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Combination of matrix solid-phase dispersion extraction and direct on-line liquid chromatography–nuclear magnetic resonance spectroscopy–tandem mass spectrometry as a new efficient approach for the rapid screening of natural products: Application to the total asterosaponin fraction of the starfish *Asterias rubens*

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Abstract

A combination of matrix solid-phase dispersion extraction (MSPD) and LC–NMR–MS hyphenation is proposed as a rapid screening method of natural products for unknown compounds. In this report, this new analytical approach is applied for the first time. MSPD represents a significant simplification compared to classical extraction procedures and is thus an excellent complement to the fast and powerful LC–NMR–MS: MSPD yields extracts suitable for LC–NMR–MS in one simple preparation step, while LC–NMR–MS yields a wealth of information in one single chromatographic run. The suitability of this technique to characterise glycosidic compounds in the molecular mass range of 1200 to 1400 a.m.u. is demonstrated. The information on the number of exchangeable protons provided by an additional back-exchange experiment proved to be particularly valuable for structural elucidation. The possibility of semi-quantitative LC–NMR measurements through methyl signals H_3-18 and 19 of the steroidal skeleton is demonstrated and is ensuingly used to provide relative quantitative data of the steroid oligosaccharide fraction.

Keywords: Starfish; Matrix solid-phase dispersion extraction; Nuclear magnetic resonance spectroscopy; Hyphenated techniques; Natural products; Asterosaponins

1. Introduction

The search for new biologically active compounds from natural sources has been of resurgent interest over the last decade. The vast diversity of chemical structures found in nature still exceeds even that of the largest combinatorial library. Thus there is an increasing demand for rapid methods for structural elucidation applicable to mixtures [1,2]. Such applications require, on the one hand, improvements in the effectiveness of sample preparation (extraction,

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clean-up and separation) and, on the other hand, the ready and reliable availability of spectroscopic information of individual compounds at the earliest possible stage without necessarily providing the full structural information.

Conventional off-line strategies of sample preparation are usually aimed at obtaining pure compounds from the crude sample, which are then submitted to NMR and MS examinations. That is why the extraction of the crude sample material with organic solvents, often taking several hours, is usually followed by a series of preparation steps such as gel chromatography, counter current chromatography or preparative column chromatography. As natural products often contain a large number of similar and thus difficult to separate compounds, some of which have been identified previously while many constituents are still unknown, the search for new compounds using the classical approach may become very tedious and time-consuming.

Matrix solid-phase dispersion (MSPD) is a sample preparation technique that combines both sample homogenisation and extraction of compounds of interest in one single step starting from the intact sample material [3–5]. Thus, it simplifies the extraction and clean-up steps, reduces the sample manipulation and is much faster than conventional techniques. It should therefore be very useful for a rough separation of extracts into classes of compounds of similar polarities. Even though it is most widely used for extraction of residues from biological samples, its suitability for separation of natural products has been reported before [5] and it has been proposed recently in connection with LC–NMR [6].

LC–NMR hyphenation [7] combines a separation step with the acquisition of spectroscopic data of individual compounds. In the past, this technique has been used for speeding up the structural elucidation of compounds in more or less complex mixtures. Several applications have been reported in the field of natural product analysis [7–21], but the great value of this technique for a systematic screening of compound classes has been recognised only recently [22–25].

The addition of a mass spectrometer to an existing LC–NMR set-up is a further important step in the progress of hyphenated techniques. It leads to a comprehensive analytical system providing the complementary information of both NMR and MS in a single chromatographic run. Since the first reports on LC–NMR–MS in 1995 [26,27], a number of further contributions have been made to pharmaceutical problems [28] but there have been few reports on natural product analysis [29–31].

Very recently, an LC–NMR–MS method to characterise subfractions of toxic glycosidic constituents of starfish (asterosaponins) which were not sufficiently separated by classical preparative column chromatography has been developed by us [25].

Asterosaponins are $\Delta^{9(11)}$-3β,6α-dioxygenated steroids with a sulfate group attached at C-3 and an oligosaccharide chain containing five or six sugar units at C-6. Several pharmacological properties are reported in literature such as: hemolytic activity, in vitro cytotoxicity towards tumour cells, antiviral activity as well as anti-inflammatory and analgesic properties [32]. Individual representatives of this homogeneous class differ in their steroidal sidechain or their sugar moiety, while the steroidal nucleus is common to all asterosaponins. Approximately 100 different combinations of sugar moiety and sidechain are described in the literature [32,33].

A chemical screening by LC–NMR–MS allows the recognition of novel asterosaponins at an early stage of the analytical process. In this way candidates justifying further preparative isolation and a detailed and comprehensive NMR examination can be reliably selected in order to avoid re-isolation of known compounds.

However, the time-consuming classical extraction and separation techniques initially used contrast to some extent with the fast and powerful LC–NMR–MS screening approach [25]. Furthermore, there is no need for a preparative scale work-up of starfish specimens, as LC–NMR–MS screening requires only sample amounts in the 1–2 mg range. The sample preparation should therefore be substituted by faster small-scale extraction procedures, such as MSPD.

Since the final separation of asterosaponins takes place on the analytical HPLC column of the LC–NMR–MS system, we looked for a rapid extraction procedure that provides a fraction containing all closely related asterosaponins, but which at the same time is clean enough to be directly submitted to LC–NMR–MS examination.

