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Analytical Methods

Rapid, cost-effective and accurate quantification of *Yucca schidigera* Roezl. steroidal saponins using HPLC-ELSD method

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ABSTRACT

Yucca GRAS-labelled saponins have been and are increasingly used in food/feed, pharmaceutical or cosmetic industries. Existing techniques presently used for *Yucca* steroidal saponin quantification remain either inaccurate and misleading or accurate but time consuming and cost prohibitive. The method reported here addresses all of the above challenges. HPLC/ELSD technique is an accurate and reliable method that yields results of appropriate repeatability and reproducibility. This method does not over- or under-estimate levels of steroidal saponins. HPLC/ELSD method does not require each and every pure standard of saponins, to quantify the group of steroidal saponins. The method is a time- and cost-effective technique that is suitable for routine industrial analyses. HPLC/ELSD methods yield a saponin fingerprints specific to the plant species. As the method is capable of distinguishing saponin profiles from taxonomically distant species, it can unravel plant adulteration issues.

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1. Introduction

Well before their chemical structures were elucidated, *Yucca* saponins were utilised by American Indians in a series of applications, including soap. Multiple uses of a range of compounds originate from different parts of 35–40 *Yucca* (Agavaceae) species. *Yucca* are mainly distributed in the deserts of Central and Northern America and have evolved over centuries. Presently, *Yucca schidigera* syrup (concentrated juice; *Yucca* extract), and dried and finely powdered logs (*Yucca* powder), both abundant in steroidal saponins, are of particular interest to cosmetic, pharmaceutical and beverage industries as well as animal nutrition. Those GRAS-labeled products possess foaming features that are of particular interest in soft drinks (root beer), cosmetic (Tanaka, Tamura, Masuda, & Mizutani, 1996), food and feed industries (Fenwick & Oakenfull, 2006). The very foaming activity of *Yucca* extract is due to the abundance of saponins (~10% dry matter) (Oleszek et al., 2001). Such high saponin contents are the reason why, *Y. schidigera,* together with *Quillaja saponaria*, are the two major commercial sources of saponins that happen to be misused, one instead of the other, as plant adulterants (Cheeke, 2000).

The most abundant *Y. schidigera* stem/bark saponins are steroidal saponins. They differ in the structure of their aglycon, according to which, they are classified into spirostane- or furostane-type derivatives. Primary saponins are glycosides of three C-25 epimeric pairs of sapogenins: sarsapogenin and smilagenin, markogenin and samogenin, gitogenin and neogotogenin. In both, spirostane and furostane derivatives, the C-3 carbohydrate chains are typically branched oligosaccharides with pentapyranosyl and/or hexopyranosyl units. As far as furostane bidesmosides are concerned, C-26 linked carbohydrate usually corresponds to a hexopyranose. It should be noted that derivatives of other sapogenins occur as minor compounds within *Y. schidigera* stem/bark.

The above summarised complexity of *Y. schidigera* stem/bark steroidal saponins has remained for a longtime an obstacle in their identification and quantification. Indeed, the inability of quantifying steroidal saponins from *Y. schidigera* has been an industrial challenge and constraint, as it directly reflects the incapacity of standardization in steroidal saponins. Should such standardization be possible and available in the industry, it would answer an essential question related to the extract quality, as steroidal

Abbreviations: GRAS, Generally Regarded as Safe; HPLC/ELSD, High Performance Liquid Chromatography/Evaporative Light Scattering Detector; HPLC/MS, High Performance Liquid Chromatography/Mass Spectrometry; L/L, liquid/liquid; NMR, Nuclear Magnetic Resonance; OD, Optical Density; RC, regenerated cellulose; SPE, Solid Phase Extraction; TOF, Time of Flight; YSS, *Yucca* Saponins Standard.

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saponin levels could be directly correlated with features held by these compounds, for instance, with the foaming capacity.

Classical methods presently used in measurement of steroidal saponins include spectrophotometric measurements (Baccou, Lambert, & Sauvaire, 1977; Uematsu, Hirata, Saito, & Kudo, 2000), foam height measurements (Ross & Miles, 1941) or gravimetric method (Wina, Muetzel, & Becker, 2005). Neither of these methods satisfies accuracy or reliability requirements that necessitate industrial quality control. For example, a gravimetric technique, which is based on gravimetric determination of butanol fractions, and the spectrophotometric method, both lack specificity, for they measure, besides steroidal saponins, all compounds present in the butanol fraction, including polyphenols and therefore could overestimate steroidal saponin levels. Moreover, a poor affinity of butanol for highly polar saponins is another source of imprecision in the estimation of steroidal saponins by this method. Furthermore, due to such a lack of specificity, gravimetric or spectrophotometric method cannot distinguish steroidal saponin profiles originating from different plant species.

Identification of different sapogenins was initiated by Kaneda, Nakanishi, and Staba (1987) and Miyakoshi et al. (2000) and further characterised by Piacente, Pizza, and Oleszek (2005) and Kowalczyk, Pecio, Stochmal, and Oleszek (2011). In their last report, Kowalczyk et al. (2011) successfully identified, characterised and quantified certain steroidal saponins present within *Y. schidigera* concentrated juice using the method of HPLC/MS. Although this method is very precise and robust, it remains expensive and time-consuming; therefore it is not used in the food industry.

