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Short communication

Multidimensional profiling of components in complex mixtures of natural products for metabolic analysis, proof of concept: Application to *Quillaja* saponins

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ABSTRACT

A method for separation and detection of major and minor components in complex mixtures has been developed, utilising two-dimensional high-performance liquid chromatography (2D-HPLC) combined with electrospray ionisation ion-trap multiple-stage mass spectrometry (ESI-ITMS^n). Chromatographic conditions were matched with mass spectrometric detection to maximise the number of components that could be separated. The described procedure has proven useful to discern several hundreds of saponin components when applied to *Quillaja saponaria* Molina bark extracts. The discrimination of each saponin component relies on the fact that three coordinates (x, y, z) for each component can be derived from the retention time of the two chromatographic steps (x, y) and the m/z-values from the multiple-stage mass spectrometry (z_n, $n = 1, 2, \ldots$). Thus an improved graphical representation was obtained by combining retention times from the two-stage separation with +MS^1 (z_1) and the additional structural information from the second mass stage +MS^2 (z_2, z_3) corresponding to the main fragment ions. By this approach three-dimensional plots can be made that reveal both the chromatographic and structural properties of a specific mixture which can be useful in fingerprinting of complex mixtures.

1. Introduction

Fingerprinting complex mixtures have become of increasing importance in studies of natural components used in medical applications. A method that also gives information on the structures and visualises them according to these should be an important tool in studies of these complex mixtures.

The medical use of *Quillaja* saponin fractions, which contain a large number of components, calls for rapid identification of both major and minor components. [1–5] The main method used for profiling saponins in extracts is high-performance liquid chromatography (HPLC) with MS or UV detection. [6–9] To date, the structures of ~70 *Quillaja* saponins (Fig. 1) have been characterised [10–17] and most of these consist of quillaic acid as the triterpene. However, several other triterpene structures in minor components are known and thousands of theoretical saponin structures are possible as a result of different combinations of substituents. Postulating that the extracts contain hundreds of saponin components, the previously reported methods provide a resolution that only allows for screening of a limited number of the components in a biological sample of *Quillaja saponaria* Molina. Thus a method capable of providing information for cataloguing and comparing a larger number of components is needed *e.g.* for metabolic studies.

In this work a method suitable for screening both major and minor components in complex mixtures has been developed utilising information from 2D-HPLC–ESI-IT-MS^n. The compounds are either detected by MS^1 providing information related to molecular mass and concentration or by MS/MS analysis which offers the possibility to address not only analytical but also structural issues [6,7,9,10,18]. The information obtained on each of the saponin components is the retention time, given as fraction number (x), in the first chromatographic step, retention time from the second chromatographic step (y) and m/z-values for $[M+Na]^+$, $[A+Na]^+$ and $[B+Na]^+$ ions (z_1, z_2, z_3). In this study these coordinates (x, y, z_n) provide means to discern more than 400 saponin components in a complex mixture.

2. Experimental

2.1. Chemicals and materials

The *Quillaja* saponin bark extract was obtained from Berghausen (Cincinnati, OH, USA). Acetonitrile, methanol (LiChrosolv®), trifluoroacetic acid (Uvasol®) and ammonium acetate (p.a.) were obtained from Merck (Darmstadt, Germany) and the water was produced in house by a Milli-Q system (Millipore Corporation). Isolute™ 10-g

