Two-dimensional qNMR of anthraquinones in *Frangula alnus* (*Rhamnus frangula*) using surrogate standards and delay time adaption

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ABSTRACT

In the absence of adequate reference material, a problem often encountered in natural product chemistry, we investigated the use of surrogate standards in two-dimensional qNMR for the quantification of anthraquinones in the bark of alder buckthorn (*Frangula alnus*). Using the integrals of cross signals in the HSQC spectrum obtained from commercial standards rutin and duroquinone and adapting the delays for the ^{1}JCH coupling, we quantified the total amount of anthraquinones and anthraquinone glucosides, as well as the content of the value-determining glucofrangulins and frangulins. Thereby, duroquinone was used as an external standard to establish the calibration curve for the methylated anthraquinone scaffold, whereas calibration curves for the glycosides were obtained using the anomeric proton signals of the rutinose disaccharide. The method was validated for accuracy, precision, specificity, linearity and limit of quantitation and shows clear advantages over the method of the European Pharmacopeia, especially in terms of specificity and meaningfulness of the results. Apart from being a useful alternative in the quality control of alder buckthorn, the presented approach demonstrates, moreover,the versatility of sophisticated 2D measurements in quantitative NMR.

Keywords: rutin, duroquinone, quality control, natural product, quantitative NMR, surrogate analytes

1. Introduction

For the quality control of drugs derived from natural products, e.g. plant extracts or herbal preparations, both the detection of possible adulterations as well as the standardization to the amount of active ingredients are of utmost importance. However, in natural products chemistry, reference standards for quantification often are not commercially available or difficult to obtain, due to their low amount in the respective plant material, their low stability in pure/isolated form or the abundance of structurally similar components. One way to overcome this problem is the use of surrogate standards with similar physicochemical properties with respect to the analytical technique, e.g. similar UV absorption for spectrophotometric methods [1, 2], or the use of deuterated reference compounds in mass spectrometry [3, 4]. In the present study, we investigated the use of surrogate standards in two-dimensional NMR, quantifying the active principles in the bark of *Frangula alnus* Mill. (syn. *Rhamnus frangula* L.), Rhamnaceae.

The bark of *Frangula alnus*, which is commonly known as alder buckthorn, is used to treat constipation and other complaints in which a soft stool is desirable [5–8]. The compounds responsible for the laxative effect are anthraquinone glycosides, especially glucofrangulins A and B (Fig. 1), but also other anthranoids, such as frangulins A and B and additional 8-O-glucosides (Fig. 2) [5–9]. In order to increase its tolerability, the drug must be stored for at least one year or alternatively heated under a stream of air, thus converting genuine anthrones and dianthrones into its oxidized forms [5–7]. At the same time, storage leads to deglycosylation, converting glucofrangulins into frangulins, and further into frangula-emodin, the respective aglycone (Fig. 2) [5, 6]. Therefore, appropriate methods for the quality control of buckthorn bark are required.

In the monograph of the European Pharmacopeia, anthraquinones are extracted with a mixture of methanol-water (70% v/v) and the genuine aglycones are removed by liquid-liquid extraction [10]. The remaining glycosides are subsequently hydrolysed and the aglycones

thereby obtained are measured using a spectrophotometric assay (after repeated liquid-liquid extraction). Apart from being nonspecific and time-consuming, this method does neither reveal accurate values for the desired glucofrangulins and frangulins nor does it give any information on possible degradation processes. This important information can only be obtained by additional quantification of the respective aglycones [5, 6].

Another method described in the literature is an HPLC/UHPLC assay with an RP18 phase for the determination of glucofrangulins A and B and frangulins A and B, respectively [8]. Due to the lack of commercial reference substances for glucofrangulins, quantification of these compounds was accomplished using the calibration curves of the respective frangulins, resulting in a bias of around 8%, due to different absorption coefficients. However, when comparing the results with the values obtained by the photometric assay of the European Pharmacopeia, the observed deviation was exceeding 50%. The authors hypothesize that the occurrence of bianthrones (which have a strong influence on photometric analysis) might be responsible for the increased values obtained with the photometric assay. Though providing more information than the photometric assay, the HPLC/UHPLC assay does not describe the content on aglycones present in buckthorn bark. To this end, another UHPLC method, using both a different HPLC column as well as another solvent system, was published [11].