The industry remains in need of an analytical method specific to steroidal saponins enabling quantifications at reasonable costs. Here we present a rapid, specific, cost- and time-effective HPLC/ELSD method, which accurately measures total *Yucca* steroidal saponins. Although this method has been developed for steroidal saponins from *Y. schidigera*, it reveals a specific chemical fingerprint profile and therefore distinguishes extracts originating from different saponin-containing species. Hence, it can also be used as an investigation tool for steroidal saponin plant adulteration analyses.

2. Material and methods

2.1. Chemicals and materials

Three samples of *Y. schidigera* juice were purchased from three suppliers of the Baja California state (samples named A, B, and C). Spray drying of sample (B), in the presence of inverted sugar, yielded a *Yucca* powder D (NATUREX processed). Authenticated *Yucca* trunk raw material was supplied by Compania Agroindustrial Baja (Mexican state) and the shampoo containing *Yucca brevifolia* root was supplied from Shea Moisture website. A standard of *Y. schidigera* saponin mixture was prepared during this study using *Yucca* syrup (extract) produced by NATUREX. High purity of saponins (>95%) was controlled using multiple test method. HPLC-grade solvents were purchased from Biosolv Chimie (Dieuze France); Deionized water was obtained using a Milli-Q Elix system (Merck-Millipore, France). All other chemicals were purchased from Sigma-Aldrich. Antifoam FD30 K was supplied by Basildon Chemical Company (Abingdon, England). The Amberlite FPX68 was purchased from Rhom & Haas Company (Chauny, France). SPE AFFINIMIP Phenolic cartridge 100 mg was purchased from Polyintell (Val-de-reuil, France). Nitrogen generator using a brezza system was from GENGAZ (Wasquehal, France).

2.2. Preparation of purified steroidal saponin standard

Most of studies involving saponin quantification by chromatography use a commercial saponin standard (Budan et al., 2014). Such commercial standard comprises only one type of saponins, the structure of which is different from the majority of saponins comprised in *Yucca* extracts. Using only one saponin as a standard originating from a distant plant family is thus in most cases inappropriate and insufficient for quantification of a mixture of *Yucca* saponins. Clearly, a need for a standard comprising a mix of relevant saponins remained. We explored a new way of quantifying saponins by purifying a mixture containing two types of major saponins of *Yucca schidigera*, *via* two distinct purification steps, as described hereafter.

2.2.1. Purification of a mix of saponins of type 1 (SAPONINS 1)

Crude, *Yucca* juice concentrate (300 mL, 38°Brix) was repeatedly extracted with 200 mL of ethanol (100%) three times. Ethanolic phases were pulled and concentrated to dryness. The powder obtained was repeatedly washed with 200 mL of ethyl acetate and 200 mL of hexane under reflux for 30 min yielding a brown viscous residue. Subsequently, the residue was partitioned between water phase and 1-butanol phase. Following concentration of the organic phase under vacuum, a brown powder (BP_7.5 g) was obtained. The obtained powder (5 g) was fractionated using column chromatography (FPX-68; 600 g; 1.5 L of water; 1.5 L of water/ethanol 7:3; 1.5 L water/ethanol 3:7). The water/ethanol 7:3 v/v fraction was concentrated in a freeze dryer and yielded 1.2 g of powder. All powder samples were subjected to SPE AFFINIMIP. After activation of the cartridge with water and methanol, the sample was loaded and washed with water. Saponins were recovered by water/acetonitrile (10:90 v/v), and the collected fraction was freeze-dried. It resulted in pure saponins, which were precipitated in methanol/ether (20:80 v/v). Following the subsequent freeze-drying step, the first pure mixture of saponins was obtained, termed SAPONINS 1 (Supplementary material, Fig. 1).

2.2.2. Purification of a mix of saponins of type 2 (SAPONINS 2)

A quantity of 500 mg of the brown powder (BP_7.5 g) resulting from the first liquid/liquid extraction was supplemented with 3 mL of water and 800 μL of thus obtained solution was injected onto preparative HPLC system (Agilent, 1200 series equipped with a 126 wells plate fraction collector). An Agilent preparative C_{18} column (30 × 150 mm, 10 μm) was used. The mobile phase was composed from two solutions (A and B), where A was 0.1% formic acid (v/v) in water and B 0.1% formic acid in acetonitrile (v/v). The gradient profile was the following: 0–10 min linearly from 90% A to 70% A; 10–20 min linearly from 70% A to 20% A. The flow rate was set at 25 mL/min, and the temperature of the column was set at 25 °C. Fractions containing saponins that were eluted from 15 to 20 min were collected and concentrated before freeze-drying. Thus obtained saponins were then dissolved in methanol and precipitated by ethyl ether. After repetition of several injections according to the process described above we finally obtained a second pure saponin mixture SAPONINS 2 (Supplementary material, Fig. 1).

