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Liquid Chromatography-Electrospray Ionization Mass Spectrometry Analysis of Quil-A: an Aqueous Extract from the Bark of *Quillaja saponaria* Molina

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ABSTRACT

Quil-A, a commercial product of the aqueous extract of *Quillaja saponaria* Molina bark, indigenous to Chile has been widely reported for its immuno-modulatory and adjuvant properties. The saponin constituent of the plant extract, which is responsible for these properties, is commonly separated using liquid chromatography techniques. However, the application of this technique is impaired by the non-saponin constituents of the aqueous matrix, leading to the shortening of the column life. Therefore, the study was to develop a reverse-phase liquid chromatography technique to improve the resolution of the aqueous matrix. Applying the reverse phase liquid chromatography using optimized gradient solvent mixtures of water, acetonitrile, and 10 mM ammonium acetate, components from the sample were eluted on a C5 reverse phase column. Liquid chromatography peaks at 214 nm, and electrospray ionization mass spectrometer signals were identified as per the NIST-MS data library. The finding indicates the presence of ions of molecules previously reported. Also, ions of high molecular weight saponin-like molecules, which are yet to be reported, were eluted at early retention times of the chromatography elution. The study suggests the possible presence of molecules, which could be novel and contribute to the ultimate benefits of Quil-A.

Keywords: Adjuvant, chromatography, extract, saponin, spectroscopy.

Introduction

Quil A is a commercial product of the aqueous extract of the saponin rich bark of *Quillaja saponaria* Molina, which has been filtered for virtual clarity or purified using gel filtration or other methods.[1] The plant is indigenous to Chile and belongs to the Quillajaceae family, although previously considered to belong to the Rosaceae family.[2,3] It is known for its foaming or/and emulsifying activity. Due to this soap-like effect, it is used by natives to wash cloths, hence the common name "Quellen" which means "to wash" in the local dialect. Saponaria was derived from the Latin word "sapo", which means soap.

Extract of the bark of *Quillaja saponaria* has been studied extensively. Available data shows that the extract has found relevance in the food, cosmetics, agricultural and pharmaceutical industries.[4,5] The avalanche of saponins in the extract has generated interests among researchers due to the immune modulatory potential. The immuno-stimulatory activity is both humoral and cell-mediated and, as such, it is used as an adjuvant in a variety of veterinary vaccines.[1,6-9] Due to the later propensity, it has found use in the fight against coronavirus disease of 2019 (COVID-19).[10] It is a component of the immuno-stimulating Complex (ISCOM) of vaccines used to treat the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), an avian virus responsible for the COVID-19 disease.[10,11]

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The earlier Quil-A extract as prepared by Dalsgaard (1970), is a complex mixture of saponins and non-saponin constituents.[12,13] Following the success story of the extract, some scholars engaged other methods of preparation of the extract in order to reduce the complexity of the matrix. Higuchi *et al*., prepared the extract from the Quillaja bark using methanol, and achieved purification through droplet counter current and reverse phase column chromatography.[14,15] Also, Steinbeck *et al*. in 1995 employed the use of the bark of young trees found to be less heterogeneous.[16]

The complexity of Quil-A is partly due to the presence of over 60 different saponin and non-saponin components.[17] As a practice, separation of these components, is commonly carried out using reverse phase liquid chromatography.[17-19] In recent times, the hyphenated technique has become one of the acceptable approaches to separate, purify, or identify phytoconstituents.[20] Exploring this technique, prior studies to provide better knowledge of the characteristics and structure of the quillaja saponins have been carried out, by notable scholars.[19, 21] In some of the studies, C18 and C8 chromatography columns were used after prior purification of the aqueous matrix to achieve moderate to good separation.[19] Typical of the reverse phase chromatography columns is their non-polar character due to the hydrocarbon moiety of the parked column. Solutes, in the liquid mobile phase, are retained by this silica-bonded moiety through hydrophobic and van der Waal force interactions. Consequently, the octadecyl silane C18 column may be suitable for the purification of peptides, while octyl silane (C8) or pentyl silane (C5) columns would be preferable for the separation of longer molecular structures like proteins. This study, therefore, was aimed at developing a liquid chromatographic technique using the C5 reverse phase column, to improve the resolution of the aqueous matrix without prior purification.