In the present study, two-dimensional quantitative NMR spectroscopy (qNMR) is applied for the determination of anthraquinones, anthraquinone glucosides as well as glucofrangulins and frangulins. Quantitative one-dimensional NMR spectroscopy, mainly of the ^{1}H nucleus, is a primary analytical method because of a direct proportionality between signal integral and the number of protons and thus the concentration of the analyte [12–14]. However, one-dimensional qNMR requires separated signals for single compounds or groups of compounds. In the case of complex multicomponent mixtures, such as plant extracts, two-dimensional qNMR techniques were employed and proved applicable [15–19]. In contrast to one-

dimensional qNMR, two-dimensional qNMR is an indirect quantification method and needs suitable standard compounds for calibration, thus facing similar problems as e.g. chromatographic techniques. In order to overcome this problem, we considered duroquinone, a 1,4-benzoquinone derivative, and rutin, a naturally occurring and easily available flavonoid, as suitable surrogate standards, showing similar molecular features as the compounds of interest (Fig. 1).

2. Materials and methods

2.1. Chemical Reagents and Material

Dried and cut plant material (Lot 30079) was obtained from Alfred Galke GmbH (Bad Grund, Germany). Deuterated DMSO (Lot Q2981) for NMR spectroscopy was purchased from Euriso-top GmbH, Saarbrücken, Germany, and conventional 5 mm sample tubes were obtained from Rototec-Spintec GmbH, Griesheim, Germany. Duroquinone TraceCERT for quantitative NMR (Lot BCBR5528V) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA), rutin trihydrate (Lot 232180216) and frangulin A (Lot 4809527) were obtained from Carl Roth GmbH (Karlsruhe, Germany) and frangulin B (Lot 0429/0) was purchased from Extrasynthese SAS (Genay, France). Acetonitrile, methanol, and ortho-phosphoric acid (85%) were purchased from VWR International GmbH, Darmstadt, Germany. Water was doubly distilled in-house.

2.2. General experimental procedures

Photometric measurements were accomplished using a Shimadzu UV Mini 1240 spectrophotometer. UHPLC analyses were performed on a VWR-Hitachi Chromaster Ultra RS equipped with a 6170 binary pump, 6270 autosampler, 6310 column oven, and a 6430 DAD, using a Phenomenex Luna Omega C18 column (100 × 2.1 mm, 1.6 µm particle size). Solvent system, gradient and flow rate were as follows: solvent A: 1.25 mL/L phosphoric acid (85%) in water, solvent B: AcN/MeOH (20:80), flow rate: 0.2 mL/min, gradient: 34% B in 15 min to 34% B, in 1 min to 50% B, in 10 min to 76% B, in 0.50 min to 98% B, in 2 min to 98% B, in 0.5 min to 34% in 11 min to 34%.

NMR spectra were recorded using a Bruker Avance III 400 NMR spectrometer operating at 400 MHz for the proton channel and 100 MHz for the ^{13}C channel using a 5 mm PABBO broad band probe with a z gradient unit. Measurements were performed at 295 K and the temperature was calibrated with a methanol-d_4 solution. For each sample automatic tuning and

matching of the probe was performed as well as automatic shimming of the on-axis shims (Z to Z^5). The automatic receiver gain adjustment mode was employed, affording the maximum value in each measurement. The Bruker Topspin software 3.6.0 was used. The pulse program *hsqcedetgpsisp2.3* with multiplicity editing and adiabatic shaped 180° pulses of the manufacturer's pulse program library was employed. The setting of the three relevant delays in this pulse program for the ^1JCH coupling were either calculated for 145 Hz or 170 Hz. ^{13}C decoupling was performed using a GARP broadband decoupling sequence. For the proton (F2) channel 1024 data points were recorded, with a spectral width of 8.0 ppm corresponding to an acquisition time of 0.16 s; for the ^{13}C (F1) channel 256 increments with a spectral width of 165 ppm were selected. Using non-uniform sampling only 75 % of the randomly chosen increments were measured. 2 scans were collected per increment with an optimized inter-scan delay of 20 s. Total measurement time for each HSQC spectrum was 2 h 15 min. For the determination of the coupling constants a simple HMBC pulse program without decoupling (hmbcgpndqf) was used.