2.2.3. Characterisation of purified steroidal saponin standard

The pure mix called *Yucca* Saponins Standard (YSS) was obtained by combining two times as much of SAPONINS 1 with SAPONINS 2.

Mass data enabling YSS characterization were collected on Agilent 6230 accurate-mass TOF_HPLC/MS with an electrospray interface using Agilent Jet stream (AJS) Thermal Focusing technology for a better sensitivity. The ionisation conditions were adjusted as

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follows: a capillary voltage 3500 V and a nebulizer temperature of 300 °C. Nebulization was aided with AJS using a Nitrogen sheath gas flow (10 L/min) and sheath gas temperature 300 °C. The full scan mass acquisitions in negative mode were performed by scanning from 100 up to 1500 *m*/*z* range. NMR analysis was conducted on Bruker DRX500 instrument with a Probe head of 5 mm BBI with a dilution in deuterated water.

Solvent analysis was performed on Agilent 7890A coupled with 5975C Mass detector (simple quad) from Agilent Technologies. Water content was determined with coulometric Karl fisher method (KF C20 model from Mettler Toledo).

2.3. Steroidal saponin analysis

2.3.1. YSS solution preparation

The above purified steroidal saponins YSS were weighed in order to produce a standard solution used for the development of a calibration curve in the range 500–3500 μg/mL (total saponins). All solutions were diluted with water and filtered through RC 0.45 μm disc filter without excessive agitation in order to avoid foaming. The standard solutions prepared could be stored at 4 °C for no longer than two weeks, and used for further calibration assays.

2.3.2. Sample preparation and HPLC-ELSD analysis

Y. schidigera juice extracts A, B and C were obtained in several steps of extraction from the raw *Yucca* trunk material. i) Step one: fresh trunks of *Yucca* were mechanically pressed to produce a first juice. ii) Step two: the raw material from the first extraction was extracted again with water and the juice thus obtained was combined with the first juice and adjusted to produce a juice of 13–16° Brix. This 13–16° Brix juice was concentrated up to 50°Brix, resulting in *Yucca* juice sample (A). iii) Step three: the *Yucca* juice from step two (11–13° Brix) was concentrated and clarified to produce a juice of 25–28° Brix. Finally the hot juice from step three of clarification was adjusted to pH 3.5–4.0 with phosphoric acid and concentrated either to 38° Brix to produce *Yucca* juice sample (B) or to 50° Brix to produce *Yucca* juice sample (C). *Y. schidigera* powder extract (D) was obtained by spray-drying *Yucca* juice sample (B) on inverted sugar as a carrier. An estimated value of saponin content in thus obtained samples, using gravimetric method, ranged between 10 and 13%. A blend of *Yucca schidigera* (trunk)/*Quillaja saponaria* (root) extract was a mixture (50:50 v/v) of *Yucca* juice extract (A) with *Quillaja saponaria* juice extract standardized at 16% total saponins by gravimetric method. An approximate level of total saponins of thus obtained blend ranged from 14 to 15% (as measured by gravimetric method). Samples were prepared according to the following protocol prior to HPLC/ELSD analysis. *Y. schidigera* juice, powder extract or blend were weighed (~200–400 mg), dissolved in 20 mL of water containing 0.05% antifoaming FD30 K agent. The solution was filtered through 0.45 μm RC disc filter prior to HPLC/ELSD analysis.

HPLC/ELSD analysis was run according to the following method: an Agilent 1200 series liquid chromatographic system was coupled with an Evaporative Light Scattering Detector (ELSD, G4260). An Atlantis® T3 column (3.0 × 150 mm, 3 μm) (Waters, Ireland) was used. As above, the mobile phase was composed from two solutions (A and B), where A corresponds to 0.1% formic acid (v/v) in water and B 0.1% formic acid in acetonitrile (v/v). However, the gradient profile was the following: 0–25 min linearly from 98% A to 40% A; 25–35 min linearly from 40% A to 20% A. The flow rate was set at 0.6 mL/min, and the temperature of the column was set at 25 °C. Evaporation and nebulizer temperature of the ELSD were set, respectively, at 50 °C and 40 °C with a gas flow of 1.2 (arbitrary unit).

The shampoo sample containing steroidal saponins of *Yucca brevifolia* was analysed by here developed method in order to appreciate the differences in saponin fingerprints between *Y. schidigera* and *Y. brevifolia* and therefore to evaluate the applicability of this method in plant/extract adulteration. As the shampoo contained very low amounts of saponins, it was necessary to proceed initially by a purification step, prior to implementing the here developed method. The shampoo sample was therefore first submitted to liquid/liquid extraction with *n*-butanol and after concentration of the organic phase, the residue was dissolved in water, filtered and injected onto HPLC. Subsequently, the above described method was applied and resulted in a purified sample.