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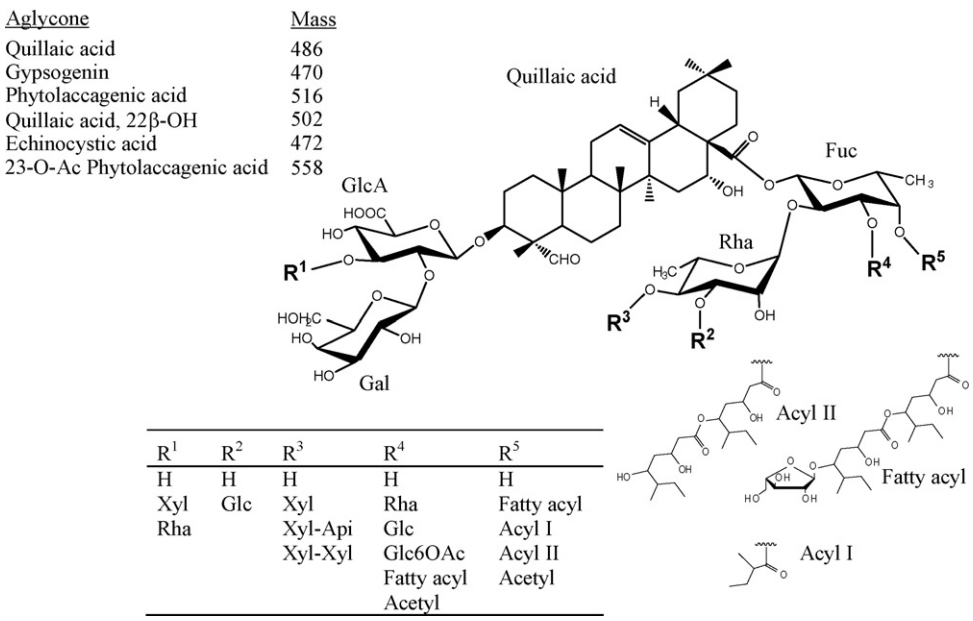

Fig. 1. Structures for reported *Quillaja saponaria* saponins.

reversed phase (RP) C18 (EC) solid phase extraction (SPE) columns were purchased from International Sorbent Technology Ltd., UK.

2.2. Solid phase extraction

The crude bark extract was first subjected to a coarse separation on an SPE column to remove non-saponin components. Bark extract (200 mg) was dissolved and applied to the pre-equilibrated column. The column was washed with 30 ml methanol/water (1:1, v/v containing 0.1% trifluoroacetic acid) and then saponins were eluted with 30 ml methanol/aqueous 30 mM ammonium acetate (9:1, v/v). This eluate was collected and concentrated to dryness yielding 46 mg of sample. The presence of saponins in the fractions was shown by ^{1}H NMR (Bruker DRX-600 spectrometer) and matrix assisted laser desorption/ionisation–time of flight (MALDI–TOF) MS (*m*/*z* 1400–2350) using a Bruker Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany) and 2,5-dihydroxybenzoic acid as matrix.

2.3. High-performance liquid chromatography

Chromatography was performed on a Kromasil 4.6 mm × 150 mm RP C-18 column (100-5C18, HiCHROM, UK) and on an Ascentis® Express RP C18 column (3 mm × 50 mm, 53811-U, Supelco, USA). Flow rate through both chromatographic systems was 1 ml/min.

Portions (2.4 mg) of SPE purified bark extract were separated on the Kromasil column and the eluate was monitored by UV detection at 210 nm and ESI-IT-MS^1 or collected as 1.5-min fractions resulting in a total of 40 fractions. A portion (40 μl) of each fraction was then injected for a second separation on the Ascentis® Express column, which allowed for a relatively short run (20 min). The eluate was monitored by UV detection at 210 nm and ESI-IT-MS^n.

In the first separation step, with the Kromasil column, the gradient consisted of aqueous 30 mM ammonium acetate and methanol with 55% methanol at start and with a linear gradient from 55% to 65% methanol in 10 min and then from 65% to 90% methanol in 50 min (Gradient 1). In the second separation step on the Ascentis® Express column the gradient consisted of aqueous 0.1% trifluoroacetic acid and acetonitrile containing 0.1% trifluoroacetic acid with 27% of the latter at start and with a linear gradient from 27% to 43% in 20 min (Gradient 2).

2.4. Mass spectrometric conditions

An Esquire LC ion-trap mass spectrometer (Ver. 4.5, Bruker Daltonik, Bremen, Germany) controlled by Esquire control software (ver. 4.0) was used for monitoring the eluate. The mass spectrometric conditions used were the same as previously described. [10,18] The flow to the ES ion source from the chromatographic system was reduced to 200 μl/min by splitting. Positive ion mode was used as it has been reported to be more sensitive compared to negative ion mode and provides information on both the C-3 and C-28 oligosaccharide. [6,10,18,19] Mass spectra were acquired in the range *m*/*z* 400–2200, whereas ions for selection were surveyed in the region *m*/*z* 1400–2200 for fragmentation in MS^2. Data dependent mode was used for selection of ions for multiple-stage MS. A maximum of two ions from each MS^1 scan could be selected and fragmented. When generating the base peak (BPC) and total ion current (TIC) chromatograms 32 spectra were averaged into a single profile spectrum used to create a data point in the chromatogram thus yielding 0.1 min between each data point.