2.3. Spectroscopic analysis

150 mg dried and powdered plant material were refluxed on a water bath for 15 minutes with 25 mL of methanol-water 70% (v/v). The extracted solution was filtrated, evaporated to dryness and re-dissolved in 1000 µL DMSO-d_6. After filtration through a 0.45 µm membrane filter, 600 µL were subjected to NMR spectroscopy. Surrogate standards rutin and duroquinone as well as the reference standards frangulin A and B were dissolved in DMSO-d_6, and diluted to the desired concentration with the same solvent.

To quantify the anthraquinones by quantitative HSQC, calibration curves for three different cross signals were established using external calibration with solutions of 2.50, 5.00, 10.0, 15.0, 20.0 and 30.0 mmol/L of rutin and duroquinone, respectively (Table 1, Fig. S1 and S2). All

solutions were prepared with DMSO-d_6. Data processing was performed using the Topspin software. The raw data matrix was zero-filled to 2048 data points in both dimensions. 2D HSQC data was processed with the algorithm for non-uniform sampling and evaluated using the manufacturer's software topspin 3.5.7. Thus, the automatic peak detection routine ("peak picking") in the 2D mode was employed in the spectral region of interest, with the following parameters: mi 0.03; maxi 1; ppdiag 1; ppresol 5; ppmpnum 50; ppiptyp parabolic; psign both. All thereby detected peaks were integrated using the automatic peak integration of the topspin software with a threshold of 0.0035 (145 Hz) or 0.007 (170 Hz), respectively.

2.4. Method validation

The method was validated for linearity, repeatability, precision, accuracy, and limit of quantification. Limit of quantification was determined by serial dilution of standard solutions. Accuracy of the qHSQC method was assessed by comparison of the obtained values with a validated UHPLC method [8] and the photometric method of the European Pharmacopeia. Precision measurements included intra- and inter-day precision as well as repeatability and were accomplished in the following way. For intra-day precision six samples were prepared and each sample measured once. Inter-day precision was assessed by preparation of another six samples in one of the following days. For repeatability, one sample was prepared and measured six-fold. Evaluation of linearity was achieved by establishing calibration curves over a range of at least 80 to 120% of the measured concentrations. Here, 6-point calibration curves were created and expressed as linear functions. As *Frangula* anthraquinones, in contrast to duroquinone, only contain one methyl group, the calibration curve was calculated for the subsequent analysis of anthraquinones and the concentrations were therefore multiplied by the factor four. Likewise, the limit of quantification is expressed for the respective anthraquinone concentration.

3. Results and discussion

3.1. Method development

In order to be able to quantify both the total anthraquinone content, as well as the amount of anthraquinone glucosides and among these, the amount of glucofrangulins and frangulins, several signals were chosen for integration (Figs. 1 and 3). These signals comprise the cross correlations for the methyl group of the anthraquinone scaffold and the anomeric signal of the glycosidically linked sugars β-D-glucose, α-L-rhamnose and β-D-apiose. The latter two sugars are structural features of frangulins and glucofrangulins, whereas β-D-glucose is a structural feature of glucofrangulins and additional anthraquinones occurring in buckthorn bark (Fig. 2). DMSO-d_6 was chosen as NMR solvent, as the chemical shifts for the compounds of interest were reported in the same solvent [9, 20]. Moreover, DMSO showed good solubility for both the pure compounds and the crude extract, and has a high boiling point, making it an ideal solvent for validation purposes.

As mentioned above, reference compounds for glucofrangulins are missing and only frangulins A and B are commercially available. Therefore, we identified rutin and duroquinone as potential surrogate standards, showing adequate chemical features (Fig. 1). Duroquinone consists of a 1,4-benzoquinone ring bearing four methyl groups, similarly to the methyl group attached to position of the anthraquinone ring. Rutin, in contrast, shows the same two sugar moieties as glucofrangulin A, namely β-D-glucosyl and α-L-rhamnosyl units. In the next step, ^1JCH coupling constants for the relevant chemical features of the surrogates were determined by a suitable HMBC experiment and compared to the corresponding features of frangulin A and B (Fig. S3–S6). The coupling constants of the methyl groups were determined with 127.5 Hz (frangulin A) and 129.0 Hz (duroquinone), respectively, and the ^1JCH coupling constant of the anomeric proton of the α-L-rhamnosyl moiety was found to be 172.3 Hz for frangulin A and 168.0 Hz for rutin, respectively. The coupling constant of the anomeric proton of the β-D-

glucosyl moiety (rutin) was determined with 166.5 Hz, and that of β-D-apiosyl (frangulin B) was 171.0 Hz. Therefore, linkage to an anthraquinone scaffold results in slightly higher coupling constants, whereas flavone-binding leads to a small decrease, compared to the coupling constants found for the free sugars [21, 22].