Materials and Methods

Materials

Quil-A was obtained from Superfos Biosector a/s (Denmark) as a sieve-filtered, off-white crystalline solid in May 2009. Ammonium acetate

A.C.S grade was obtained from Fisher Scientific Company (USA). Acetonitrile was from Anachemica Ltd. Water was distilled, deionized, and filtered using Millipore-type HA filters of 0.22 µm pore size. Millipore types HV and HA syringe filters of pore sizes 0.45 µm and 0.22 µm, respectively, were used for filtering solutions for high-performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometer (LC/MS). The vacuum manifold and pump apparatus and the solid phase extractor with C8 silica packed columns (Strata(R), USA) which has a size of 1 g packing material and 6 mL volume, were used to treat the sample.

Instrumentation

The HPLC 64 Hewlett Packard Series 1050 HPLC, equipped with a Hewlett Packard 3396 integrator and diode array detector, was used for the preliminary analysis. The electrospray ionization-mass spectrometry (ESI-MS) analysis, was carried out using the Beckman System Gold HPLC (HP 1100 series) with diode array detector, equipped with a high-pressure pump, auto-sampler, 126 solvent delivery system, 167 diode array detector, and a 406 interface analog; coupled to a mass spectrometer (MS) with ESI interface.

In all cases, analysis was done using 5 µm particle size, 300 Å pore size, and 4.6 mm x 250 mm length analytical HPLC Phenomenex Jupiter C5 reverse phase column, equipped with a 4 mm x 33 mm length, 7000 psi resistance packed guard column (having same material and particle size with the analytical column). The stationary phase was conditioned (and baselined) using acetonitrile before and after each run and kept at 35° C. With the diode array detector, peaks detected at UV absorbance wavelength (λ) of 214 nm were eluted from the column using linear gradient solvent mixtures of water, acetonitrile, and 10 mM ammonium acetate.

Procedure

Sample preparation

Three hundred milligrams per milliliter of alkaline buffered aqueous solution of Quil-A was prepared using 10 mM ammonium acetate, and distilled deionized water which was filtered with the Millipore type HA membrane filters of 0.45 µm pore size. The sonicated solution was filtered, with the Millipore types HV and HA filters of pore sizes 0.45 pm and 0.22 µm, respectively. Samples for LC ESI-MS analysis were filtered further using the HV and HA syringe filters of pore sizes 0.22 µm and applied as prepared.

Preliminary HPLC analysis

Adopting a method similar to that described by Wallace *et al*.,[22] with modification, 20 µL of the sample, buffered in alkaline pH (instead of acidic pH), was injected into the pre-conditioned analytical column kept at 35°C. Detected peaks were eluted from the column using a linear gradient mobile mixture of water, acetonitrile, and 10 mM ammonium acetate in the ratio of 15:3:2 (mixture A) and 5:4:1 (mixture B). Elution was carried out with mixture A from time (t) at zero minutes, and mixture B from t at 25 minutes. The flow rate was 1.5 mL/min at a run time of 100 minutes.

Optimization of the Analytical HPLC System

The optimized linear gradient mobile mixtures of water, acetonitrile, and 10 mM ammonium acetate prepared as mixtures A and B, and C which is acetonitrile and 10 mM ammonium acetate at 9:1 was used to elute the sample. Initial elution started with mixture A from t at zero minutes, followed by B from t at 25 minutes, and later, C from t at 50 minutes. The employed flow rate was 1 min/mL at a run time of 100 minutes.

Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (LC/ESI-MS)

Repeating the same procedure as optimized using the HP 1100 series, 10 % of each eluent at 1 min/mL flow rate was diverted into the electrospray (ES) source of the MS which was optimized, for direct infusion. The capillary voltage was -29 eV, the capillary temperature was set to 200° C, the spray voltage was set to 25 kV, and nitrogen was used as sheath gas. The MS was operated in negative ion mode and programmed to achieve metabolite identification. The LC/UV traces were recorded online using the photodiode-array detector (HP 1100 series), with integral analysis and interpretation of peaks carried out using the NIST (National Institute of Standards and Technology) data library.