2.5. Multidimensional representation of data

The chromatographic and mass spectrometric data obtained were converted into text files (.bsc) and then imported into Matlab® (The MathWorks, Inc., Natick, US, version 7.0.1) for further calculations and multidimensional representations.

2.6. Analytical characteristics

The analytical characteristics of the method were determined on a saponin solution (50 mg/ml) obtained from bark extract purified by the SPE procedure. A sample (40 μl) of this material was injected using the auto-sampler on the Kromasil 4.6 mm × 150 mm RP C-18 column and 1.5 min fractions were collected as described. The fractions were then injected on the Ascentis® Express RP C18 column. The eluate of the second step was monitored by

ESI-IT-MS^n applying conditions described in Section 2.4. The procedure was repeated two times more yielding in total three data sets.

2.7. Separation of the chromatographic fractions QH-B and QH-C

The chromatographic fractions QH-B and QH-C were first separated using the Kromasil 4.6 mm × 150 mm RP C-18 column using Gradient 1. The eluate was collected in 1.5 min fractions and these were each injected in a second separation step using the Ascentis® Express RP C18 column with Gradient 2. The eluate of the second step was monitored by ESI-IT-MS^n applying conditions as previously described [10,18].

3. Results and discussion

3.1. Solid phase extraction of crude extract

Bark extract from *Q. saponaria* Molina, obtained from Berghausen, was subjected to a crude separation step by SPE to remove most of the non-saponin components. The eluate was investigated by NMR spectroscopy and MALDI-TOF mass spectrom-

