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Characterization of quillaja bark extracts and evaluation of their purity using liquid chromatography–high resolution mass spectrometry

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ABSTRACT

In the course of development of semi-preparative liquid chromatographic methods for the isolation of individual quillaja saponins from *Quillaja saponaria* (L.), some commercially available quillaja bark extracts revealed a distinctive and characteristic pattern of additional peaks in the chromatogram that could not be attributed to saponins commonly present in quillaja. To identify these peaks, analytical procedures based on HPLC coupled with high resolution MS detection were optimized which allowed the identification of the additional saponins Mi saponin A, Mi saponin B, Mi saponin C, madhucoside A and madhucoside B. These compounds are known to be the main saponins of the Indian plant *Madhuca longifolia* (L.). Tandem MS experiments were performed for the unambiguous assignment of the sapogenin. Madhuca saponins yielded a characteristic fragment of protobassic acid, whereas quillaja saponins showed a fragment of quillaic acid as expected. In addition, samples from madhuca seed kernels were analysed to verify the origin of the characteristic chromatographic peak pattern observed frequently in commercially available quillaja bark extracts.

1. Introduction

Quillaja saponaria (L.) is an important saponin-containing plant, which is utilized in considerable quantities for various purposes. Extracts have been used since ancient times as soap, detergent, and in traditional medicines as an expectorant. Nowadays it is mostly employed industrially as a natural foaming agent in food and beverages (Rigano et al., 2009), as a supplement in the feed industry (Makkar and Becker, 2000; Holtshausen et al., 2009) and as an active surfactant in cosmetics. Highly refined quillaja bark is applied as an adjuvant for vaccines in veterinary medicine. Clinical studies have also revealed positive effects of these compounds on the immune response in humans (Kensil et al., 1996; Boyaka et al., 2001; Kirk et al., 2004; Sun et al., 2009).

Extracts of quillaja are very complex mixtures of saponins, and their actual saponin content is typically estimated by determination of the sapogenin content after hydrolysis or the assessment of the foam capability as a result of the surfactant properties of the saponins. This may not be adequate since similar foam levels can be obtained by blending quillaja extracts with low cost saponin sources, e.g. *Yucca schidigera* (L.) as reported by San Martin and Briones (2000). When chromatographic methods are used for quality control, fingerprints are compared and the saponin concentration of the sample can be estimated by comparing the area of saponin peaks of a sample to that of a highly purified quillaja bark extract (Kensil et al., 1991; San Martin and Briones, 2000). The precise quantification of individual components is routinely not done because most of the quillaja compounds are not available as pure standards.

To overcome this issue, we started the development of semi-preparative HPLC methods for the isolation of individual quillaja saponins. In the course of this activity it was observed that commercially available extracts of quillaja bark from different manufacturers (including different batches from different years), showed an additional distinctive and characteristic peak pattern in the chromatogram compared to extracts from quillaja bark prepared in our lab. The present work demonstrates the optimization of HPLC coupled to high resolution MS for structure elucidation of these unknown components in commercial samples. The benefits of HPLC/MS for quillaja saponin analysis have already been demonstrated in previous works (Kite et al., 2004; Bankefors et al., 2008), so that it seems to be an attractive approach for

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analysis of not yet identified components of commercial quillaja bark extracts.

2. Results and discussion

Commercially available extracts of quillaja bark that were analysed by HPLC showed a distinctive and characteristic chromatographic peak pattern within the retention time range of approximately 15–24 min, whereas samples of crushed quillaja bark exhibited no significant peaks in this retention range. Fig. 1 shows the basepeak chromatograms of some commercially available quillaja bark extracts (A–E) and of two extracts prepared in our lab from two samples of crushed quillaja bark of different origin (F–G). The commercial extracts show intense peaks (1, 2a, 2b) in the retention range of 15–24 min that are not related to quillaja saponins. The typical chromatographic peak pattern of quillaja bark saponins starts approximately at 25 min and shows numerous peaks up to 45 min. Quillaja bark components, QS 7, QS 17, QS 18, QS 21 as reported in literatures (Kensil, 2001; Pham et al., 2006) were identified as peaks of significant height in the commercially available extracts as well as in the crushed quillaja bark samples. The chromatographic peak patterns along with MS detection clearly shows that the commercial extracts indeed contain quillaja saponins but are mixed with components of different origin. Table 1 lists retention times and chemical formulas of typical quillaja saponins, and compares the calculated mass-to-charge ratios (m/z) to the actually measured m/z in negative ionization mode. Both singly and doubly charged species were observed but only the more abundant charge-state signals are listed in Table 1. The mass error is less than 3 ppm in all cases which is sufficient for reliable confirmation.