Solid Phase Extraction (SPE) of sample

SPE cartridges containing 1 g sorbent material were pre-conditioned with one volume (6 mL) of acetonitrile and rinsed with one volume (6 mL) of 10 mM ammonium acetate buffer solution. The sample was diluted with deionized distilled water to obtain 5 L of a 0.01 mg/mL buffered Quil-A solution. The solution was applied and aspirated through the cartridge at a flow rate of 1 to 3 mL/min by means of a vacuum manifold and pump apparatus. The cartridge was washed with I mL of the wash solution (15 % acetonitrile in 10 mM ammonium acetate solution) and dried under full vacuum for 2 to 5 minutes. The dried cartridge was eluted with 90 % acetonitrile in a 10 mM ammonium acetate solution. The collected eluent was dried, weighed, and analyzed with the optimized LC system as described above. The peaks were recorded online with the photodiode-array detector (HP 1100 series) and interpreted as recorded.

Analysis of data

Data were expressed and interpreted as in spectra identification using the NIST MS data library.

Results and Discussion

The bark of the *Quillaja saponaria* Molina tree is known for its matrix of saponins, which are of health, agricultural and cosmetic value. The saponin constituents have been reported to represent 5% of the weight of the bark and 20 % of the dried aqueous extract known as Quil-A. The report indicates that Quil-A type 1 and 2 have saponin contents of 20-26% and 70%, depending on the level of purification.[23, 24] However, these data may vary depending on location and climatic conditions.[25]

Saponins from this shrub are terpene glycosides and are commonly referred to as quillaja saponins.[22] Their elucidated basic molecular structure indicates hydrophilic (sugar or glycone) and hydrophobic (aglycone) moieties. The aglycone moiety is the triterpene which could be either quillaic acid, gypsogenin, or phytolaccinic acid.[26] Due to the bisdesmosidic nature, these saponins are commonly glycosylated at C-3 and C-28 of the aglycone by disaccharides and oligosaccharides, respectively.[19] The reported preliminary liquid chromatography screening of the extract in this study shows a constitution of highly polar components. This may explain the successes achieved in the isolation and structural elucidation of some of the pure saponin components using C8 and C18-reverse phase columns.[22, 27]

LC Analysis of the Quil-A and solid phase extracted Samples

By adopting the method for the preliminary screening of the Quil-A using the C5 analytical column, the resolution of identifiable peaks was obtained using the optimized system. At least 50 out of 88 peaks were well resolved, based on the order of their retention time (Figure 1). Typical of the identification of saponins from the herbal extract by stable froth and its absorption pattern at 214 nm ultraviolet wavelength, the obtained data provided enabling characterization of eluents as saponin-like or non-saponin. [28-31]

The non-saponin extractable molecules in Quil-A are detrimental to the LC columns, leading to the shortening of its life. This is particularly the case with C18 columns, which by their nature, portents a prolonged retention time for molecules. As such, some of the prior studies which used the C18 column had considered it necessary to eliminate these components using dialysis or gel filtration, which by this act, also provides a less complex matrix for sufficient LC separation.[22, 32] Conceptually, this is the basis for purification of the crude Quil-A extract to obtain the different types of the commercial product.[24] Because the study design was to provide an optimized system to manage the complex matrix, a preliminary purification was not applied. The obtained chromatogram prior to the SPE justified this approach. Indeed, the preliminary LC analysis of the extract indicated peaks within 10 to 30 minutes of the retention time which have similar UV absorption characteristics typical of saponin compounds.[33] However, very few

peaks along the chromatogram showed high purity, emphasizing the tendency of the components to co-elute.

It may be riveting to note that, out of 50 mg of the QuiI-A used in the SPE experiment, only 16.1 mg were retained in the filtrate. This was depicted in the obtained chromatogram, after the solid phase extraction of the extract (Figure 2), which shows the absence of peaks (molecules) eluted within the first 25 minutes of the LC analysis of the sample in Figure 1. Differences in the chromatograms may occur due to the batch-to-batch dissimilarity in the Quil-A materials. However, the differences in the obtained chromatograms prior to and after the SPE clearly substantiated the presence of saponin-like molecules lost along with non-saponins by the application of SPE.