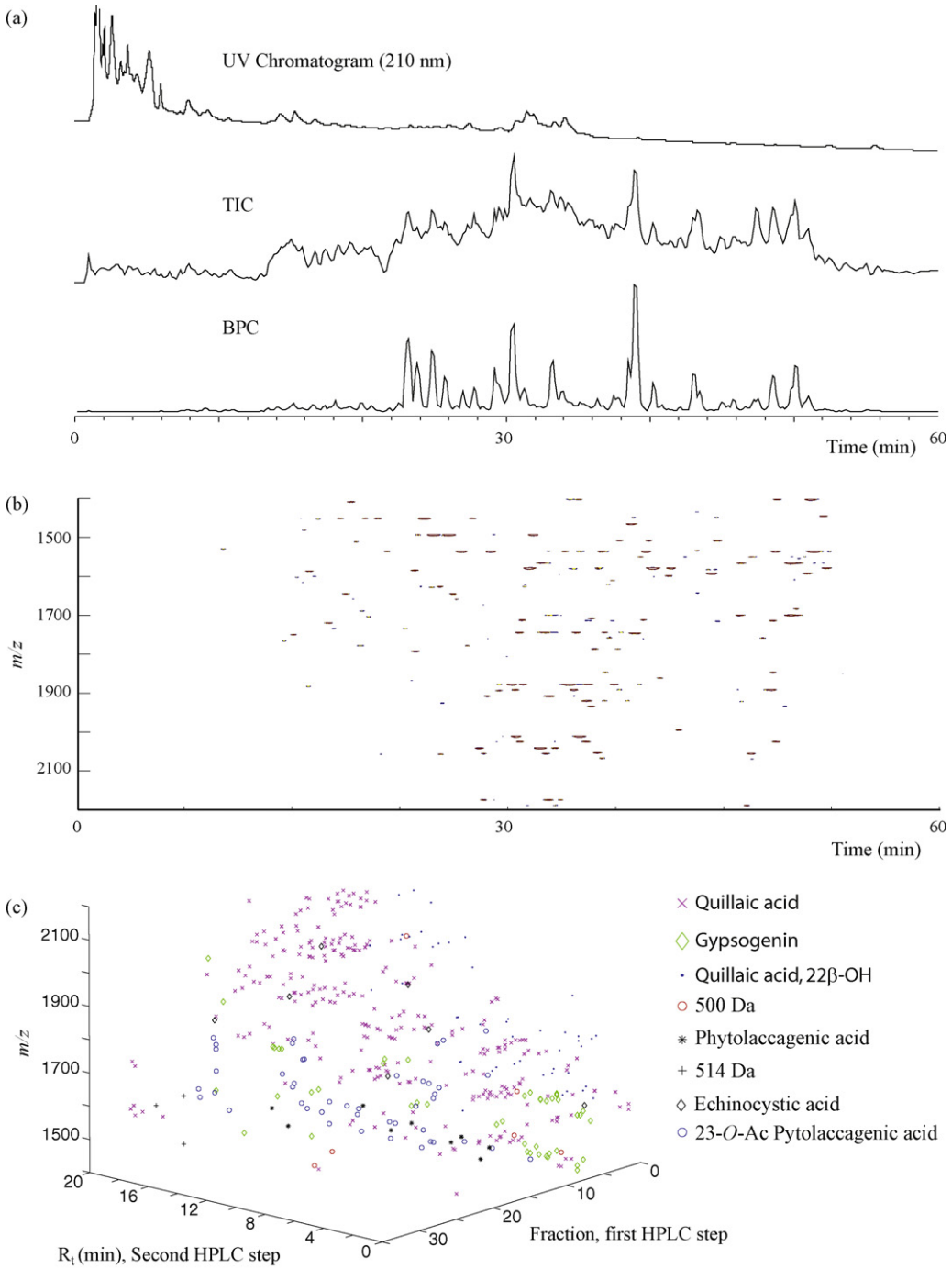

Fig. 2. Chromatograms obtained by HPLC with different detectors and one step and two steps showing the improvement in resolution and information obtained. (a) One-step HPLC obtained with UV detection, total ion current (TIC) MS detection and base peak chromatogram (BPC) MS detection; (b) map with retention time along one axis and MS^1 data along the other axis; (c) a three-dimensional map with retention times along two axes and MS^2 data along the third axis giving structural information on the separated components.

etry and only in the spectra of the 90% methanol eluate peaks from *Quillaja* saponins were observed.

3.2. Two-dimensional chromatographic separation of saponin components

The obtained separation of saponin components was evaluated by UV at 210 nm, TIC, BPC (Fig. 2a) and by plotting maps with elution time along one axis and corresponding mass spectra along the other axis (Fig. 2b). In the latter presentation the peak intensities of the $[M+Na]^+$ ions further separated the overlapping compounds by their different molecular masses. The best 2D-separation was achieved by using water and methanol at near neutral condition (Gradient 1) as solvent for the Kromasil column (first step) and water and acetonitrile at acidic conditions (Gradient 2) for the Ascentis® Express column (second step).

3.3. Multiple-stage MS analysis

The two-step chromatographic procedure in combination with automated data dependent mass spectrometry was applied on the SPE purified bark extract. The spectrometer was set to monitor, select and fragment the most abundant ions ($[M+Na]^+$) in the range m/z 1400–2200. Precursor ions ($[M+Na]^+$) were selected for MS^2 generating the sodiated fragments $[A+Na]^+$ and $[B+Na]^+$ (Fig. 3). Thus in positive ion mode isolation and fragmentation of the $[M+Na]^+$ ion provide an MS/MS spectrum containing mainly two ions referred to as $[A+Na]^+$ and $[B+Na]^+$ (Fig. 3) where the first ion corresponds to the fragment after loss of the C-3 moiety and the latter ion corresponds to the C-28 oligosaccharide. Information on the structures of the C-3 oligosaccharide and the triterpene can thus be extracted from MS/MS spectra by subtracting the m/z-values of $[M+Na]^+$–$[A+Na]^+$ and $[A+Na]^+$–$[B+Na]^+$, respectively. The triterpenoid core is most commonly a quillaic acid, but other reported structures are the 22β-hydroxy derivative of quillaic acid, gypsogenin, echinosystic acid, phytolaccagenic acid and acetylated phytolaccagenic acid [13,15]. Interpretation of this information together with the mass of the C-28 oligosaccharide (obtained from $[B+Na]^+$) provides a coarse classification of the saponin components [18]. In addition to all of the previously reported triterpene structures the mass difference of 468, 500, 514, and 648 Da between the fragments A and B indicates new triterpene structures. Furthermore, 43 new $[B+Na]^+$ ions were found corresponding to new types of C-28 oligosaccharides in 148 components previously not reported.