Initial HSQC experiments were carried out with delays for 145 Hz, recording 1024 data points for the proton (F2) channel, with a spectral width of 8.0 ppm corresponding to an acquisition time of 0.16 s. In a first evaluation of the method, repeatability for the relevant signals was determined, resulting in relative standard deviations of 2.04% (methyl), 4.68% (β-D-glucosyl), 3.10% (α-L-rhamnosyl), and 11.03% (β-D-apiosyl). The high RSD values of the latter two signals was caused by their low intensity in contrast to the greater signals of the β-D-glucosyl and the α-L-rhamnosyl moieties and, above all, the methyl group. In order to increase the signal intensity of the anomeric carbon-proton signals, the delays were set corresponding to a coupling constant of ^{1}JCH = 170 Hz. With this change, the RSD values for the measured integrals were reduced by 50 to 60% for three of the four signals (Table S1). Due to the increased signal intensity of the anomeric sugar signals a higher threshold could be applied, also leading to significantly better RSD value of the methyl group (despite lower signal intensity).

To investigate the applicability of the surrogate standards, solutions of rutin, duroquinone, frangulin A and frangulin B were measured at different concentrations (2.5 to 30 mmol/L) with delays optimized for 145 Hz and 170 Hz, respectively (Figs. S7–S10). At delays optimized for 145 Hz the regression curves for the α-L-rhamnosyl moieties of rutin and frangulin A revealed similar offsets but differences in slopes of around 6% (Fig. S7). The same accounted for the β-D-glucosyl (rutin) and β-D-apiosyl (frangulin B) moieties, with a 10% higher slope for the apiosyl unit. At delays optimized for 170 Hz the deviation between the slopes of the latter two sugar moieties increased to 26%, still showing similar offsets (Fig. S8). In contrast, regression

curves for the α-L-rhamnosyl moieties were nearly identical. Investigation of the methyl groups of duroquinone, rutin and frangulin gave a different picture. At 170 Hz, regression curves of the duroquinone methyl groups showed similar offsets as those of the anthraquinone methyl group, but a significantly higher slope, whereas at 145 Hz the slope of both curves was similar, but their offsets were differing (Fig. S9–S10). The methyl group of rutin, which was also studied as possible surrogate, did neither reveal similar offsets nor slopes to the anthraquinone methyl group and was therefore excluded.

As the slopes of the regression curves were stable over time and the offsets were differing, but then in the same manner for all compounds, the application of the selected surrogates was possible at either 145 or 170 Hz. However, because of the better repeatability at delay times optimized for 170 Hz, subsequent validation for linearity, precision, accuracy, limit of detection and limit of quantification was accomplished at 170 Hz.

3.2. Method validation

3.2.1. Linearity and quantitation limit

For validation of linearity, calibration curves of three different cross correlations were established using duroquinone and rutin as external calibration standards (Fig. 1). Rutin was used to obtain calibration curves for the anomeric correlations of glycosidically linked β-D-glucosyl and α-L-rhamnosyl moieties (CH-1´´ and CH-1´´´) [23], whereas the calibration curve for the anthraquinone methyl group was established using the cross correlations of the methyl groups of duroquinone [24]. Table 1 shows regression equations of the calibration curves obtained from the surrogate standards and those measured with frangulin reference standards. As mentioned above, the calibration curve for the duroquinone methyl group is expressed for the concentration of anthraquinones, showing only one methyl group instead of four. Thereby, integrals and resulting regression equations for the anomeric proton of the α-L-rhamnosyl

moieties were nearly identical, whereas integrals of the same proton of the β-D-glucosyl moiety were around 20% lower and those of the β-D-apiosyl moiety were slighly higher. However, a look at the regression equations suggests that because of the similar offsets the β-D-glucosyl signal of rutin is suitable for the quantification of β-D-apiose using a factor of 0.8. The same accounts for the methyl group signals, where a factor of 1.3 needs to be applied. Given the fact that the intensities of the α-L-rhamnosyl moieties are not affected from their linkage to different scaffolds, at least at delay times optimized for 170 Hz, we used the regression equation of β-D-glucose as is for the quantitation of the anthraquinone glucosides.