2.4. Gravimetric method

Yucca extract (10 g), was accurately weighed and termed (W1), and dissolved in 200 mL of water. Following 30 min of agitation in an ultrasonic bath, the solution was transferred into a separatory funnel. Supplementary 10 mL of water were used to clean the remaining solution and were also filtered as above. The obtained aqueous solution was extracted with 150 mL of 1-butanol and left to stand for 20 min until complete phase separation. The organic phase was transferred to a pre-weighed round bottom flask (the weight of which was termed W2) and evaporated under reduced pressure to dryness. The round bottom flask containing the residue was heated at 100 °C for at least 4 h and then desiccated in the desiccator. Following 30 min in the desiccator, the round bottom flask was weighed and the obtained weight was termed (W3). The saponin content was calculated using the following Eq. (1):

$$\text{Saponin } (\%) = [(W3 - W2)/W1] \times 100\% \quad (1)$$

2.5. Recovery

The above developed standard solution was combined with a *Yucca* extract sample and the mix was extracted as described in 2.3.2. Recovery was determined by comparing the difference between the sum of all saponin peak areas of the mix (sample + standard) (Cp) and the sum of peak areas of the sample alone (Cws) without the standard, divided by the sum of the saponin peak areas of the standard (Cs) (Eq. (2)):

$$\text{Recovery } (\%) = [(Cp - Cws)/Cs] \times 100\% \quad (2)$$

2.6. Analytical method assessment

The analytical method HPLC/ELSD was evaluated for linear response of the calibration curve, precision (intra-day and reproducibility), and accuracy. The calibration curve was linear over the range of 500–3200 μg/mL of saponins, which was determined using 6 solutions of the *Yucca* Saponins Standard (YSS) varying concentrations measured in duplicates. A linear regression equation was obtained by plotting the logarithms of peak area responses versus logarithms of concentrations, in μg/mL. The precision of the analytic method was determined on *Yucca* powder sample D. The precision (intra-day) was evaluated by calculating the percentage of relative standard deviation of the peak area sum of the saponins from six injections prepared following six different weighings at 100% of the test concentration. The reproducibility of the method was conducted on six injections of the *Yucca* powder sample D, run on a different day, by a different analyst and using a different piece of equipment. Accuracy, i.e. agreement of the calculated concentration with the nominal concentration, was examined on three different *Yucca* extracts at three concentration levels.

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3. Results and discussion

Several methods have been previously used and proposed to evaluate *Yucca* saponin quantification. They are all mainly based either on foaming, gravimetric, spectrophotometric measurements or on HPLC/MS quantification (Skhirtladze et al., 2011). These different methods display disadvantages that seriously impact their use, notably at industrial levels. Therefore we have developed a new, improved, rapid and cost effective method that alleviates inaccuracies, insufficiencies, inconveniences and inadequacy of the existing techniques.

In order to appreciate *Yucca* saponin levels obtained by here developed HPLC/ELSD method, we have compared them with those obtained using classical gravimetric method.

3.1. HPLC/MS characterization of YSS

HPLC/MS was used to control the YSS saponin composition. During the ionisation and transfer through a capillary, quasi-molecular ions of steroidal saponins repeatedly lose carbohydrate units from their side chains and may yield in an erroneous structural information. This is especially true when analysis is run in the positive mode. For this reason, negative ionisation was chosen to get a more important signal and improve the accuracy on $[M-H]^-$. Following this method, a more reliable determination of the molecular formula could be used. Kowalczyk et al. (2011) identified 19 compounds in crude syrup extract of *Yucca schidigera* using HPLC/MS. YSS analysed by HPLC/MS resulted in the same peaks (Fig. 1) as those previously identified by Kowalczyk et al. (2011). Interestingly, in this study, the analysis of YSS revealed additional peaks corresponding to other steroidal saponins. Those peaks were identified as saponin peaks based on their molecular formula detected by accurate TOF/MS. Future work involving HPLC coupled with triple quadrupole or NMR methods will structurally characterise those remaining steroidal saponins.

3.2. Gravimetric method

Gravimetric method is one of the first methods that was developed for saponin measurements decades ago (Harbone, 1973). It is based on the affinity of saponins for *n*-butanol. The quantification of total saponins using gravimetric method has shown a poor specificity of *n*-butanol to the most polar saponins (bidesmosidic glycoside and saponins with 3 or 4 branched glycosides), eluted between 12 and 17 min (Fig. 2A). Those saponins when extracted by *n*-butanol could hardly be seen on the chromatogram (Fig. 2B). Conversely, aqueous extraction of these polar saponins resulted in clear corresponding HPLC peaks. Data clearly show that saponins, notably polar saponins, are poorly extracted by *n*-butanol, which yields in an imprecision in this method. Moreover *n*-butanol is not specific to certain saponins. It extracts other compound classes, including polyphenols, sugars, organic acids, and therefore overestimates saponin levels.