3.4. Multidimensional representation of data

The chromatograms obtained from the two-step separation and the MS data obtained by monitoring the second separation by MS^1 result in data that can be presented as a three-dimensional plot. Each component can thus be represented by a set of coordinates (x, y, z) defining its chromatographic properties (x, y) and m/z-value (z) obtained from MS^1 (Fig. 2c). This representation of data increases the resolution due to separation in three dimensions which is often needed when complex mixtures with a multitude of components are studied. In addition, when using multiple-stage MS in monitoring the second chromatographic step, the obtained values along the z-axis are correlated to the structural elements in each component. Thus different triterpenes in the components could be indicated by the molecular masses of the triterpenes (Fig. 2c), which are calculated from the fragment ions in MS^2 ($[A+Na]^+$–$[B+Na]^+$) (Fig. 3). Alternatively, other structural features such as the C-3 or C-28 oligosaccharides could be presented in three-dimensional plots. Further multiple-stage MS will give similar representations on substructures derived from the oligosaccharide [10,18]. It is important to notice that dependent on the number of components simultaneously eluting into the ion source only a limited number of the components can be selected and analysed in MS^2 depending on instrumental performances.

3.5. Analytical characteristics

The chromatographic procedure was repeated three times on a sample of SPE purified bark extract. The chromatograms obtained in the second step from the same fractions were compared regard-

