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

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7 **Abstract**

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

17 **Keywords**

18 total saponins, plant extracts, steroidal saponin, triterpenoid saponin, chromogenic reaction,
19 spectrophotometric quantification

20 **Abbreviations**

21 UV-vis: Ultraviolet–Visible

22 HPLC: High-Performance Liquid Chromatography

23 ELSD: Evaporative Light Scattering Detector

24 MS: Mass Spectrometry

25 US: Ultrasonic Extractor

26 MAE: Microwave Extractor

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- 27 PLE: Pressurized Liquid Extractor
- 28 HPTLC: High-Performance Thin-Layer Chromatography
- 29 AU: Arbitrary Unit
- 30 RSD%: Relative Standard Deviation
- 31 LOD: Limit Of Detection
- 32 LOQ: Limit Of Quantification

33 1. Introduction

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

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

55 Individual quantification of saponins in vegetal samples has been reported in a large number of
56 studies by chromatographic methods. There are quantitative methods using chromatography such as

57 HPLC which coupled with different ultra-violet (UV), mass spectrometry (MS) or evaporative light
58 scattering (ELSD) detectors allow for rapid, precise and robust measurements of the saponins
59 content (Cheok et al., 2014; Man et al., 2010; W. A. Oleszek, 2002; W. Oleszek & Bialy, 2006). These
60 devices are however expensive and it is necessary to optimize the chromatographic conditions to
61 separate the various constituents present in each matrix plant which is time-consuming. In addition
62 to this, quantification with chromatographic devices require standards of saponins. However, for this
63 class of natural compounds, only a few representatives are commercially available as a standard,
64 making it a very restrictive situation.

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

72 Quantification of total saponins by spectrophotometry has become popular due to its simplicity and
73 better value compared to chromatographic methods. It consists in revealing the saponin pool using a
74 chromogenic agent with acid and measuring the chromophore formed by a UV-vis
75 spectrophotometer. The use of a standard calibration allows the quantification of saponins in the
76 sample. The method usually used is that presented by Hiai and their collaborators (Hiai, Oura,
77 Hamanaka, et al., 1975; Hiai, Oura, Odaka, et al., 1975). The method uses a sulfuric vanillin solution
78 to quantify triterpenoid saponins at a wavelength of 544 nm. Several standards of steroidal and
79 triterpenoid sapogenins were tested, triterpenoid sapogenins had a maximum absorbance close to
80 540 nm while for steroidal sapogenins the maximum absorbance was around 460 nm with different
81 molar absorption coefficients. In addition, the amount of sulfuric acid presents over 60% (v/v) in the
82 reactional volume and causes the formation of interference close to the wavelengths of
83 quantification, for example, rhamnose absorbs at 470 nm and sorbose at 520 nm (Hiai et al., 1976).
84 These interferences can come from the plant matrix or the glycosides present on the saponins
85 themselves, it is then necessary to carry out a pre-treatment before carrying out the quantification.
86 There is another spectrophotometric method usually used and developed initially by Baccou and
87 their collaborators (Baccou et al., 1977). They used sulfuric anisaldehyde to specifically reveal
88 steroidal sapogenins at 430 nm. The specificity of the method has the advantage of directly
89 quantifying steroidal saponins in a complex matrix without carrying out a pre-treatment. However,

90 the conditions used do not allow the triterpenoid saponins to be revealed, reducing considerably the
91 scope of this method.

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

98 2. Material and methods

99 2.1 Chemical and materials

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

110 2.2 Sample preparation

111 2.2.1 Comparison of extraction methods

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

120 of extraction (%m/m) and the total area (AU) in HPLC-ELSD corresponding to the saponins using the
121 method explained in 2.4.