The limit of quantification was determined by serial dilution of the calibration standards and resulted in concentrations of 2.25 mmol/L for α-L-rhamnose, 3.38 mmol/L for β-D-glucose, 2.60 mmol/L for β-D-apiose, and 0.3 mmol/L for duroquinone corresponding to 1.56 mmol/L anthraquinone (Table 1). The varying LOQs originate from the differences in the slopes and offsets of the respective regression lines.

3.2.2. Calculation procedure

In the next step, the calibration curves were used to obtain the following information of the buckthorn bark extracts. The total amount of anthraquinones was measured using the calibration curve for the methyl group, whereas the amount of anthraquinone glucosides was obtained from the signal of the anomeric proton of the β-D-glucosyl moeity at 5.14 ppm (Fig. 3). Furthermore, integration of the anomeric proton signal of the α-L-rhamnosyl moiety revealed the amount of glucofrangulin A plus frangulin A, whereas the corresponding β-D-apiosyl signal gave the sum of glucofrangulin B and frangulin B, respectively.

3.2.3. Precision and repeatability

After repeatability was already assessed during method development (and was found satisfying after optimizing the delays for a coupling constant of 170 Hz), the method was furthermore investigated for its inter- and intra-day precision. Table 2 shows the results for the precision measurements of the four cross correlations used for quantification in mmol/L, whereas in Table 3 the amounts of the relevant compound subclasses in the plant material given in mmol/g. The relative standard deviations were in an expected range, with values of 2.41 to 3.48% for the methyl group (total anthraquinone content), 2.93 to 5.17% for β-D-glucose (content of glucosides), 3.62 to 5.58% for α-L-rhamnosyl, and 5.58 to 8.05% for β-D-apiosyl. However, as the concentrations from the β-D-apiosyl and α-L-rhamnosyl moieties are summed up to the total amount of glucofrangulins and frangulins, the high deviations are relativized, indicated by acceptable RSD values of 3.34 to 4.78% (Table 3).

The unit mmol/g has already been discussed in one of our previous studies as an ideal unit for the evaluation of medicinal plants and herbal medicinal products, focussing more on the pharmacological potential of the active ingredients than on their mass fractions [25]. However, regarding the decomposition of glucofrangulins into frangulins and subsequently to frangula-emodin, the proposed unit also allows easier evaluation of degradation processes.

3.2.4. Accuracy and comparability

For the determination of accuracy two reference methods were applied, a validated UHPLC assay and the spectrophotometric method of the European Pharmacopoeia. The UHPLC method was used to determine the amounts of glucofrangulins A and B as well as frangulins A and B in the plant material used for this study (Fig. S11–S12). As displayed in Table 4, the amount of glucofrangulin A and frangulin A show only a slight deviation from the values obtained by UHPLC (recovery rate of 101.2%). In contrast, the recovery rate for glucofrangulin B and

frangulin B is somewhat higher (107.6%), but still in an acceptable range. Thus, the value-determining constituents of alder buckthorn were accurately quantified.

Additionally, the method of the European Pharmacopoeia was employed to obtain the total amount of glycosides, which we compared to the amount of anthraquinone glucosides measured by qNMR (Table 4). The method of the European Pharmacopoeia does not distinguish between glucofrangulins and other anthraquinone glycosides in alder buckthorn, but quantifies the total amount of glycosides expressed as glucofrangulin A. In this assay, the minimum amount of 7.0% is necessary to comply with the specifications of European Pharmacopoeia [10]. Using the spectrophotometric assay, the value for buckthorn bark was determined with 8.38 ± 0.63% and thus was 14% above the corresponding value obtained by qNMR (7.35 ± 0.34%). However, as frangulins A and B are lacking a glucose moiety, those compounds are not included in the amounts measured by qNMR. Therefore, lower values obtained with the qNMR method had to be expected.