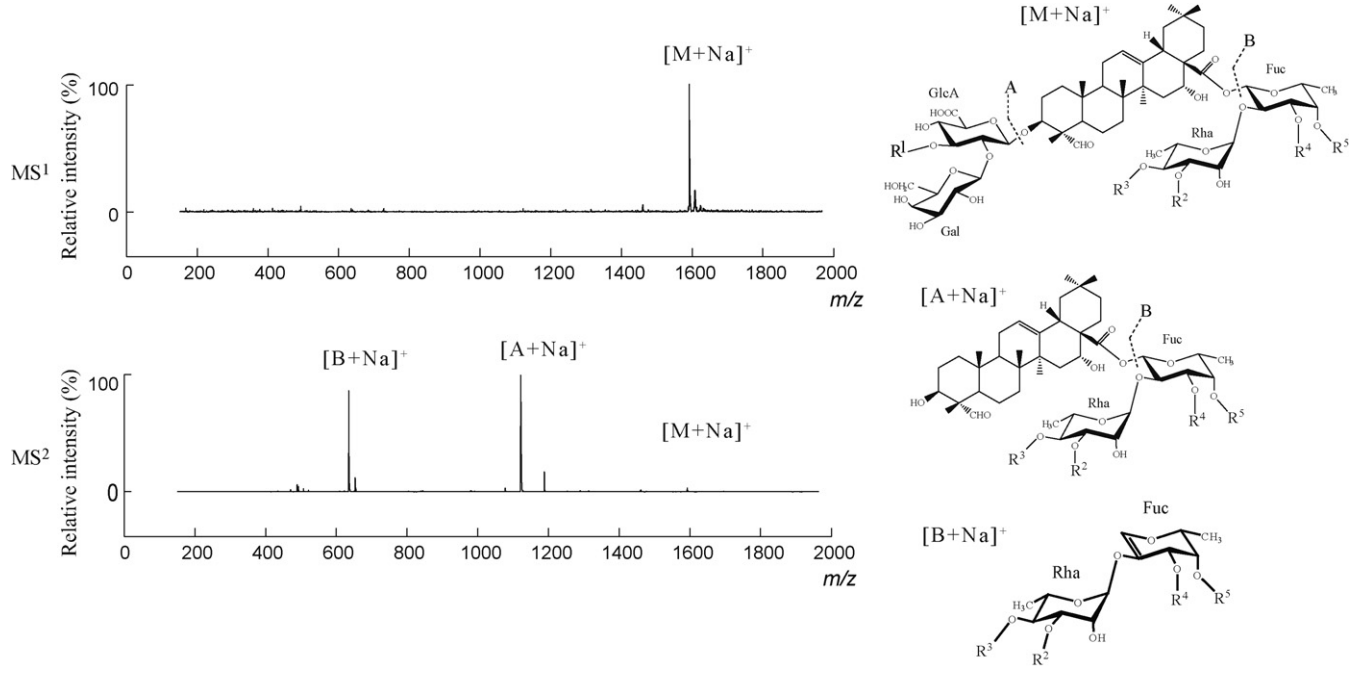

Fig. 3. MS^1 and MS^2 spectra obtained for compound S1. The MS^2 spectra were obtained when the $[M+Na]^+$ ion was isolated and fragmented. Glycosidic cleavages are visualised in the structure resulting in the fragment ions $[A+Na]^+$ and $[B+Na]^+$ in MS^2.

ing retention times and peak areas of saponin components. Each of the three base peak chromatograms from the same fraction contained peaks with similar retention times and areas (Fig. 4). A more careful evaluation of the characteristics performed on a number of components showed a deviation in retention time between runs of less than 0.1 min, corresponding to the time between data points. The calculated %RSD of peak areas from corresponding components was between 1% and 17% for the 20 thoroughly investigated components. Further evaluation of the peaks with high %RSD values showed that the inaccuracy was correlated to splitting of the component into two successive fractions in the first chromatography step. The rather coarse separation in the first step caused some differences in the relative proportions of the component in the two fractions between different runs. However, by adding the peak areas calculated for such components in the chromatograms from both these fractions the %RSD value decreased to <10% with an overall average of 5%.

3.6. Application of the method on the chromatographic fractions QH-B and QH-C

Two chromatographic fractions, QH-B and QH-C, used in vaccine production [3,20] were analysed with the described chromatographic method. Each of the chromatographic fractions were analysed separately and the combined results are presented in a three-dimensional plot (Fig. 5). The QH-B components have a slightly higher mobility in both chromatographic steps but most of the components in the two fractions overlap, although these chromatographic fractions have been obtained by reversed phase chromatography as two well-defined fractions. This depends on the migration of the fatty acyl group between fucose O-3 and O-4 generating two compounds with different chromatographic mobility. This migration has occurred after the preparative fractionation and it causes problems in the separation of *Quillaja* saponins as, in each separation step, two components will occur from both the fucose O-3 and O-4 substituted compounds. Thus both compounds will be present in each fraction or separation in spite they were completely separated in previous steps.