Overall, due to its poor specificity and its poor affinity for the extraction solvent of *Yucca* saponins, the gravimetric method clearly both under- or over-estimates saponins, depending on their polarity, and therefore, does not seem to be utterly adequate for accurate saponin measurements. However, despite disadvantages of the gravimetric method, the precision was investigated. Relative standard deviation (RSD%) measured on 6 independently prepared samples of *Y. schidigera* juice extract yielded in 7.2%. Relative high RSD value indicates that such imprecision would negatively reflect and impact the manufacturing productivity. Moreover, use of gravimetric method is a long process. Indeed, the analysis of each sample requires five hours. Hence, gravimetric method requires five times as much time as compared to here developed HPLC/ELSD method. It is important to note that such time consuming sample preparation is inappropriate for use at the industrial level, more precisely in use for industrial quality control purposes (Table 1). Finally, gravimetric method is not considered to be "environmentally friendly" because it requires large amounts (~400 mL) of petrol-originated organic solvents per each sample.

3.3. HPLC/ELSD

In order to alleviate insufficiencies, inadequacy and disadvantages of the existing techniques, and notably of the gravimetric method, we have developed a new, reliable, accurate, rapid and cost effective method for quantitative measurements of *Yucca* steroidal saponins that could be applied at industrial level. This method was applied to various *Y. schidigera* extracts. The liquid chromatography method coupled with ELSD detector is a fast and reliable method for the quantification of non chromophoric molecules, including steroidal saponins (Liang et al., 2007; Libo,

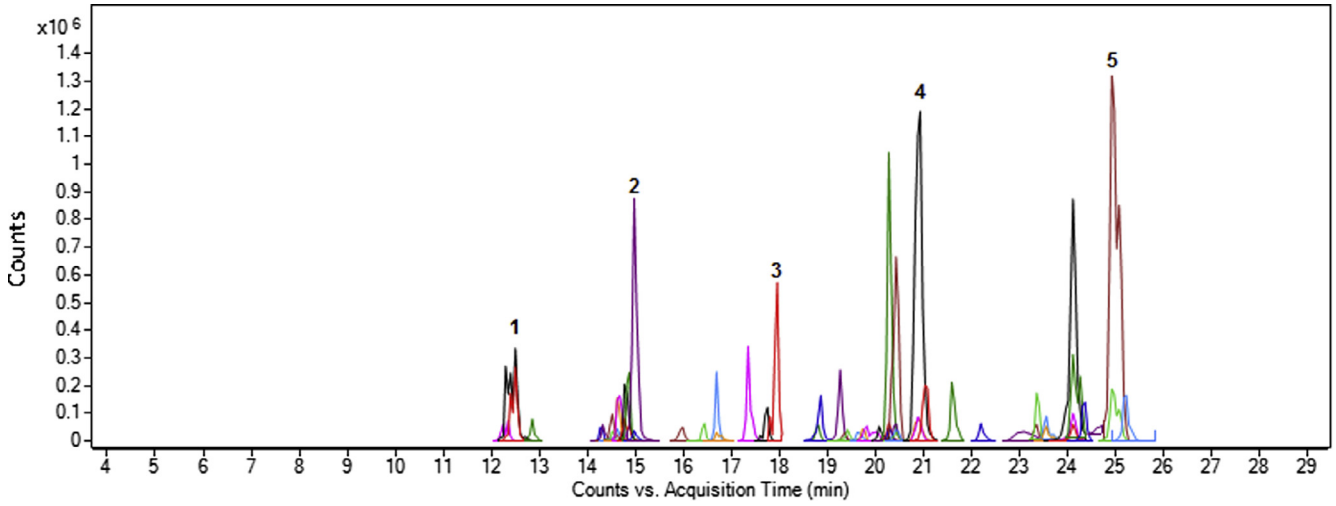

Fig. 1. Extracted compound chromatogram (ECC) of YSS showing more than 19 compounds related to steroidal saponin and identification of 5 majoritary compounds: **1** $[M-H]^-$ at *m/z* 1065.5119 ($C_{50}H_{82}O_{24}$, Δppm 0.86 ppm), **2** $[M-H]^-$ at *m/z* 1051.5356 ($C_{50}H_{84}O_{23}$ Δppm 2.09 ppm), **3** $[M-H]^-$ at *m/z* 1033.5250 ($C_{50}H_{82}O_{22}$ Δppm 1.27 ppm), **4** $[M+COOH]^-$ at *m/z* 931.4585 ($C_{44}H_{70}O_{18}$ Δppm 4.4 ppm), **5** $[M-H]^-$ at *m/z* 871.4681 ($C_{44}H_{72}O_{17}$ Δppm 0.29 ppm). This analysis used an Agilent 1260 Infinity LC System and an Agilent 6230 accurate-mass TOF_LC/MS System.