Liquid Chromatography/Electrospray Ionization-Mass Spectrometry (LC/ESI-MS)

The LC/MS analysis and online characterization of peaks were intended to provide clarity to the identity of possible saponin-like molecules. LC/MS analysis chromatogram of the sample, as obtained for the diode array and ESI-MS spectra data (Figure 3), showed some peaks for ions which eluted within 5 minutes of the retention time. This was evidenced by the extracted ion chromatogram (EIC) in figure 4. Though the peaks appear to be intriguing, some did not show the typical saponin UV absorption characteristics. Also, some of the saponin-like peaks, identified to have been eluted within 25 minutes of the chromatogram, were indicated to be molecules with very high molecular weight. The overall evaluation of the chromatogram spectra of the LC/ESI-MS revealed the presence of peaks that appeared within a retention time of 30 minutes, showing profound saponin-like characteristics. The EIC provides a clearer overview of the lag time between these obtained DAD and MS signals.

Though the actual stability of the obtained MS molecular ions could not be determined, obtained indicated the presence of some of the molecules reported in previous literature (Table 1), and other molecules which are yet to be reported (Table 2).[19,22] Prior literature shows that the Quillaja saponin with the highest molecular weight (2321) is QS-17. Interestingly, this report identified molecules with higher molecular weight.[18,19]

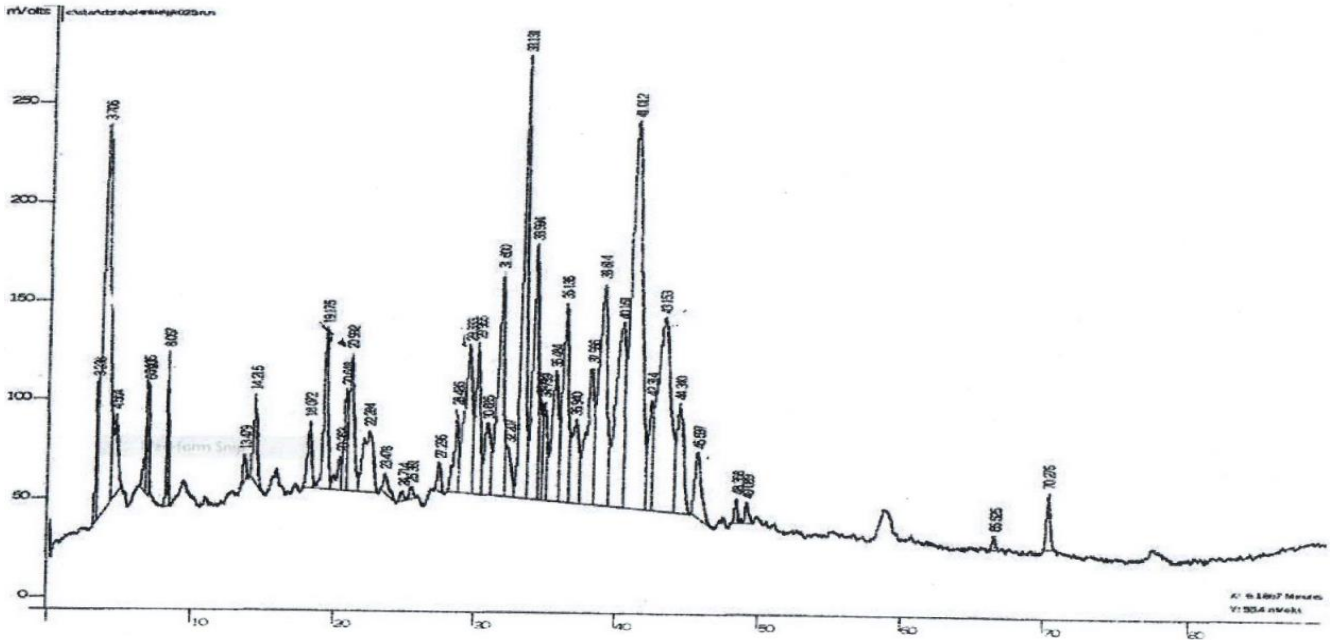

Figure 1: Chromatogram of sample (Quil A) indicating peaks of saponin or saponin-like compounds at the optimized LC system.