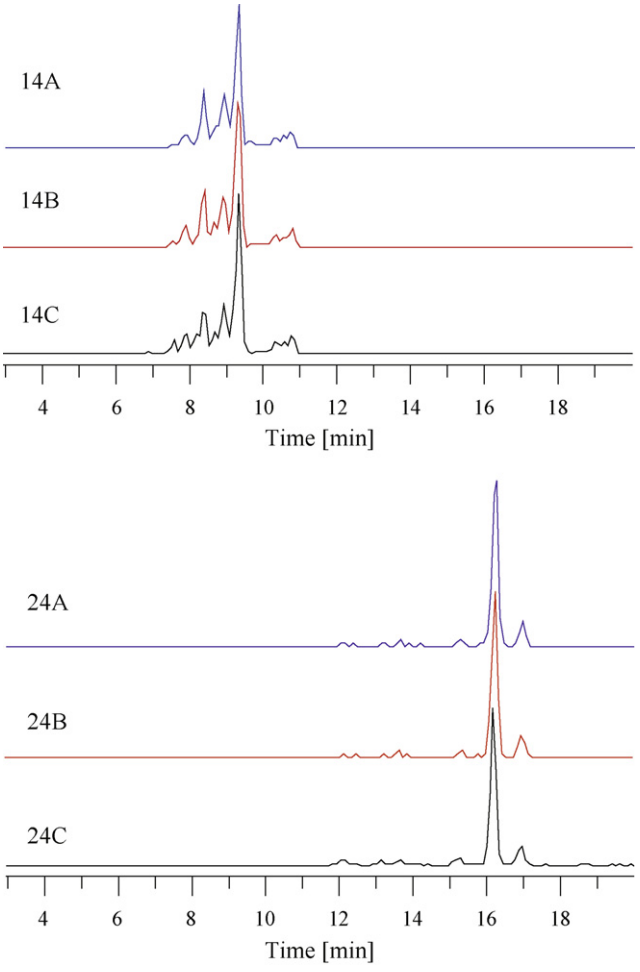

Fig. 4. To obtain reproducibility data a sample (40 μl) of the SPE purified material was injected on the Kromasil column and 1.5 min fractions were collected. These fractions were then injected on the Ascentis® Express column for a second separation and monitored by ESI-IT-MS^n and base peak chromatograms (MS^1) or extracted ion chromatograms were evaluated. The illustrated chromatograms are base peak chromatograms obtained from fractions collected between 20.5 and 22 min (fraction 14) and 35.5 and 37 min (fraction 24). The procedure was repeated yielding in total three sets of fractions (a–c).

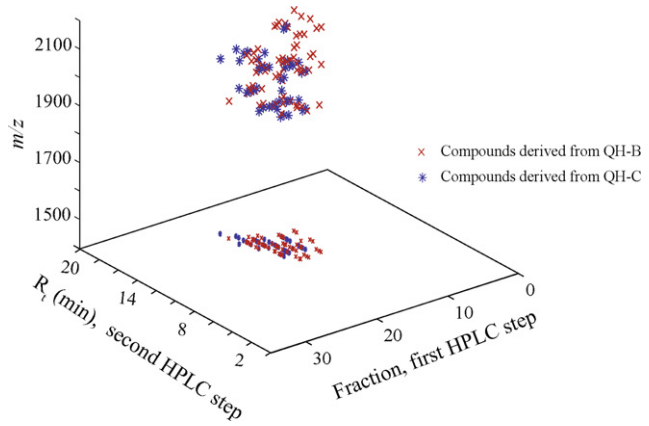

Fig. 5. A three-dimensional map with retention times along two axes and MS^2 data along the third axis on the separated components from QH-B and QH-C.

When comparing the three-dimensional plots with the separation results of the complete bark extract (Fig. 2c) with that of the chromatographic fractions QH-B and QH-C (Fig. 5) the components from the latter two fractions are observed in a region free from saponins with other aglycones than quillaic acid. Thus analysis of the three-dimensional plot is valuable when compounds with certain structures or structural elements should be selected and isolated from a mixture or the composition of compounds in several chromatographic fractions should be compared.

4. Conclusion

The general purpose of this study is to describe an HPLC-MS method suitable for fingerprinting complex mixtures. The method should also give information on structural elements in the components and visualise them according to these.

This method was carried out on a bark extract from *Q. saponaria* Molina and two chromatographic fractions, QH-B and QH-C, with selected structures. As the components in the bark extract have very similar structures and thus similar chromatographic properties the extract is a good test concept. The investigation described here provides a chromatographic system useful for analysis of complex mixtures and metabolic fingerprinting. Furthermore, combination of chromatographic and multiple-stage mass spectrometric data gave a resolution feasible to discern several hundreds of saponin components, and in the proposed method each component obtains coordinates derived from the two chromatographic steps (x, y) and the different mass spectroscopic data (z_n). To interpret the complex mixture of saponins and to visually compare different mixtures three-dimensional plots were applied using the three coordinates to mark each compound in the plot that in combination with sign-coding, *e.g.* type of triterpene, proved very useful (Figs. 2c and

5). The method achieves a higher number of discerned saponin components from a bark extract compared to previously reported methods [6,9] and it also gives structural information on each component.

The reported study is a further step towards metabolic profiling based on LC–MS. As precision is of importance for these analyses, utilising chromatography for identification, the reproducibility shown by the three replicate runs allows for use of the method in metabolic studies.

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