MSPD promises to complement our approach of a

rapid structure guided screening: while LC–NMR–MS speeds up the separation and structural elucidation of unknown compounds, MSPD simplifies the extraction and clean-up steps.

Therefore, it is the objective of this paper to demonstrate that MSPD can provide extracts of natural products which are suitable for direct LC–NMR–MS examinations and to illustrate the efficiency of the on-flow LC–NMR–MS approach in terms of an adequate separation of closely related compounds as well as the quality of spectroscopic information. Furthermore this is the first time that a total asterosaponin fraction rather than subfractions is submitted to LC–NMR–MS analysis.

Another objective of the study is to provide information on the relative amounts of asterosaponins in the sample, which is difficult to obtain by classical methods since the signal response in UV- or mass spectrometric detection depends strongly on the chemical nature of the compounds and may vary considerably between isomers. NMR spectroscopy offers a universal method of detection, depending only on the relaxation behaviour of the nuclei under study. It has been shown that LC–NMR allows the quantification of compounds for which no reference standard is available [34].

2. Experimental

2.1. Animal material

Specimens of *Asterias rubens* (approx. 6 cm in diameter) were collected in Fredericia, Denmark in Summer 1998 and were deep-frozen until examination.

2.2. MSPD

Intact specimens of the starfish (5 g) were cut into pieces of approx. 3 mm and mixed with 20 ml water and 9 g RP-18 material (LiChroprep RP-18, 25–40 μm; Merck, Darmstadt, Germany). The mixture was then ground in a mortar for 5 min until a homogenous paste was obtained. The blend was transferred into a column (2 cm I.D.) filled with a bed of another 9 g RP-18 material and covered with a piece of filter paper. After washing the slurry with 100 ml water, compounds were eluted with increasing amounts of acetonitrile. Elution was assisted by a slight excess pressure of nitrogen. Subsequently, the fractions were evaporated to dryness under a stream of nitrogen. The fraction containing the asterosaponins (10 ml) eluted at a composition of H_2O/CH_3CN 50:50, v/v. From 5 g starfish material, 11 mg of the asterosaponin fraction were obtained. At 0.2% the yield of the fraction containing the asterosaponins is higher than that obtained using our initial extraction procedure (0.1%) [25].

The separation was monitored by thin layer chromatography (Alugram RP-18 W from Macherey u. Nagel (Dueren, Germany); H_2O/CH_3CN (50:50, v/v); detection: 5% H_2SO_4 in MeOH; R_F values of asterosaponins: 0.78, 0.69–0.63).

2.3. LC–NMR–MS hyphenation

The LC system consisted of a Bruker Saxonia LC-22 pump (Bruker, Leipzig, Germany), a Rheodyne 7725 i injection valve (Cotati, USA), a Bischoff Lambda-1010-UV-detector (Bischoff, Leonberg, Germany) at 210 nm and a Bruker BPSU-36 peak sampling unit. At the outlet of the BPSU, an LC-Packings ICP-4-20 flow splitter (LC-Packings, Amsterdam, The Netherlands) was attached, which allowed the transfer of 95% of the flow to the NMR spectrometer and 5% to the MS. Water was added to the MS flow in the ratio of 4:1 via a PEEK T-piece and a syringe pump for D–H-back-exchange experiments. Due to the residual D_2O in the eluent, the most abundant peak in the D–H-back-exchange experiments was $[M+2D]^-$, however $[M]^-$ was clearly distinguishable in all cases. A YMC J'sphere ODS L80 HPLC column (4.6×250 mm, YMC, Schermbeck, Germany) was used with 20 mmol ammonium formate in D_2O/CH_3CN (65.5:34.5, v/v) as eluent. The eluent flow was 0.05 ml/min for on-flow LC–NMR–MS experiments and 0.5 ml/min for the (MS triggered) stopped-flow experiments.

A 50 mg/ml solution of the sample in the eluent was injected using a 20 μl injection loop after filtration through an Eppendorf pipette tip stuffed with cotton wool (corresponding to an absolute amount of 1 mg injected onto the column).

A Bruker Avance DRX 600 spectrometer (Bruker, Rheinstetten, Germany) equipped with a 4 mm *z*-gradient-LC probe head (active detection volume 120 μl) was used. On-flow spectra were recorded using

the following conditions: ^{1}H frequency, 600.13 MHz; temperature, 300 K; sweep width, 8400 Hz; 16 k data points, zero filled to 32 k; exponential multiplication LB 1 Hz; 108 scans per row, pulse repetition time 2 s. Solvent suppression was achieved by the WET sequence with ^{13}C decoupling during the WET pulse train. Chemical shifts were referenced to acetonitrile which was set to 2.00 ppm. To extract 1D ^{1}H-NMR spectra from the on-flow NMR chromatogram, up to four rows of a peak were summed up. Integration of the methyl signals was performed using the standard 2D integration routine of the Bruker XWinNMR software package. 2D WET-TOCSY spectra were recorded in stopped-flow mode. ^{13}C decoupling was applied during the WET pulse train and acquisition. Spectra size was 4 k× 512 data points, zero-filled to 4 k×2 k data points. TOCSY mixing time was 20 and 100 ms, respectively.