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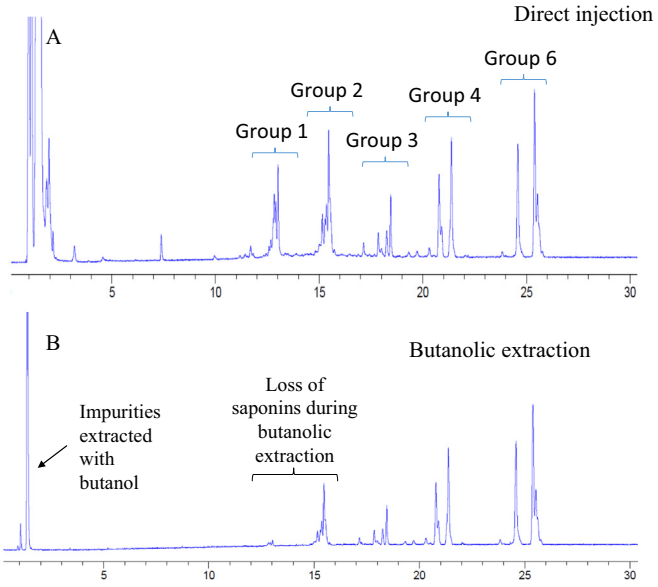

Fig. 2. Comparative HPLC/ELSD chromatogram between (A) direct injection of *Yucca schidigera* sample (group 1–5 corresponding to quantified saponins) and (B) butanolic phase that resulted from the extraction by gravimetric quantification.

Table 1
Characteristic between HPLC/ELSD method and gravimetric method.

Analyte	Gravimetric method	HPLC/ELSD method
Specificity	NOK	OK
Time consuming (hours)/analysis	5	1
Solvent consume (mL)	360	50
Intra-day precision% (n = 6)	7.20	2.79

Zhihong, & Daofeng, 2012). The main advantage of this method as compared to the ultraviolet or mass detector, seems to rely on its response factor, which is less influenced by the molecular structure and the nature of the mobile phase. Thanks to this response factor, the developed standard containing a pool of *Yucca* saponins successfully allows the integration, and therefore the quantification, of all major steroidal saponins, irrespectively of their polarity. Hence, this new method does not require the use of an internal standard.

It should be noted that here presented method alleviates the complexity of the quantification of steroidal saponins from *Yucca* and successfully measured *Yucca* extract steroidal saponin compounds that require an appropriate detection mode and adequate markers which set the detection range. As *Yucca* steroidal saponins do not have chromophore moiety, UV detection mode is clearly not suitable for their measurement, while ELSD mode is capable of detecting such compounds. A question remained regarding the possibility of having a standard comprising several *Yucca* steroidal saponins with different polarities. The extreme saponins of the standard set the detection range of saponins of different polarity, and therefore the detection limits, within which, a sample of *Yucca* steroidal saponins could be measured. Similar questions were raised regarding *Aesculus hippocastanum* aescins. Like saponins in *Yucca*, several aescins occur concomitantly within the horse chestnut. As those aescins absorb within the UV range, European pharmacopeia suggested HPLC/UV method for their quantification (Monograph ESCOP, 1999). As to the integration mode, according to the European pharmacopeia, all peaks within the range delimited by methyl salicylate and the ibuprofen correspond to aescins and are quantified as such. Similarly, as ELSD detector does not provide neither spectral nor mass information, it was very important to generate a standard comprising differing *Yucca* steroidal saponins, the extremes of which would delimit the integration range within which would all steroidal saponins be quantified. Similar procedure described by San Martin and Briones (2000) is accepted and used for *Quillaja* saponin quantification (Food and Agricultural Organisation of the United states FAO 2004, 61st Joint Expert Committee for Food Additives JECFA).

As structural identification and purification of each single saponin is time-consuming, we generated a standard that contained a majority and, more importantly the extremes, of *Y. schidigera* saponins. Compounds detected by HPLC/ELSD of the purified standard all corresponded to saponins that elute between 12 and 24 min with small impurities detected before 12 min (0.9%, expressed in area percent integration). No interfering peaks in UV detection range (200–400 nm) could be observed.

In order to better characterise the purified *YSS* we have quantified i) the residual solvent content by GC/MS head space method (<0.05%); ii) the water content by coulometric method (4.1%), and iii) the absence of residual phenolic compounds by ^{1}H NMR determination (focusing on aromatic proton detection range). All of these analyses lead to the absolute purity of 95.0% for the new here prepared standard.

Here developed method presents a slight downside. In this method, the aim of having a mixture of saponins within the same standard answers the requirement of the measurement of all *Yucca* saponins, within a range of detection and retention time defined by the extreme compounds contained within the very standard. Because the standard is a mixture of saponins, it cannot specifically quantify each saponin separately. The detection limit of the standard mixture is much higher than a detection limit of a single purified saponin. Therefore, this method is not applicable to very low contents in saponins. However, it should be noted that quantification of saponins in the industry mainly relates to products with significant levels in saponins that approximate 10%. Overall, as the standard contains a broad range of *Yucca* saponins, as confirmed by further recovery data, the overall result yields in an accurate values. Moreover, qualitative plant adulteration evaluation seems to be possible at even low concentrations in saponins, provided saponin concentration remains within the detection limit.