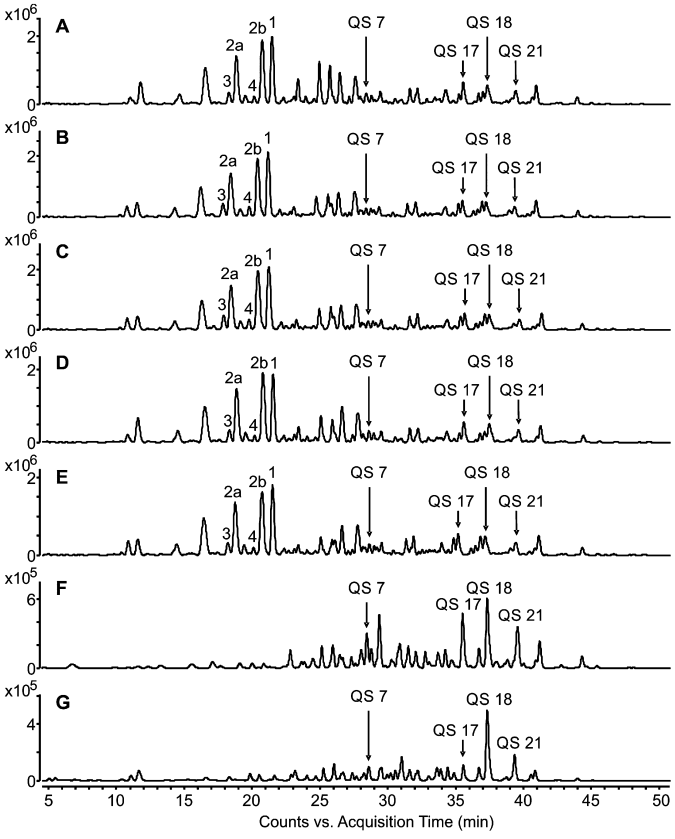

Fig. 1. Chromatograms of commercially available quillaja bark extracts (different suppliers A–E), crushed sample of quillaja bark (F–G) (F, chilenean bark; G, pharmacopoeia quality).

In order to identify the compounds that do not originate from quillaja, MassHunter software was used to calculate molecular formulas for each distinctive peak of the additional characteristic pattern. Subsequently, the Chemical Abstracts database was used to list possible molecules with the calculated molecular formulas.

Almost all of these molecules were of plant origin, namely saponins present in several different plants. It turned out that among these plants only *Madhuca longifolia* (L.) (following the taxonomic concept of Govaerts et al., 2001) belonging to the family Sapotaceae and common in India contained all the saponins identified according to Chemical Abstract.

The two major varieties of *M. longifolia* found in India are *M. longifolia* var. longifolia (L.) and *M. longifolia* var. latifolia (L.). These two are so closely related that no distinction is made in the trade of their fruit or fat. The seed kernels are pressed to obtain the oil, and the press residue (mahua seed cake) can be used as a low cost source for saponins.

In Fig. 1, the identified saponins are labelled as follows: Mi saponin A (1), Mi saponin B (2a, 2b), Mi saponin C (3) and madhucoside B (4) (Kitagawa et al., 1975, 1978; Pawar and Bhutani, 2004). The peaks 2a and 2b have almost identical MS spectra which indicates that they might be isomers. Table 1 lists retention times, molecular formulas and compares the calculated m/z to the actually measured mass m/z for madhuca saponins. Single charged molecular ions $[M-H]^-$ were detected in the negative ionization mode for each identified madhuca saponin. In addition to the peaks labelled in Fig. 1, madhucoside A was identified at a retention time of 19.6 min, but its intensity was very small.

To ensure that the peaks 1–5 belong to madhuca saponins, tandem MS (MS^2) experiments were carried out selecting the deprotonated molecular ion as precursor. Protobassic acid, the sapogenin of all madhuca saponins measured in this work, was clearly detected as one of the fragments (m/z 503.3378) in each MS^2 spectrum for these five peaks, whereas quillaja saponins show quillaic acid (m/z 485.3272) as a characteristic fragment. Fig. 2 presents MS^2 spectra of Mi saponin A and QS 21 originating from the singly deprotonated molecular ion selected as precursor. Besides the characteristic sapogenin fragments for madhuca saponins (protobassic acid) and for quillaja saponins (quillaic acid) various other fragments can be observed as expected.

Finally, the correct assignment of signals to madhuca saponins in commercially available quillaja bark extracts was verified by the analysis of samples from the seed kernels and the deoiled seed cake of the genuine madhuca plant. As expected, seed kernels and the seed cake of madhuca showed the characteristic chromatographic pattern of Mi saponin A, B, C and the madhucosides, which is an additional confirmation of the blending of some commercially available quillaja bark extracts with material from madhuca. In contrast, none of these peaks could be detected in quillaja bark samples of pharmacopoeial quality purchased in an Austrian pharmacy or in a sample of quillaja bark provided from a chilenean company. Fig. 3 shows a comparison of chromatograms of a blended commercial quillaja bark extract and a crushed chilenean quillaja bark versus the analysed samples of seed kernels and the press residue (mahua seed cake) of madhuca.

In conclusion, this work demonstrated that HPLC is well suited for separation of saponins present in different plants. In the example studied here the hyphenation with high resolution MS allows the clear confirmation of identity of different saponins. Therefore these techniques can be used routinely for quality control of quillaja bark extracts. Commercially available extracts are often labelled with the CAS number 8047-15-2, which indicates that the product consists of saponins without an explicit reference to the plant source. Therefore mixing saponins from quillaja and madhuca does not result in a product that would contradict the CAS number. On the other hand, some commercially available