethanol in the chromogenic reaction reduced drastically the interferences measured at 425 nm. In the same experiment as before, the interferences from Camellia at the quantification wavelength corresponded to an overestimation of only 3% of the steroidal saponins content of Fenugreek.

Coupling this modified method with the method presented in this paper could permit obtaining both steroidal saponins content and the triterpene saponins content in a mixture of both saponins types.

To verify if the method is fit for this purpose, Camellia and Fenugreek extracts were mixed in several ratios: 20/80, 50/50, 80/20 (m/m).

Steroidal saponin content was assayed with the spectrophotometric method at 425 nm for each mixture. Then, the triterpenoid saponin content was determined by subtracting the total saponins content at 600 nm determined in 3.4.2 by the steroidal saponins content found previously. Values obtained are compared against calculated values with bias % and presented in Table 4.

Table 4
Comparison of results for total saponins (600 nm), steroidal saponins (425 nm) and triterpenoid saponins (600–425 nm) in blends.

	Total saponins found (%m/m)			Steroidal saponins found (%m/m)			Triterpenoid saponins Found (%m/m)		
Ratio Camellia/ Fenugreek (m/m)	Calculation	Spectrophotometric method (600 nm)	Bias %	Calculation	Spectrophotometric method (425 nm)	Bias %	Calculation	Subtraction (600–425 nm)	Bias %
20/80	26.8	27.1	1.2	5.9	5.9	0.0	20.9	21.2	1.4
50/50	27.8	26.9	−3.2	14.7	14.8	0.7	13.1	12.1	7.6
80/20	28.7	28.0	−2.6	23.5	23.2	−1.3	5.2	4.8	7.7

From the combination of two spectrophotometric methods (600 nm and 425 nm), values obtained were relatively close to those found by calculation with a bias% which did not exceed 7.7%. This is the first time that a method permits quantifying in a complex matrix the contents of each saponin type. Compared to other methods, these separate results would be difficult to obtain by chromatographic technics. Indeed, the upstream work for identifying and distinguishing the steroidal saponins from triterpenoid saponins would require significant work by LC-MS. Besides, both types of saponins may have close physicochemical properties, their retention range could overlap and so become hard to integrate.

Because we found interferences at 425 nm during the first studies, the applicability of this method should be undergone with other matrices to be fully demonstrated. This application has great potential in the industry by example for the standardization of plant material constituted of two saponins types or to unravel plant adulteration issues.

4. Conclusion

In this study, a new, simple, fast and inexpensive spectrophotometric method for the total quantification of saponins in plant samples was developed. The proposed method exhibited good performance in terms of linearity, precision and accuracy.

The specificity of the method was demonstrated in several plant extracts indicating that the total quantification is reliable in complex extracts with no interference. Therefore, sample preparation could be performed with a simple, cost-effective extraction step by using an ultrasonic bath reducing drastically the time of analysis.

Moreover, the method exhibited identical absorbance responses for both steroidal and triterpenoid saponins permitting to use of any saponin standard available to quantify saponins in samples. In this study we demonstrated that a mixture of escin saponins, commercially available and less expensive than purified saponins have similar performance to the latter, this standard can be a good alternative for the determination of total saponins contents in samples.

Finally, we demonstrated new potential applications: total saponins quantification for mixtures constituted of both types of saponins but also the quantification of each saponins type in mixtures by a combination of another spectrophotometric method specific for steroidal saponins.

The spectrophotometric method developed here has been patented under the registration number EP3742153A1.

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CRediT authorship contribution statement

Maxime Le Bot: Conceptualization, Methodology, Validation, Investigation, Writing – original draft. **Juliette Thibault:** Methodology, Investigation. **Quentin Pottier:** Investigation. **Severine Boisard:** Conceptualization, Methodology, Writing – review & editing, Supervision. **David Guilet:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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