3.3.1. Linearity

Here developed HPLC/ELSD method was developed using external standardization. Because ELSD results in a non-linear response, the calibration curve was plotted in Log/Log scale. Linearity of the response is acceptable in the range between 500 and 3200 μg/mL (corresponding to total saponins injected and 6 levels of concentration). Linearity in this range was confirmed. More precisely, the measure of goodness-of-fit (r^2) was higher than 0.995 (Slope of the calibration curve was at 1.671). Due to the non-linearity and because all the saponins are not at the same concentration in the here developed standard, difficulties may persist in case of samples containing very low amounts of saponins. In such case, estimation of the detection limit and quantification may prove difficult. This would be a minor issue as the contents in total saponins generally range from 1% to 20% as exemplified by *Yucca* juice extracts A, B and C and *Yucca* powder D.

3.3.2. Precision

To determine repeatability, *Y. schidigera* powder extract sample (D) that contained ~6% total saponins was analysed in six replicates and the RSD% value observed for total saponin quantification was at 2.79%. Therefore the method is precise for multiple sample injections within the same sequence. As far as the intra-day

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variation is concerned, six replicates corresponding to six weights of samples of the same *Yucca* powder extract sample (D) were analysed on a different day and by 3 laboratories. The inter-day precision and reproducibility RSD% were calculated from the area sum of the total saponins and yielded respectively 3.02% and 4.73%. Such RSD% level corresponds to an adequate precision level required for industrial control.

3.3.3. Quantification of total saponins in Y. schidigera extract samples and recovery tests

In order to check the suitability and the applicability of the developed method, we performed the measurement of saponins in three different samples of *Yucca schidigera* juice extract. These measurements resulted in different LC/ELSD profiles (Fig. 3). The influence of diluent containing 0.02% of antifoam was investigated by analysing the diluent containing 0.02% of antifoam alone, the sample in water, and the sample with 0.02% antifoam in water. No peaks were detected on the LC/ELSD chromatogram of the diluent containing antifoam. The difference of data obtained between samples in water and samples with 0.02% antifoam in water remained negligible. In conclusion, the antifoam at this concentration has no influence on the overall measurement of *Yucca* saponins. This indication is an advantage as the supplementation with the antifoam facilitates the accurate filling in of laboratory material, including flasks, as it alleviates the formation of the foam while the *Yucca* sample containing saponins is poured, and therefore enables more precise measurements. Meanwhile it is recommended to wash the column with 100% acetonitrile after each sample injection.

The sample analysis for recovery test was performed in triplicates with a concentration approximating the 100% of the calibration curve. Typically, 300–500 mg of *Yucca* sample that contained ~10–13% total saponins was dissolved in 25 mL of water containing 0.02% antifoam and analysed. To evaluate the influence of the repartition of saponins and validate the method of quantification, we tested the recovery by spiking the *Yucca* juice samples A, B, and C with known amount of YSS and have shown a recovery in the range of 80–120% (Table 2). We concluded that the composition of the *Yucca* extract has no impact on the overall result and on the response provided by other compounds present within the sample.

Here developed method enabled accurate quantification of saponins contained within *Yucca* samples originating from different geographic areas and prepared from plant material harvested during different year and season (Fig. 3). However, as mentioned above, here developed method is not applicable to very low contents in saponins. Indeed, very low levels in saponin contents *in planta* could occur in case of (e.g. seasonal changes), as described by Szakiel, Pączkowski, and Henry (2011).

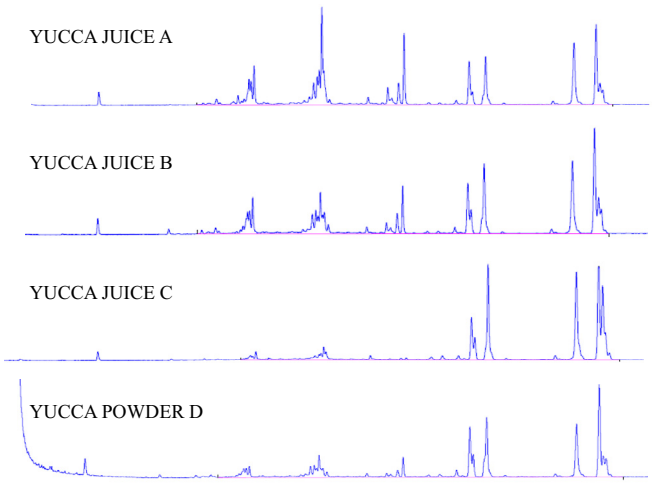

Fig. 3. HPLC/ELSD chromatogram corresponding to three sources of *Yucca* juice extract (A, B and C) and the corresponding spray dried extract with inverted sugar (D) from *Yucca schidigera* juice extract (B).

Table 2
Accuracy of the HPLC/ELSD method.

Yucca juice (HPLC/ELSD)	Amount added (%)	Amount detected (%)	Recovery (%)
A (15% saponins)	5.3	5.2	98.1
	10.4	9.8	94.2
	13.5	12.8	94.8
B (10% saponins)	5.2	4.5	86.5
	10.3	9.8	95.1
	16.4	14.8	90.2
C (12% saponins)	6.0	5.5	91.7
	15.0	13.9	92.7
	18.9	17.8	94.2

As reported previously, the gravimetric method could not provide an insight as to the saponin composition of the residue of extraction. The newly developed LC/ELSD method answered this issue.