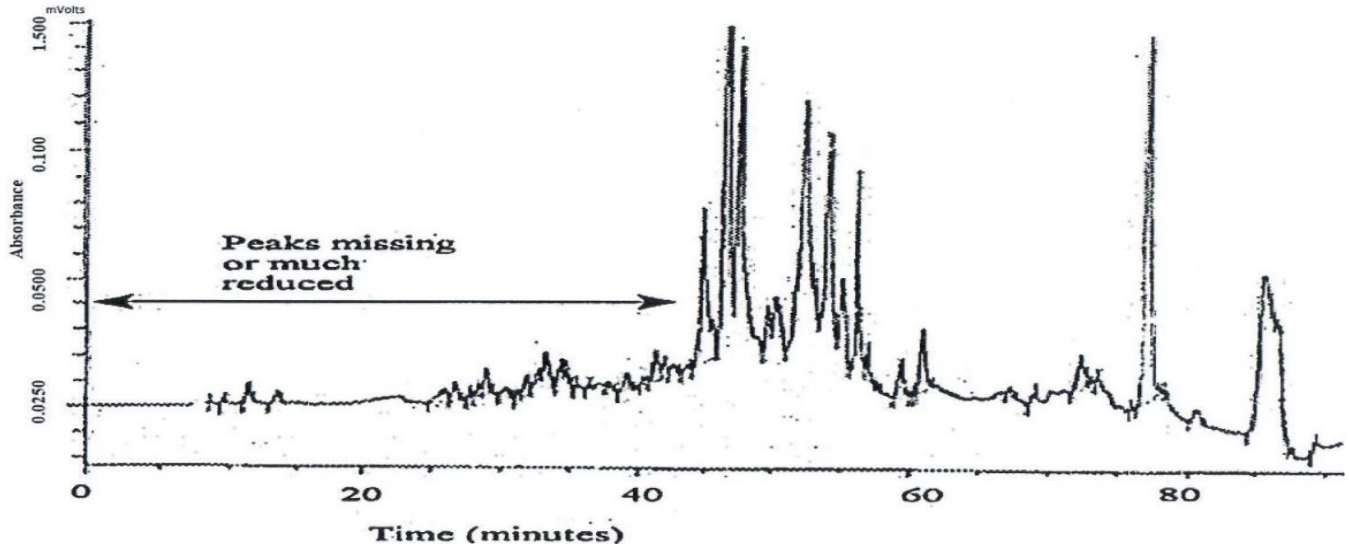

Figure 2: Chromatogram of sample (Quil A) after SPE indicating peaks of saponin or saponin-like compounds at the optimized LC system.

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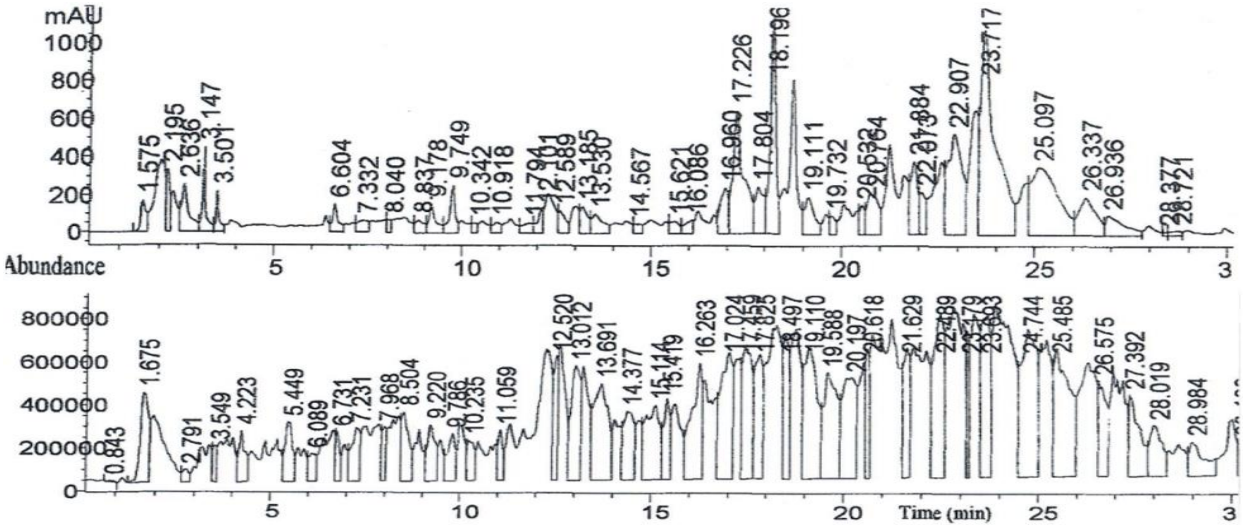