122 2.2.2 Extraction method for the quantification for analysis of saponins

123 Sample preparation was carried out in triplicate to obtain Camellia, Quinoa, Fenugreek and Yucca
124 extracts. A weight of 500 mg of dried and ground saponin plant was solubilized in 50 mL of
125 dichloromethane. The solution was extracted with an ultrasonic bath Elmasonic S30(H) (Elma
126 Schmidbauer, Germany) set at 50 °C for 5 minutes then filtered, the residue was extracted twice with
127 dichloromethane. The residue is then solubilized with 50 mL of methanol and extracted with an
128 ultrasonic bath set at 50°C for 5 minutes and again filtrated, the residue is extracted twice with
129 methanol. Filtrates were recovered and evaporated under reduced pressure with a rotary evaporator
130 for dryness. The extraction yields were 18, 45, 22 and 28% (m/m) for Camellia, Quinoa, Fenugreek
131 and Yucca samples respectively. Camellia and Fenugreek extracts were blended till obtaining
132 mixtures corresponding to 20/80, 50/50 and 80/20 (m/m). Extract solutions were prepared in
133 methanol at 10 mg.mL⁻¹. After centrifugation at 10 000 g for 10 minutes, supernatants were used for
134 analysis.

135 2.3 HPLC- MSⁿ characterization

136 Camellia and Fenugreek extracts were analysed with an LC Waters 2795 with a UV detector (Waters,
137 USA) coupled with a mass spectrometer Esquire 3000 plus composed of electrospray ionization and
138 an ion trap analyser (Bruker, USA). Mass data was recorded with the following ionization conditions:
139 a capillary voltage 3500 V and a nebulizer temperature of 340°C together with a nitrogen sheath gas
140 (207 kPa). The full scan mass acquisitions in the positive and negative mode were performed by
141 scanning from 400 up to 1600 m/z range with a target value of 1100 m/z. Mass data were compared
142 with databases and literature to tentatively identify compounds and saponins present in Camellia
143 and Fenugreek extract.

144 2.4 HPLC-ELSD method

145 The total saponins content in Camellia, Quinoa, Fenugreek and Yucca extracts was determined by
146 HPLC-ELSD according to the following method: an LC-2030C 3D with diode array detector (Shimadzu,
147 Japan) was coupled with an Evaporative Light Scattering Detector LT-ELSD Model 90LT (Sedere,
148 France). A Capcell Pak C₁₈ AQ (4.6 × 150 mm, 3µm) (Osaka Soda, Japan) was used. The mobile phase
149 was composed of two solvents (A and B), where A corresponds to 0.1% formic acid (v/v) in water and
150 B to acetonitrile. The gradient used was: 0-20 min from 0 % to 100 % B and held for 5 min. The flow
151 rate was set at 1 mL.min⁻¹, and the temperature of the column was set at 25 °C. The nebulizer
152 temperature of the ELSD was set at 50 °C with a gas pressure of 350 kPa. A volume of 20 µL of the

153 supernatant obtained during sample preparation (2.2) was injected. Areas corresponding to saponins
154 were integrated with the ELSD and the total saponins content in the extract was determined by a
155 standard calibration curve.

156 2.5 Gravimetric method

157 The total saponins content in Camellia and Fenugreek extracts was determined with the gravimetric
158 method according to (Budan et al., 2013). Extract (100 mg) was accurately weighed and dissolved in
159 50 mL of water. The solution was transferred into a separatory funnel and supplemented with 15 mL
160 of ethyl acetate. The mixture was then shaken and the aqueous layer was recovered after the
161 complete separation phase and partitioned twice again with ethyl acetate. The aqueous solution was
162 then extracted with 10 mL of n-butanol and left to stand for complete separation. The aqueous
163 phase was then extracted twice with 10 mL n-butanol and the combined organic phases were
164 evaporated under reduced pressure to become dry and freeze-dried for 12h. The saponin content
165 was calculated as the mass of dried butanolic extract.

166 2.6 HPTLC analysis

167 Extracts of Camellia and Fenugreek were analysed by High-Performance Thin-Layer Chromatography
168 (HPTLC). 50 µg of methanolic extracts and 0.4 to 8 µg of protodioscin and escin standards in
169 methanol were spotted on a C₁₈ plate Alugram® RP-18 TLC W/UV₂₅₄ with an autosampler 4 (CAMAG,
170 Switzerland). Migration was performed with a mixture of methanol/water/formic acid 65/35/1
171 (v/v/v). The plate was derivatized by spraying 0.5% (v/v) of *p*-anisaldehyde in a sulfuric methanol
172 solution according to Jork (Jork et al., 1990) and heated at 150 °C with a TLC Plate Heater 3 (CAMAG,
173 Switzerland) until visualization of the spots. Scans and UV spectra of the plate were recorded with a
174 TLC Scanner 3 and TLC Visualizer (CAMAG, Switzerland). The spectra were processed with the
175 software WinCats version 1.4.4.