However, the actual amounts of the value-determining glucofrangulins and frangulins were determined with 5.78 ± 0.23% (Table 3) and were clearly below the values measured with the method of the European Pharmacopoeia and, moreover, below the minimum value of 7.0%. Rosenthal et al. also reported lower amounts of anthraquinone glycosides, but the values determined by UHPLC and HPLC were only ranging from 2.2 to 3.5% in the plant material [8]. The explanation that the higher values obtained with the method of the Pharmacopoeia derives from occurrence of bianthrones might influence the spectrophotometric method, in our opinion, falls too short, as they should be removed with the liquid-liquid partitioning step. Furthermore, Rosenthal et al. only quantify the glucofrangulins and frangulins and do not consider additional glucosides, which, as demonstrated by our results, contribute significantly to the total glycoside content.

3.2.5. Specificity and selectivity

One of the main advantages of the present method in comparison to the method of the European Pharmacopeia is its specificity, which results from using anomeric cross correlations of the β-D-glucosyl, α-L-rhamnosyl, and β-D-apiosyl moieties. Due to their characteristic shift values, these signals are clearly separated from the remaining signals in the crude extract and from the free sugars (Fig. S11) and can therefore be unambiguously quantified. As these shift values derive from the glycosidic bond to specific aglycones, signals from the respective sugars linked to other scaffolds (e.g. rutin) would thus not interfere with the measurements (Fig. 4). Therefore, these signals could also be used as markers in order to exclude adulterations with other, easier-to-obtain, anthraquinone glycosides, or for identification purposes. Here, especially the cross-correlation signals of the α-L-rhamnosyl and β-D-apiosyl moieties would be of interest, as glucofrangulins/frangulins are restricted to the genus *Rhamnus* s.l., and the co-occurrence of (gluco)frangulin A and B is, so far, only known from *Frangula alnus*.

4. Conclusion

In this study, we present a quantitative NMR method for the quality control of buckthorn bark. The method allows the quantitative determination of the total anthraquinone content, the content of anthraquinone glucosides and the sum of glucofrangulins and frangulins, in the crude extract with a single measurement. Although HPLC/UHPLC methods exist for the quantitation of anthraquinones, they either focus on the amount of aglycones or on the amount of glucofrangulines and frangulines, rendering them less comprehensive. As shown for buckthorn bark, the contents of both aglycones and glycosides, are necessary to draw conclusions on the correct storage/treatment of the drug. A fact that is also neglected in the method of the European Pharmacopeia. Furthermore, the method of the Pharmacopeia does not differentiate between

the value-determining glucofrangulins and frangulins and additional anthraquinone glycosides, making the method less specific and thus unable to detect potential adulterations.

The example of buckthorn bark shows another major problem in natural product chemistry, which is the availability of commercial reference standards. In order to overcome this problem, we investigated the use of surrogate standards with similar chemical features and their applicability in two-dimensional qNMR. Following measurements of the relevant coupling constants and optimizing delay times, we successfully quantified the abovementioned compound classes. Validation of the method revealed good linearity, precision and accuracy, along with clear advantages in specificity and meaningfulness of the results, in comparison to previous methods. The present study moreover shows the potential of two-dimensional qNMR as a comprehensive quantitation method, at the same time giving quantitative information for compounds with different polarities, such as aglycones and their glycosides, and additionally revealing qualitative information, provided by the characteristic shift values of e.g. anomeric proton signals.