For relaxation experiments, a solution of 1 mg forbeside B in 1 ml D_2O/CH_3CN (65.5:34.5, v/v) was injected into the probehead and ^{1}H spectra with varying relaxation delays D1 were recorded employing WET solvent suppression. The number of scans was 24; number of dummy scans 8.

The mass spectrometer was a Bruker Esquire-LC ion trap mass spectrometer from Bruker Daltonics, Bremen, Germany, equipped with an ESI ionisation source. Ionisation parameters were as follows: negative ion mode; capillary voltage 3150 V, end plate voltage 2350 V; dry gas was 11 l/min nitrogen at 300°C; nebulising gas was nitrogen at 30 p.s.i.. Scan from m/z 50 to 1600; accumulation cut-off m/z 85; 125 to 150 averages per spectrum in on-flow LC–NMR–MS mode; 20 averages in stopped-flow mode. MS/MS spectra were recorded in auto-MS/MS mode. The fragmentation amplitude was set to 2 V.

2.4. Reagents

Acetonitrile (quality HPLC ultra gradient grade) was purchased from Baker (Deventer, The Netherlands) and (quality NMR Chromasolv) from Riedel-de-Haen (Seelze, Germany); deuterium oxide (99.9 atom%) was obtained from Deutero GmbH (Kastellaun, Germany); water for HPLC purposes was prepared using a Milli-Q purification system from Millipore (Milford, CT). Acetonitrile-*d3* (99.8 atom%) and deuterium oxide (100 atom%) were purchased from Euriso-top (Gif-sur-Yvette, France). All other chemicals and reagents were of analytical grade or equivalent.

2.5. LC–NMR data of 4 and 8

4: ^{1}H-NMR spectrum of the sugar moiety identical to ovarian asterosaponin 1 (**3**). ^{1}H-NMR (D_2O/NH_4 formate/acetonitrile): 5.31 ppm (br d, A-H-11), 5.00 (d, J=9.1 Hz, A-H-24), 4.60 (d, 7.7 Hz, Qui II-H-1), 4.56 (d, 7.7 Hz, Qui I-H-1), 4.47 (d, 7.7 Hz, Fuc I-H-1), 4.47 (d, 7.7 Hz, Fuc I-H-1), 4.44 (d, 7.2 Hz, DXU-H-1), 4.37 (m, A-H-23[1]), 4.13 (m, A-H-3), 1.68 (s, A-H-27), 1.64 (s, A-H-26), 1.49 (A-H-20[1]), 1.49/1.24 (A-H_2-22[1]), 1.35 (d, 6.1 Hz, Qui I-H_3-6), 1.25 (d, 6.1 Hz, Qui II-H_3-6), 1.18 (d, 6.7 Hz, Fuc I-H_3-6), 1.16 (d, 6.7 Hz, Fuc II-H_3-6),1.14 (d, 6.7 Hz, DXU-H_3-6), 0.93 (s, A-H_3-19), 0.89 (d, 5.1 Hz A-H_3-21), 0.54 (s, A-H_3-18).

8: ^{1}H-NMR spectrum of the sugar moiety superimposable with ovarian asterosaponin 1 (**3**); steroidal sidechain methyl resonances identical to those of asteroside C (**6**). ^{1}H-NMR (D_2O/NH_4 formate/AcCN): 5.31 ppm (br d, A-H-11), 4.60 (d, J=7.7 Hz, Qui II-H-1), 4.56 (d, 7.7 Hz, Qui I-H-1), 4.47 (d, 7.7 Hz, Fuc I-H-1), 4.47 (d, 7.7 Hz, Fuc I-H-1), 4.44 (d, 7.2 Hz, DXU-H-1), 4.12 (m, A-H-3), 1.35 (d, 6.1 Hz, Qui I-H_3-6), 1.25 (d, 6.1 Hz, Qui II-H_3-6), 1.18 (d, 6.7 Hz, Fuc I-H_3-6), 1.16 (d, 6.7 Hz, Fuc II-H_3-6),1.14 (d, 6.7 Hz, DXU-H_3-6), 1.28 (s, A-H_3-21), 0.92 (s, A-H_3-19), 0.90 (d, 7.3 Hz, A-H_3-28), 0.86 (d, 7.3 Hz, A-H_3-26), 0.77 (d, 7.3 Hz, A-H_3-27), 0.69 (s, A-H_3-18).

3. Results and discussion

3.1. MSPD

MSPD extraction (see experimental section) represents a significant simplification of the sample work-up. Whereas our initial procedure [25] consisted of a two-fold 16 h acetonitrile extraction, ultrasonic treatment, centrifugation and (two-fold) preparative chro-

[1]Signals assigned by TOCSY experiments.

matographic purification, the MSPD procedure results in an asterosaponin extract suitable for direct LC–NMR–MS examination in one single step. The extraction yield is better than that of the method used originally (see Experimental section). The fraction containing the various asterosaponins, having comparable polarities, reproducibly elutes at a composition of water/acetonitrile (50:50, v/v).

Furthermore, MSPD allows a miniaturisation of the extraction step, complementing the analytical scale LC–NMR–MS hyphenation. Thus it is possible to work up a small sample of animal material employing MSPD and, on the basis of the LC–NMR–MS data obtained, decide whether the sample contains compounds of interest, justifying a larger-scale preparative isolation (see below, compound **14**).