3.3.4. LC/ELSD as plant adulteration control method

Thanks to the LC/ELSD saponin profile, it became possible to distinguish saponins originating from different *Yucca*, or other, taxonomically distant species, and even to check for possible cross-species contaminations. We exemplified this by analysing different extracts containing saponins: *Y. schidigera* juice extracts obtained according to different methods A, B, and C, *Y. schidigera* dried trunk (identified by TLC), *Yucca brevifolia* root extract that was contained within a shampoo and a *Yucca schidigera* (trunk)/*Quillaja saponaria* (bark) extract blend. Following 100% water extraction for 24 h at room temperature of the *Y. schidigera* dried trunk, the LC/ELSD method showed a typical chromatogram with high concentrations in the most polar steroidal saponins (first two groups Fig. 2A). This indicates that the process of aqueous extraction from dry plant material yields in higher amounts of highly polar saponins rather than mechanical pressing of fresh raw material. All 5 groups of saponins were detected in all three types of *Yucca* samples: A, B and C. Indeed, differences in the saponin composition are explained by the distinctions in the extraction or clarification process that may involve hydrolysis, including the use of phosphoric acid to adjust pH with hot *Yucca* juice concentrate, of the most polar steroidal saponins and the use of differently treated plant material prior to extraction: either fresh or dried trunk.

Secondly, we showed that spray drying of *Yucca* juice sample B with inverted sugar had no influence on the saponin repartition (Fig. 3D). This is an important finding that guarantees the process and the origin of *Yucca* even after spray drying and that shows a successful quantification of the ratio of *Yucca* saponins when blended with saponins originating from another species, including *Quillaja*.

Thirdly we analysed the *Yucca*/*Quillaja* blend. HPLC/ELSD chromatogram (Fig. 4A) clearly shows the presence of *Quillaja* saponins interfering with the most apolar saponins from *Yucca*. This method clearly can be used for analysing plant adulteration issues of saponins originating from taxonomically close and distant species. A range of existing methods, including spectrophotometric, foam height or gravimetric techniques are incapable of addressing thus type of issues.

Finally, we report the data obtained following the extraction of a shampoo containing *Yucca brevifolia* root extract. This ingredient

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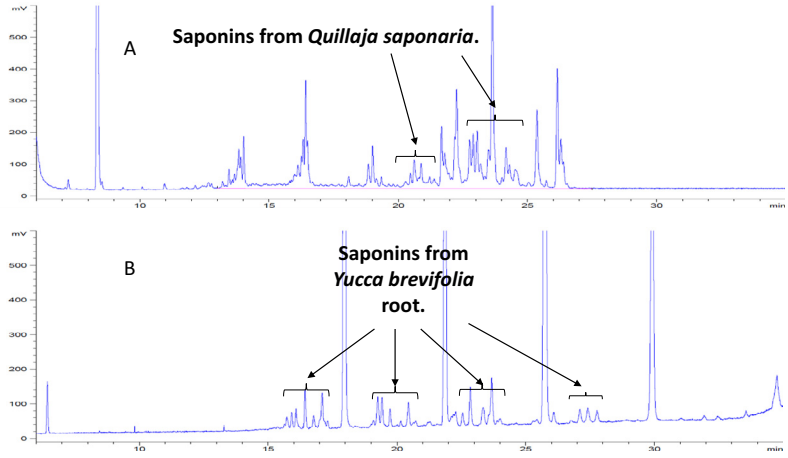

Fig. 4. HPLC/ELSD chromatogram corresponding to: (A) *Quillaja saponaria* saponin extract; (B) spiked on *Y. schidigera* juice extract (B) *Y. brevifolia* root saponins from shampoo sample.

is used for foaming and as a hair growth promoter. The HPLC/ELSD profile showed clearly a different saponin composition and profiles between *Y. brevifolia* and *Y. schidigera* (Fig. 4B).

This method clearly can be used for analysing plant adulteration issues of saponins originating from taxonomically close and distant species. A range of existing methods, including spectrophotometric, foam height or gravimetric techniques are incapable of addressing these types of issues.

3.4. Concluding remarks

Thus, we have developed a new HPLC/ELSD method performed on a reversed-phase C18 column with gradient elution of an aqueous acetonitrile system that rapidly, accurately and reliably quantifies total steroidal saponins in *Yucca schidigera* extracts. Overall, the proposed method exhibited a satisfactory linearity, reliable accuracy (recovery) and adequate reproducibility for quantitative determination of total saponins in *Yucca schidigera* extracts. Compared to the existing techniques, including gravimetric method, time and solvent requirements are respectively divided by 5 and 7 (Table 1) and sample preparation does not need any pre-purification. Finally, one of the strengths of the method relates to reliable authentication of species abundant in steroidal saponins that are typically used in commercial extracts.

Conflict of interest

Author Mathieu Tenon declares that he has no conflict of interest.

Author Nicolas Feuillère declares that he has no conflict of interest.

Author Marc Roller declares that he has no conflict of interest.

Author Simona Birtić declares that she has no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

Not applicable.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.11.033.

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