Figure 3: Peaks at 214 nm for DAD signal (top) and MSD signal (bottom) as obtained from LC/ESI-MS analysis of Quil-A, indicating MSD signal lagging behind the DAD signal.

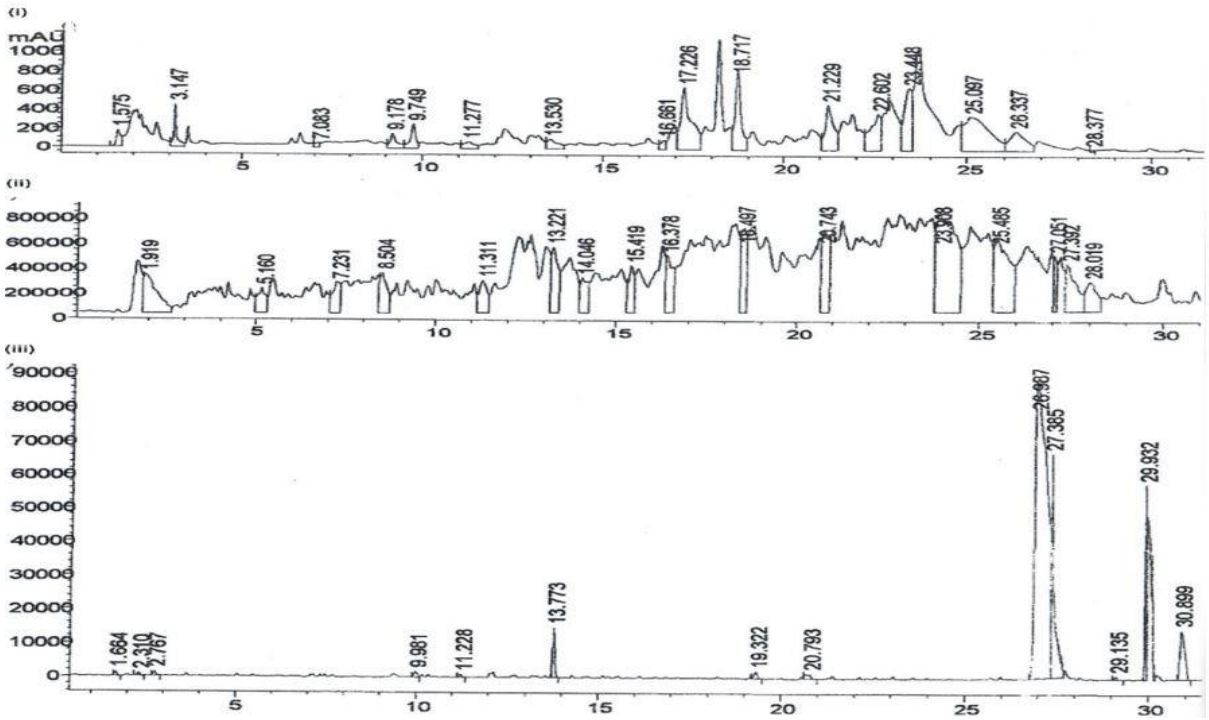

Figure 4: Extracted ion chromatogram (EIC) for saponin-like molecular ions 1989 and 1560 m/z signals at 25.485 (ii) and 26.987 (iii) minutes of the MSD spectra are concentrated at the peaks eluted at 25.097 and 26.337 minutes of the DAD signals (i) respectively.