3.2. LC–NMR–MS screening — structural information

Fig. 1 shows the on-flow NMR chromatogram of the total asterosaponin fraction obtained from MSPD extraction (1 mg was injected onto the column). For clarity, only the chemical shift ranges of the methyl resonances and of the anomeric resonances are shown.

As a complete structural elucidation of compounds as complex as asterosaponins cannot be achieved by one-dimensional ^{1}H-NMR provided by on-flow LC–NMR, two approaches have proven to be effective in the case of asterosaponins:

(a) Analysis of easy-to-assign resonances characteristic of certain structural features e.g. olefinic protons, methyl groups or anomeric protons of sugar units and (b) comparison of subspectra of unknowns with those of known compounds or with those of other unknowns to recognise structural analogies or differences.

In the investigated sample we were able to identify 17 asterosaponins (two of which only tentatively) by means of a LC–NMR–MS screening. Table 1 gives an overview of the compounds identified and their MS data. While ruberosides A to F had been isolated earlier from different fractions of *Asterias rubens* and the other given asterosaponins have been described in various starfish, ruberoside G as well as the tentatively assigned compounds **4** and **8** have not been isolated before.

As an example of the valuable information obtained from an on-flow LC–NMR–MS chromatogram, the ready identification of the new compound **14** (ruberoside G) from this MSPD sample shall be discussed in more detail:

In the LC–NMR chromatogram (Fig. 1) the conformity of the sugar methyl signals (1.1–1.4

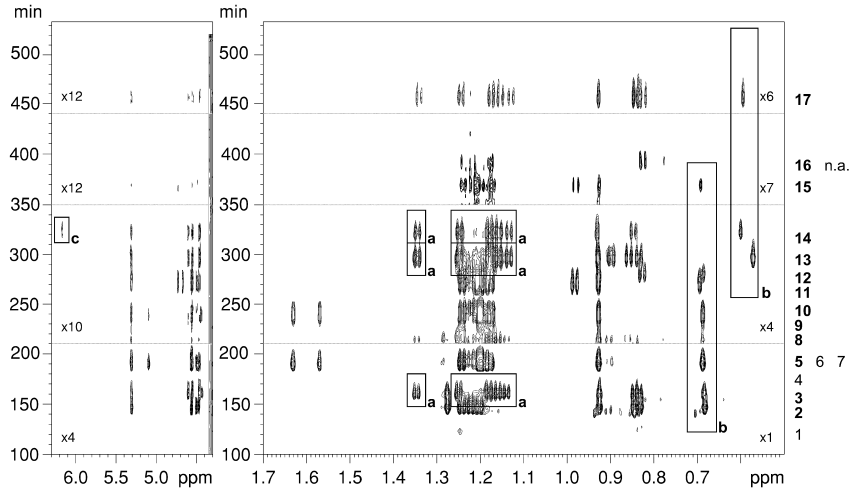


Fig. 1. On-flow LC–NMR chromatogram of the asterosaponin fraction obtained from MSPD extraction. Expansion of the anomeric and the methyl proton resonances. Frames indicate exemplary structural analogies between compounds. Individual segments of the chromatogram were scaled up for clarity. Compounds 1, 4, 6 and 7 only appear at larger scalings. n.a., compound not assigned.

Table 1
Asterosaponins identified from the MSPD extract from *Asterias rubens*[a]

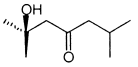
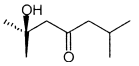
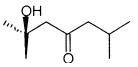
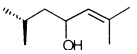
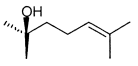
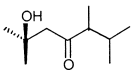
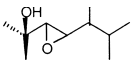
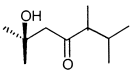
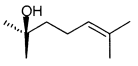
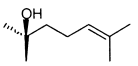
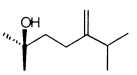
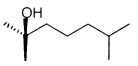
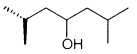
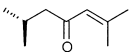
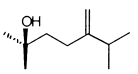
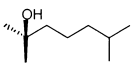
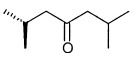
No.	Retention time (min)	m/z[b] (D_2O)	m/z[c] (D–H-back exchange)	MS/MS mass loss	Relative amount[d]	Saccharide chain[e]	Steroidal sidechain	Compound
1	128.5	1421	1405	−101	0.38	G-$_3$F-$_2$G-$_4$X$_{2-3}$Q-Q		versicoside A, forbeside A [32,33]
2	150.7	1256	1243	−101	3.65	Q-$_2$G-$_4$X$_{2-3}$Q-Q		glycoside B_2, forbeside B [32,33]
3	164.0	1270	1257	−20 −101	4.64	F-$_2$F-$_4$Q$_{2-3}$D-Q		ov. asterosaponin 1, forbeside C [32,33]
4[f]	176.8	1254	1241	−20 −148 −148 −20 −2×148 −2×148 −20 −757	0.25	F-$_2$F-$_4$Q$_{2-3}$D-Q		[36]
5	192.7	1388	1373	−165 −148 −148 −148 −134	5.36 sum of **5** and **6**	G-$_3$F-$_2$F-$_4$X$_{2-3}$Q-Q		asteriidoside C [37]
6		1270	1257			Q-$_2$G-$_4$X$_{2-3}$Q-Q		asteroside C [32,33]
7								ruberoside F [36]
8[f]	220.9	1284	1271	−20 −115		F-$_2$F-$_4$Q$_{2-3}$D-Q		
9	240.9	1223	1211	−148 −148 −148 −134	0.95 sum of **9** and **10**	F-$_2$F-$_4$X$_{2-3}$Q-Q		solasteroside A [32,33]
10	251.3	1223	1211	−148 −148 −148 −134		Q-$_2$F-$_4$X$_{2-3}$Q-Q		ruberoside E [36]
11	272.8	1402	1387	−165 −148 −148 −148 −134	0.51	G-$_3$F-$_2$F-$_4$X$_{2-3}$Q-Q		asteriidoside B [37]
12	283.3	1390	1375	−165 −148 −148 −148 −134	0.85[g]	G-$_3$F-$_2$F-$_4$X$_{2-3}$Q-Q		ruberoside C [25]