176 2.7 Spectrophotometric method

177 UV spectra of protodioscin and escin IB standards were recorded in methanol respectively at 0.2 and
178 0.03 mg.mL⁻¹ with an Evolution 60 Spectrophotometer (Thermo Fisher Scientific, United States).
179 Maximum wavelengths and molar absorption coefficients were determined.

180 2.7.1 Total saponins quantification (600 nm)

181 Total saponins contents were determined in Camellia, Quinoa, Fenugreek, Yucca and mixture
182 extracts by spectrophotometric analysis. A volume of 100 µL of the supernatant obtained during
183 sample preparation (2.2) was transferred into a capped test tube with 100 µL of a 50% *p*-
184 anisaldehyde solution in methanol (v/v) and 2 mL of 50% aqueous sulfuric solution (v/v). This step

185 was followed by the solution being immediately heated at 60 °C in a water bath for 20 minutes.
186 Finally, the chromogenic reaction was stopped by transferring the capped test tube into an ice-water
187 bath for 10 minutes. The reactional solution was transferred into a Cary 60 UV-Vis
188 spectrophotometer (Agilent Technologies, United States). The absorbance measurement was
189 performed against a blank at 600 nm. The total saponins content in the extract was determined by
190 using a standard calibration curve. To avoid drift of the absorbance between chromogenic reactions,
191 standard solutions and samples were performed during the same chromogenic reaction batch.

192 2.7.2 Analytical method assessment

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

197 2.7.3 Total steroidal saponins quantification (425 nm)

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

207 2.7.4 Total triterpenoid saponins quantification

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

$$211 \text{ Total triterpenoid saponins content} = \text{Total saponins content} - \\ 212 \text{ total steroidal saponins content (1)}$$