Some ions of the same molecular mass were seen eluting at different points on the profile. A probable explanation could be that some ions of the same compound, probably isomers, are showing differences in affinity property to the column.[21] It is worth noting that isomers of the Quillaja saponins, which are all immunologically active, have been reported by previous scholars.[22, 34] An example of such is the previously reported QS-21 with a molecular weight of 1989 and different isomeric forms as identified in *Quillaja saponaria* and *Quillaja brasiliensis*. In recent times, this saponin has found relevance as an adjuvant in the manufacture of the COVID-19 vaccine.[35,36]

Conclusion

Increasing interest in saponin has prompted scholars to start probing fractions with high molecular weight compounds. Molecules with m/z ions ranging from 800 to 3000 and visible stable frothing during slight agitation could attract attention. Hence, high molecular weight molecules eluted within 30 minutes of the chromatogram in this study may be of novel interest. Provided structural identity limits in this study notwithstanding, obtained data strongly supports further probe of components lost during the purification of the extract.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 1: Identified molecular ion of saponin or saponin-like compounds which have been reported in previous studies.

S/N	Molecular ion Mass ($^m/_z$)	Compound name (where known)	Elution Times (min)	
			MSD	DAD
1	956	QS-L1	7.817-8.504	7.587-8.20
2	1512	DS-1	13.012	13.184
			13.221	19.110
			19.110	
3	1560	S13	26.387	26.937
4	1626		10.485	14.684
			14.684	14.560
5	1640		19.110	19.110
6	1696	DS-2	4.844	
			17.825	4.698
			20.743	17.804
7	1788		30.199	
			18.497	18.480
			19.110	19.110
			21.233	21.229
			22.124	22.073
8	1862		17.459	17.226
			17.825	17.804
9	1869		17.459	17.226
			26.575	26.337
10	1876		17.459	17.226
			17.825	17.804
11	1886	QS-7	18.770	18.718
			19.585	19.577
12	1989	QS-21	24.744-5.485	23.971-25.097
13	2018		10.818	10.520
			17.459	17.226
			22.661	22.602
14	2150	QS-18	23.693	23.448
			23.908	23.717
15	2296	QS-III	8.364	8.258
			22.124	22.073
			22.823	
			23.179	
16	2321	QS-17	6.624	6.604

Table 2: Identified molecular ions of saponin or saponin-like compounds which have not been reported

S/N	Molecular Ion Mass ($^m/_z$)	Elution Times (min)		UV Absorption Characteristics
		MSD	DAD	
1	2438	17.825	17.804	Saponin like
2	2447	5.160	5.047	Saponin like
4	2453	7.231	7.083	Saponin like
6	2509	5.722	5.695	Saponin like
8	2520	11.059	10.918	Saponin like
9	2547	17.825	17.804	Saponin like

11	2567	7.231	7.083	Saponin like
14	2613	3.682 15.419	15.395	Saponin like
15	2624	8.504	8.449	Saponin like
18	2677	3.549	3.501	Saponin like
20	2698	1.675	1.575	Saponin like
21	2705	6.089	5.945	Saponin like
22	2713	6.089	5.945	Saponin like
23	2727	3.549	3.501	Saponin like
24	2746	6.089 14.684	5.945 14.560	Saponin like
25	2771	3.994	3.846	Saponin like
27	2785	7.817	7.587	Saponin like
28	2793	4.493	4.462	Saponin like
29	2794	9.996	9.976	Saponin like
30	2801	6.383	6.216	Saponin like
32	2848	12.610	12.589	Saponin like
34	2872	8.364	8.258	Saponin like
35	2912	7.968 16.378	7.923	Saponin like
36	2935	5.160 4.844	5.047 4.698	Saponin like
38	2954	9.220 9.498	9.178	Saponin like

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