Table 1. Continued

No.	Retention time (min)	m/z[b] (D_2O)	m/z[c] (D–H-back exchange)	MS/MS mass loss	Relative amount[d]	Saccharide chain[e]	Steroidal sidechain	Compound
13	300.1	1256	1243	−20 −148 −148 −20 −2×148 −2×148 −20 −757	1	F-$_2$F-$_4$Q$_2$-$_3$D-Q		ruberoside A [25]
14	326.3	1251	1239	−20 −148 −148 −20 −2×148 −2×148 −20 −757	0.56	F-$_2$F-$_4$Q$_2$-$_3$D-Q		ruberoside G [38]
15	374.9	1237	1225	−148 −148 −148 −134	0.15	Q-$_2$F-$_4$X$_2$-$_3$Q-Q		ruberoside D [25]
16	399.6	1225	1213	−148 −148	≈0.05	Q-$_2$F-$_4$X$_2$-$_3$Q-Q		[25]
17	463.9	1253	1241	−20 −148 −148 −20 −2×148 −2×148 −20 −757	0.55	F-$_2$F-$_4$Q$_2$-$_3$D-Q		ruberoside B [25]

[a] Q, quinovose; X, xylose; F, fucose; D, deoxy-xylo-hex-ulose; G, galactose.
[b] Molecular anion after H–D-exchange.
[c] Molecular anion after D–H-back-exchange.
[d] Relative to **13**.
[e] Abbreviated representation, all glycosidic linkages are (1→X)-linked.
[f] Compound tentatively assigned.
[g] Contains unresolved minor compound.

ppm, Fig. 1 frames a) as well as the pattern of the anomeric proton resonances suggests that **14** possesses the same oligosaccharide chain as the previously isolated ruberoside A (**13**) [25] or the frequently found ovarian asterosaponin 1 (**3**) [32,33]. Superimposable sugar ring proton signals provide further evidence for this assignment of the oligosaccharide chain (Fig. 2).

Most asterosaponins carry a hydroxy function at C-20. Their H_3-18 methyl signals appear at approximately 0.7 ppm. The distinct upfield shift of the H_3-18 methyl resonance of compound **14** indicates the absence of a hydroxy function at the C-20 position of the steroidal sidechain, as in the previously isolated ruberosides A and B (**13**, **17**) (Fig. 1, frames b). An additional olefinic resonance at low field (6.16 ppm, Fig. 1, frame c and Fig. 2) indicates the presence of a double bond in the steroidal sidechain in proximity to a deshielding functional group such as a carbonyl group. A methyl singlet at 1.85 ppm indicates a methyl group (H_3-26) attached to the double bond, while an expected H_3-27 signal is obscured by the acetonitrile hump in the spectrum. The chemical shifts of these assigned signals are in