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

214 3. Results and discussion

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

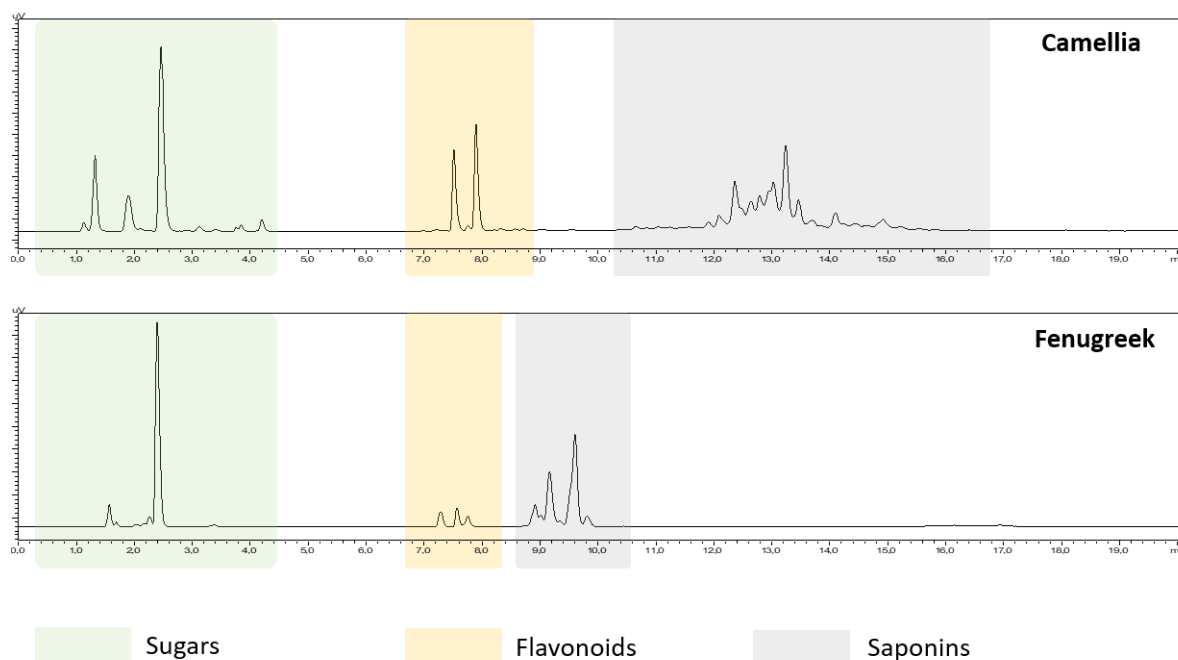
221 3.1 Sample preparation

222 Preliminary extraction experiments have been carried out to ensure the complete extraction of
223 triterpenoid and steroidal saponins from plant materials. *Camellia oleifera* (Camellia) was used as a
224 triterpenoid saponin plant and *Trigonella foenum-graecum* (Fenugreek) was used as a steroidal
225 saponin plant. The experiments consisted in comparing several extraction procedures with different
226 extraction modes: pressure liquid extraction (PLE), microwaves assisted extraction (MAE), ultrasonic-
227 assisted extraction (UAE). The extraction performance was evaluated with the extraction yield (%)
228 obtained as well as the integrated area in HPLC-ELSD (AU) corresponding to the saponins. The results
229 showed that utilization of methanol at 50°C gave the best results in terms of extraction yield as well
230 as the integrated saponins area for both plants. Moreover, PLE, MAE and UAE gave similar results in
231 terms of extraction yields and integrated area which ranged from [31.7 – 32.7%] and [6.93 – 6.94 AU]
232 respectively for Fenugreek and from [19.5 – 21.7%] and [6.95 – 6.98 AU] respectively for Camellia.
233 These similar results testify to the exhaustiveness of saponins extraction in these matrices.
234 Therefore, ultrasonic-assisted extraction was chosen for its “easy to use” and relatively better value
235 aspects. The chosen sample preparation for this study consisted in performing three extraction cycles
236 with methanol and evaporating to dry the filtrate after filtration to obtain an extract usable for the
237 determination of total saponins content. During the development of the spectrophotometric method
238 and as a precaution, a delipidation step with dichloromethane was added to eliminate the
239 compounds which may cause interferences on the measurement such as triterpenoid and steroidal
240 compounds. It should be noted that if these compounds are not present or have no impact on the
241 spectrophotometric measure, the extraction step can be drastically simplified by using a single 10
242 minutes extraction cycle with methanol followed by centrifugation. The quantification of the
243 saponins is then carried out with an aliquot of the supernatant.

244 3.2 Usual methods

245 3.2.1 HPLC-ELSD method

246 A liquid chromatography method coupled with an Evaporative Light Scattering Detector (HPLC-ELSD)
247 was developed in-house to quantify saponins present in Camellia, *Chenopodium quinoa* (Quinoa),
248 Fenugreek and *Yucca schidigera* (Yucca) extracts. This method is very reliable and was used as a
249 reference to compare total saponins content obtained by the gravimetric and the
250 spectrophotometric methods. Saponins are molecules with weak chromophores, their quantification
251 with UV-vis detector remains difficult because of the maxima wavelengths close to the cutoff of the
252 solvent and their various intensities. Indeed, escin IB and protodioscin, two of the standards used in
253 this study, have only one maximum at 205 nm with a respective molar absorption coefficient of
254 22172 and 3693 L.mol⁻¹.cm⁻¹. The use of an ELSD detector is well suitable for the quantification of
255 these molecules because neither the optical properties of the compounds nor their capacity to form
256 charged species has an impact on the ELSD response. The extracts obtained during the sample
257 preparation are constituted of various metabolites. Because the ELSD detector gives no spectral
258 information about molecules, preliminary work is, therefore, necessary to identify saponins from
259 other constituents by using detectors suitable for their identification. Previous works in HPLC-MSⁿ
260 were therefore carried out on extracts, the Fig. 1 shows ELSD chromatograms for Camellia and
261 Fenugreek. Extracts are complex matrices mainly constituted of sugars, flavonoids and saponins. The
262 area corresponding to saponins was integrated and the total saponins content was determined by
263 external calibration. Quantification by ELSD is almost independent of the structure and therefore any
264 saponin standard could be used with the result given as equivalent. For this study, similar standards
265 to saponins found in studied plants were used. For Camellia and Quinoa, escin IB, a triterpenoid
266 saponin was chosen. Whereas for Fenugreek and Yucca, protodioscin, a steroidal saponin was
267 chosen. The total saponins content determination performed in triplicate by specifically calculating
268 the sum of the areas of saponins for the four extracts is given in Table 1. The HPLC-ELSD method
269 developed here permitted quantifying saponins in several extracts with a relative standard deviation
270 (RSD%) which does not exceed 5% corresponding to a good level of precision for a method based on
271 ELSD detector (Tenon et al., 2017). This method meets all of the criteria for its use in industry.
272 However, the device is expensive and upstream work must be done to identify the saponins present
273 in the samples, which can be time-consuming in the case of different matrices' analyses.