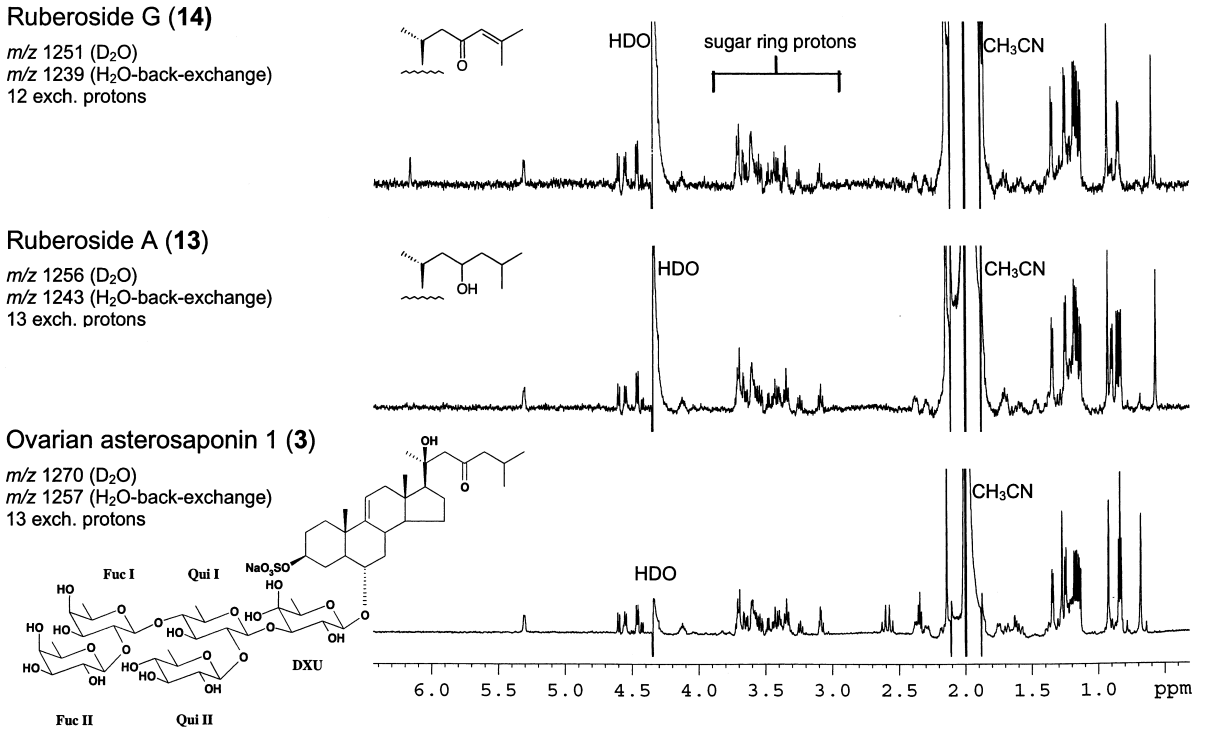


Fig. 2. One-dimensional ^{1}H-NMR spectra of compounds **3**, **13** and **14**, extracted from the on-flow NMR chromatogram. The conformity of the sugar-ring proton signals indicates identical oligosaccharide moieties in all three compounds. The additional olefinic resonance at 6.16 ppm of **14** suggests a double bond in proximity to a carbonyl function. The ESI MS data and information from D–H-back exchange experiments support this assignment.

excellent agreement with ^{1}H-NMR data published for nipoglycoside B [35] indicating a Δ^{24},23-keto-sidechain.

The MS data provided by the LC–NMR–MS run support this assignment:

The molecular ion of **14** after H–D-exchange (see Experimental section) is found to be *m*/*z* 1251, i.e. five mass units less than that of the similar ruberoside A (**13**), which elutes directly prior to **14**. The loss of two mass units corresponds to the additional double bond in the sidechain of **14**, while the remaining difference of three units is in conformity with the exchange of a hydroxy function for a carbonyl function in the sidechain of **14**. This information from the LC–NMR–MS experiment is of particular advantage when the experiment is performed in eluents containing deuterium oxide (see Experimental section): it allows the further differentiation of hydrogen equivalents by means of the analysis of exchangeable protons. The number of exchangeable protons in both compounds, obtained from the D–H-back-exchange experiment is in conformity with the assignment made above (**14**: 12 exchangeable protons; **13**: 13 exchangeable protons; see Fig. 2 and Table 1).

As the LC–NMR–MS analysis indicated compound **14** to be a new asterosaponin not yet described in literature, it was preparatively isolated by RP–HPLC and submitted to two-dimensional NMR examinations. The statements made by LC–NMR–MS were confirmed without exception establishing the structure shown in Table 1. The detailed examination together with ^{1}H and ^{13}C-NMR data will be presented elsewhere [38].

In an analogue manner two novel minor compounds (**4**, not shown in Fig. 1, visible only in enlarged scaling of the contour plot, and **8**) with an oligosaccharide chain identical to that of ruberoside

G were identified in the sample by signal comparison. While the steroidal sidechain of **8** could also be assigned by signal comparison (sidechain resonances identical to those of asteroside C, **6**) two additional stopped-flow 2D TOCSY experiments, one with a short mixing time of 20 ms, one with 100 ms, were necessary in the case of **4**.

These spectra revealed the connectivity of the olefinic sidechain resonance H-24 at 5.00 ppm to a resonance at 4.37 ppm (H_3-23) and to two upfield methyl singlets at 1.68 and 1.64 ppm (H_3-26/27). The spin system further extends via signals at 1.49 and 1.24 ppm to the H_3-21 methyl doublet at 0.89 ppm. This indicates the presence of a Δ^{24},23-hydroxy-sidechain.

Compounds **4** and **8** were tentatively assigned the structures indicated in Table 1 as preparative RP–HPLC yielded not enough sample to obtain ^{13}C-NMR data [38]. ^{1}H-NMR data are given in the Experimental section.

3.3. Advantages of the on-flow LC–NMR approach

The on-flow screening approach provides not only a straightforward overview of the sample — but even signals of partial coeluting compounds can be assigned to individual compounds, which is much more difficult in spectra obtained under stopped-flow conditions or in off-line NMR spectra.

Furthermore, minor chemical shift differences (e.g. H_3-18 signals of compounds **11** and **12** in Fig. 1) can be reliably recognised under isocratic on-flow conditions, since each spectrum is recorded under identical conditions.

The on-flow LC–NMR–MS experiments are performed as overnight experiments at an eluent flow-rate of 0.05 ml/min. At this low flow-rate a broad van-Deemter minimum is found, as shown for compound **15** in a preliminary test. The eluted peak volume (peak width at half height×flow-rate), which is crucial for the sensitivity, decreases from 400 μl at 0.8 ml/min over 360 μl (0.4 ml/min) to 280 μl at 0.1 and 0.05 ml/min. No deterioration of the peak shape was observed at low flow-rates using isocratic conditions. However, we experienced deteriorations of the peak shape recorded in the NMR flow cell when employing gradient elution, for unknown reasons.