274

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

276 Table 1

277 Analysis results and comparison between HPLC-ELSD method and gravimetric method

Extracts	HPLC-ELSD 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	-	-

278 ^aValues given as escin IB equivalent for HPLC-ELSD method

279 ^bValues given as protodioscin equivalent for HPLC-ELSD method

280 3.2.2 Gravimetric method

281 The saponins content in Camellia and Fenugreek extracts was estimated by the gravimetric method.

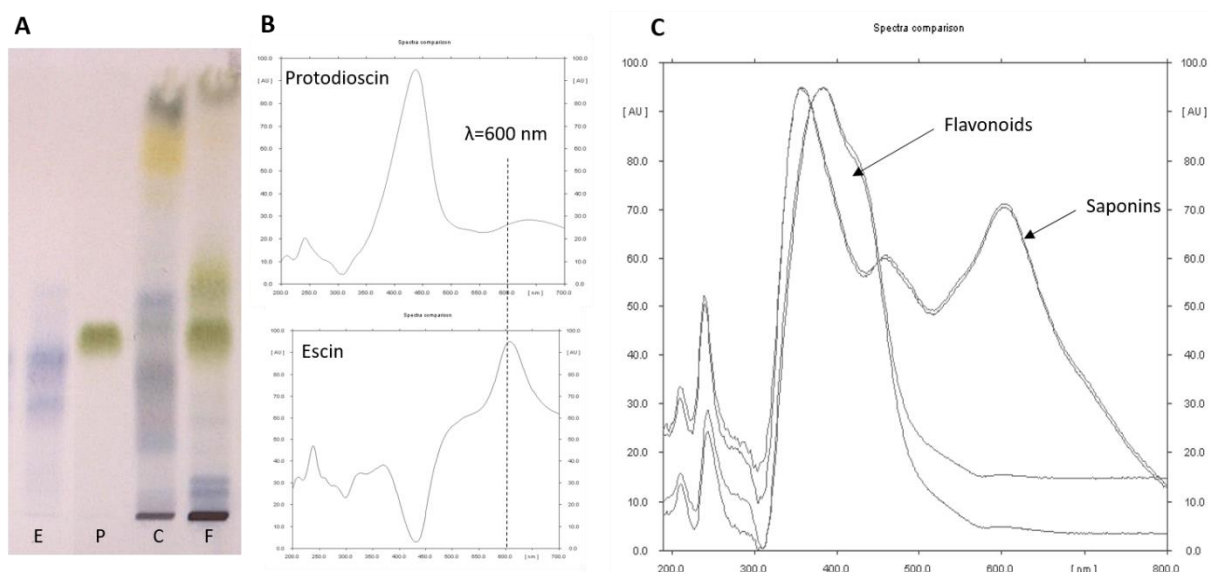
282 This method widely used is based on the affinity of saponins with the butanol to specifically extract
 283 these latter. The results for the method presented in Table 1, show a saponin content for Camellia
 284 and Fenugreek of 58 and 51% respectively (m/m). These values compared to those obtained with the
 285 HPLC-ELSD method lead to overestimations of up to 93%. These overestimations of the saponin
 286 content are due to other compounds still present in the butalonic extracts resulting from the low
 287 specificity and affinity of the saponins to butanol. To this lack of accuracy, is added a lack of

288 precision, resulting in relatively high RSD% as well as an excessively long process compared to a
289 method such as HPLC-ELSD.