3.4. LC–NMR–MS double hyphenation: structural information

The complementary structural information of both NMR and MS detection proved to be particularly valuable in the case of complex glycosidic compounds, such as asterosaponins: Fig. 3 shows the ESI-mass spectra of compounds **9** and **10** as an example. Both compounds have the molecular mass m/z 1223 (after H–D exchange) and show identical MS/MS fragments at m/z 1075, 927, 779 and 645, corresponding to the sequential loss of three methyl pentoses and one pentose. Nevertheless mass spectrometry yields the information that the two compounds differ from one another, since the intensities in the MS/MS spectra, recorded under identical conditions, are different. However, for further information on the two isomers, NMR data are needed:

Comparison of sidechain methyl resonances from the NMR chromatogram readily indicates that both compounds possess the same steroidal sidechain as the recently isolated asteriidoside C [37], while analysis of the sugar resonances indicates that the oligosaccharide chain of **10** is identical to that of **15** and **16** and thus an asterosaponin not previously isolated. Preparative isolation and extensive two-dimensional NMR examination resulted in corroboration of the structure of **10** deduced from LC–NMR, (ruberoside E) [36]. Compound **9** was identified as solasteroside A [32,33]. Thus both compounds differ only in the terminal sugar unit of the oligosaccharide sidechain: fucose in **9** and quinovose in **10**, i.e. in the arrangement of the hydroxy group at C-4 of the terminal sugar unit.

3.5. Semi-quantitative analysis

To acquire a deeper insight into secondary metabolism, the quantitative distribution of individual compounds within a class of closely related compounds is also of interest.

Since the suitability of NMR as a detection method for quantification depends strongly on the relaxation properties of the nuclei of interest, a curve of signal integrals vs. pulse repetition time has been recorded (Fig. 4) of the common asterosaponin forbeside B. It shows clearly that the steroidal methyl signals H_3-18 and H_3-19 (not shown) are

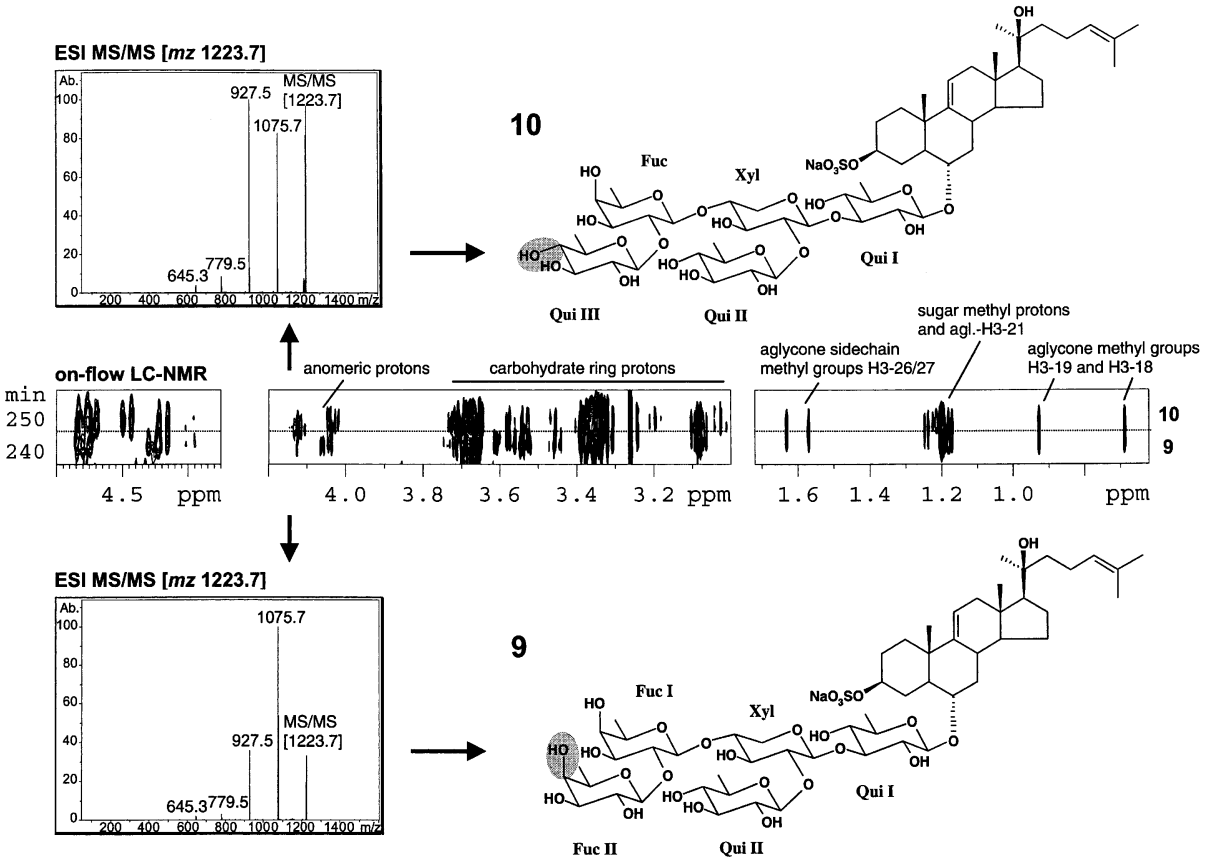


Fig. 3. The MS/MS spectra of **9** and **10**. Both MS/MS spectra show identical mass fragments. The corresponding expansion of the LC–NMR chromatogram indicates that both compounds show the same steroidal sidechain resonances, but differ in their carbohydrate signals. Thus, **10** was readily identified as novel asterosaponin ruberoside E. It differs from **8** only in the arrangement of the hydroxy group at C-4 of the terminal sugar unit.