290 3.3 Spectrophotometric method

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

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



308

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

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

324 Other constituents like polyphenols and polysaccharides are also present in the methanolic extract of
325 saponin plants. The electron-rich aromatic rings of polyphenols like flavonoids react with *p*-
326 anisaldehyde to form reddish chromophores (Xu & Liu, 2021). Absorbance is therefore around 300-
327 500 nm and does not interfere with saponins as observed in Fig. 2. Sugars could react by a double
328 bond via hydroxyl dehydration leading to a blue chromophore as stated for saponins. However,
329 dehydration of sugar is more demanding in energy than saponins. Controlled conditions such as
330 percentage of sulfuric acid and heating temperature, could avoid the development of these potential
331 interfered chromophores.

332 A colorimetric method in solution was thus developed and the reaction conditions were optimized:
333 concentration of *p*-anisaldehyde (2.3% v/v), sulfuric acid (45.5% v/v), reactional solvent (water),
334 temperature (60 °C) and time of incubation (20 minutes). This colorimetric method allows the
335 development of a sapogenin chromophore with a maximum absorbance at 600 nm. The molar
336 absorptivity obtained at this wavelength is similar for steroidal and triterpenoid saponins making it
337 possible to quantify them in a mixture and with any standard of saponin. Moreover, absorption at
338 600 nm is only specific for sapogenins, making it possible to quantify directly in the presence of other
339 compounds such as sugars and polyphenols, and so considerably reduces the stages of sample
340 preparation. Validation studies for the proposed method were carried out and described in this
341 paper, comprehensive results from the method validation were given in Table 2.

342 3.3.1 Selectivity

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

355 3.3.2 Linearity

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

361 **Escin IB:** $Abs. = (5.95 \pm 0.24) \times 10^{-3}C(\mu\text{g}\cdot\text{mL}^{-1}) + (22.5 \pm 1.2) \times 10^{-3}$ (2a)

362 With regression coefficient of r^2 : 0.9934

363 **Protodioscin:** $Abs. = (7.30 \pm 0.31) \times 10^{-3}C(\mu\text{g}\cdot\text{mL}^{-1}) + (46.1 \pm 1.2) \times 10^{-3}$ (2b)

364 With regression coefficient of r^2 : 0.9918

365 **Escin:** $Abs. = (5.08 \pm 0.07) \times 10^{-3}C(\mu\text{g}\cdot\text{mL}^{-1}) + (55.4 \pm 0.7) \times 10^{-3}$ (2c)

366 With regression coefficient of r^2 : 0.9973

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

370 3.3.3 Limit of detection

371 Limit of detection (LOD) was defined as the lowest analyte concentration that can be determined by
372 the analytical process. LOD was determined with procedural blanks consisting of repeating six times a

373 blank solution without analyte over the whole procedure under repeatability conditions. The
374 quantity of saponins in blanks is determined from the calibration curve with each standard: escin IB,
375 protodioscin and escin. LOD was calculated with the following equation Eq. (3):

$$376 \quad LOD = 3 \times s_{blank} \quad (3)$$

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

380 3.3.4 Limit of quantification

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

$$384 \quad LOQ = 10 \times s_{blank} \quad (4)$$

385 According to the IUPAC, the LOQ was expressed in terms of relative standard deviation, which is the
386 maximum tolerated value traditionally equal to 10%. The LOQ was determined to be 19, 18 and 20
387 $\mu\text{g} \cdot \text{mL}^{-1}$ for escin IB, protodioscin and escin.

388 3.3.5 Molar absorption coefficient

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

$$393 \quad \text{Escin IB: } \varepsilon_{600nm} = 2744 \pm 99 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \quad (5a)$$

$$394 \quad \text{Protodioscin: } \varepsilon_{600nm} = 2745 \pm 84 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \quad (5b)$$

$$395 \quad \text{Escin: } \varepsilon_{600nm} = 2630 \pm 159 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1} \quad (5c)$$

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

398 The major advantage of this method is that any saponin standard can be used to quantify the
399 saponins in a sample, whether it consists of triterpenoid, steroidal or both saponins. In addition,
400 purified saponins are quite expensive like escin IB and protodioscin, the use of a mixture of saponins
401 like escin standard could be an inexpensive alternative to quantify saponins in samples. Because the

402 chromogenic reaction can cause a drift of the absorbance between runs, it is preferable to quantify
403 saponins in samples with a standard performed on the same chromogenic reaction.

404 3.3.6 Precision

405 The precision of an analytical method describes the variation in the results under stipulated
406 conditions. In this study, precision was defined under repeatability conditions and intermediate
407 precision. The study was performed on Camellia and Fenugreek extracts with escin IB and
408 protodioscin respectively as standard. Repeatability was determined by measuring each sample six
409 times under the same operating conditions according to the analytical method. Intermediate
410 precision was determined by repeating the repeatability over two days. Precision was expressed as
411 relative standard deviation RSD%. Values obtained under repeatability conditions were 1.7 and 1.2%
412 for Camellia and Fenugreek extracts. Under intermediate precision conditions, the RSD% were 2.6
413 and 2.3% respectively for Camellia and Fenugreek extracts, which were acceptable results for a
414 spectrophotometric method.

415 3.3.7 Accuracy

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

$$423 R\% = \frac{C_{0+S} - C_0}{C_s} \times 100 \quad (6)$$

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

429 Table 2

430 Analytical characteristics of the spectrophotometric method

Triterpenoid saponins

Steroidal saponins

Parameters	Standard escin IB	Standard escin	Standard protodioscin
Regression equation	Abs. = $(5.95 \pm 0.24) \times 10^{-3}$ $(\mu\text{g.mL}^{-1}) + (22.5 \pm 1.2) \times 10^{-3}$	Abs. = $(5.08 \pm 0.07) \times 10^{-3}$ $(\mu\text{g.mL}^{-1}) + (55.4 \pm 0.7) \times 10^{-3}$	Abs. = $(7.30 \pm 0.31) \times 10^{-3}$ $(\mu\text{g.mL}^{-1}) + (46.1 \pm 1.2) \times 10^{-3}$
Correlation coefficient (r^2)	0.9934	0.9973	0.9918
Linear working range ($\mu\text{g.mL}^{-1}$)	19 – 230	20 – 230	18 – 230
LOD (n = 6, $\mu\text{g.mL}^{-1}$)	6	6	5
LOQ (n = 6, $\mu\text{g.mL}^{-1}$)	19	20	18
$\epsilon_{600\text{nm}}$ ($\text{L.mol}^{-1}.\text{cm}^{-1}$)	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

431 3.4 Applications of the spectrophotometric method

432 3.4.1 Determination of total saponins content in samples

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

442 Table 3

443 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

444 ^aValues given in escin IB equivalent

445 ^bValues given in protodioscin equivalent

446 The total saponins content found for the four extracts were in the range of 9.5 – 30.5% (m/m), these
447 values are quite similar to those found by the HPLC-ESLD method developed in-house (Table 1).

448 These results show that the spectrophotometric method proposed here possesses all criteria to be
449 used in total saponins measuring processes. Therefore, it is not necessary to carry out upstream work
450 on the samples because of the specificity of the method for quantifying sapogenins in complex
451 matrices.

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

456 3.4.2 Determination of total saponins content in saponins mixtures

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

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

471 Table 4

472 Comparison of results for total saponins (600 nm), steroidal saponins (425 nm) and triterpenoid
473 saponins (600 – 425 nm) in blends

Ratio	Total saponins found (%m/m)			Steroidal saponins found (%m/m)			Triterpenoid saponins Found (%m/m)		
	Calculation	Spectrophotometric method (600 nm)	Bias%	Calculation	Spectrophotometric method (425 nm)	Bias%	Calculation	Subtraction (600 – 425 nm)	Bias%
Camellia/Fenugreek (m/m)									
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

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

478 3.4.3 Determination of steroidal and triterpenoid saponins contents in mixtures
479 Previous works carried out by (Baccou et al., 1977) have shown that soft conditions of *p*-
480 anisaldehyde (0.13% v/v) and sulfuric acid (12.5% v/v) permit to react only with the spirostan and
481 furan structure present in steroidal saponins regardless of the C3 hydroxyl function. Their
482 spectrophotometric method made it possible to selectively quantify steroidal saponins from complex
483 matrices at 425 nm.

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

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

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

496 Steroidal saponin content was assayed with the spectrophotometric method at 425 nm for each
497 mixture. Then, the triterpenoid saponin content was determined by subtracting the total saponins

498 content at 600 nm determined in 3.4.2 by the steroidal saponins content found previously. Values
499 obtained are compared against calculated values with bias% and presented in Table 4.

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

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

512 4. Conclusion

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

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

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

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

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

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