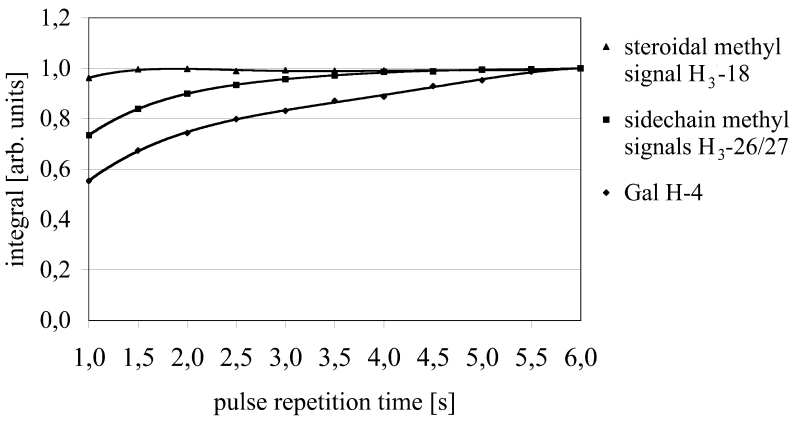


Fig. 4. Integrals (arbitrary units) of selected signals of an asterosaponin (forbeside B) as a function of the pulse repetition time.

completely relaxed, beginning at a pulse repetition time of about 1.5 s, while the sidechain methyl proton signals reach their maximum intensity at ca. 4 s and the sugar proton Gal-H-4 has not reached its full integral at 6 s. Thus the integrals of the steroidal methyl signals can be used for quantification, as the pulse repetition time in the on-flow experiments was 2 s. Due to the rigid arrangement in the steroidal skeleton, these fast relaxing signals represent a probe well suited for LC–NMR quantification as most other proton resonances show longer relaxation times [34]. However, since the spectral dispersion of H_3-18 resonances may be small, coeluting signals may not be distinguished, but when the H_3-18 resonances are spectrally dispersed, quantification of new compounds can be achieved even without standard compounds available [34].

Generally, there are two possibilities to perform quantitative measurements in LC–NMR: by adding a standard to the eluent or by injecting a standard substance together with the sample onto the column. However, small organic molecules added to the eluent usually relax more slowly than the steroidal methyl groups and are thus not suited as a quantification standard. That is why the addition of an internal standard to the sample appears to be the method of choice.

As no asterosaponin of known content is commercially available, and as this method is subject to further cross-validation, we so far present relative semi-quantitative data in Table 1. However, we believe that the potential of on-flow LC–NMR for quantitative measurements has been demonstrated.

3.6. LC–NMR–MS double hyphenation: further advantages

Apart from the gain in time and sample efficiency, the most important advantage of the double-hyphenated LC–NMR–MS set-up over stand-alone LC–MS and LC–NMR set-ups is according to our experience the unequivocal assignment of the MS data to the NMR peaks as LC–MS and LC–NMR chromatograms of the same sample are sometimes difficult to correlate because slightly different chromatograms are obtained in the two systems. This may be due to effects arising from the higher amounts of injected sample in LC–NMR, the different chromatographic behaviour of deuterium oxide compared to water or different eluent gradient-forming units in the chromatographic systems.

The possibility of MS triggering the stopped-flow experiments permits the reliable detection of peaks of interest and even provides an additional feature: it is now possible to monitor the peak purity as a triggering criterion instead of just the peak maximum in conventional UV-triggered experiments.

D–H-back-exchange experiments may also be performed in conventional LC–MS set-ups but in LC–NMR–MS they are self-evident. The relevance of this information has been shown.

4. Conclusion

The combination of MSPD extraction with on-flow LC–NMR–MS is a powerful approach for a rapid chemical screening of natural products, as shown here for a class of closely related glycosidic compounds. Within 2 days of work, an overview of the composition of the asterosaponin fraction was obtained, starting from the intact animal material — while with the initial procedure approx. 4 days were needed. Furthermore, the small scale of the work-up allows an examination of the asterosaponin distribution of individual starfish specimens e.g. to compare individuals of different subspecies, sex or environmental growth conditions.

As LC–NMR–MS information is used to guide further preparative work, it largely facilitates the identification of unknown compounds in the presence of known compounds and thus prevents unnecessary re-isolation. LC–MS data alone would not yield sufficient information due to identical molecular mass and MS^2 fragmentation patterns of asterosaponins.

Relative quantitative data obtained by LC–NMR provide representative information on the composition of a sample and thus complement other methods of quantification in cases where no standard compounds are available (which is often the case in natural product analysis in search of new compounds).

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