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Figure Captions

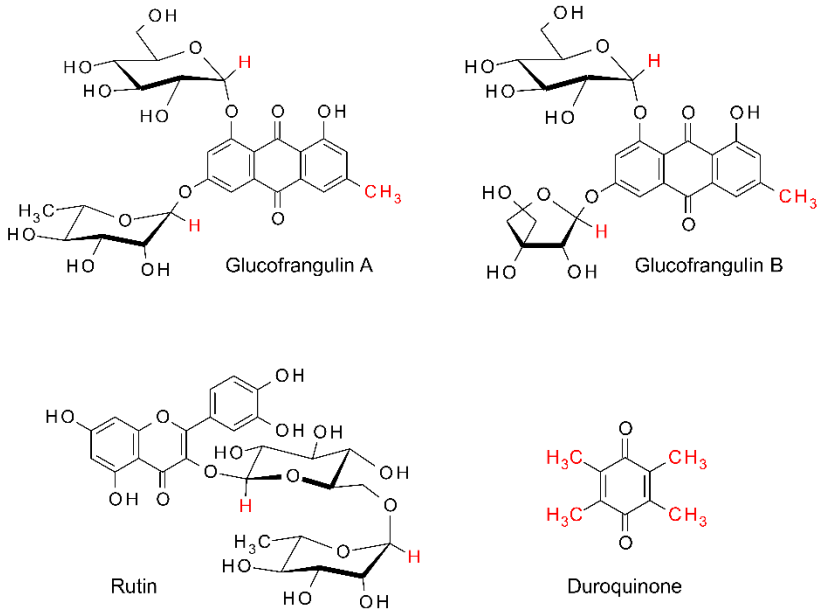
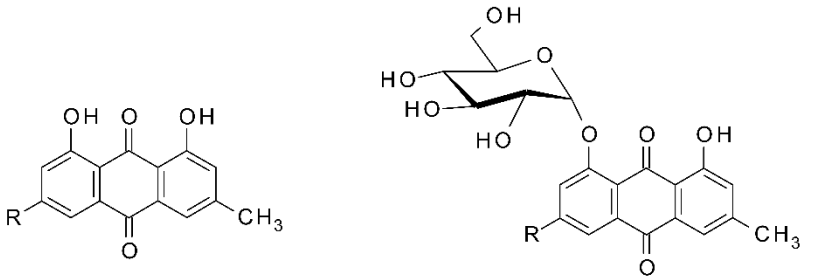


Fig. 1: Chemical structures of glucofrangulins A and B, and surrogate standards rutin and duroquinone. Signals used for quantitative NMR are highlighted in red.



R=H	Chrysophanol	R=H	Chrysophanol-8-*O*-glucoside
R=OH	Frangula-emodin	R=OH	Frangula-emodin-8-*O*-glucoside
R=OCH_3	Physcion	R=OCH_3	Physcion-8-*O*-glucoside
R=α-L-Rha	Frangulin A	R=α-L-Rha	Glucofrangulin A
R=β-D-Api	Frangulin B	R=β-D-Api	Glucofrangulin B

Fig. 2: Chemical structures of anthraquinones reported from buckthorn bark.

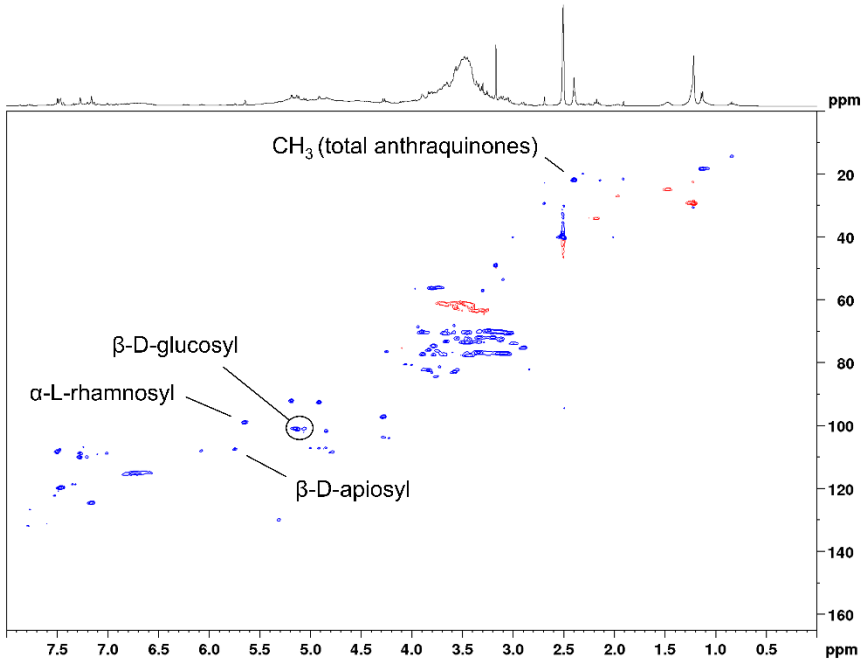


Fig. 3: HSQC diagram of buckthorn bark extract in DMSO-d_6 with delays optimized for 170 Hz.

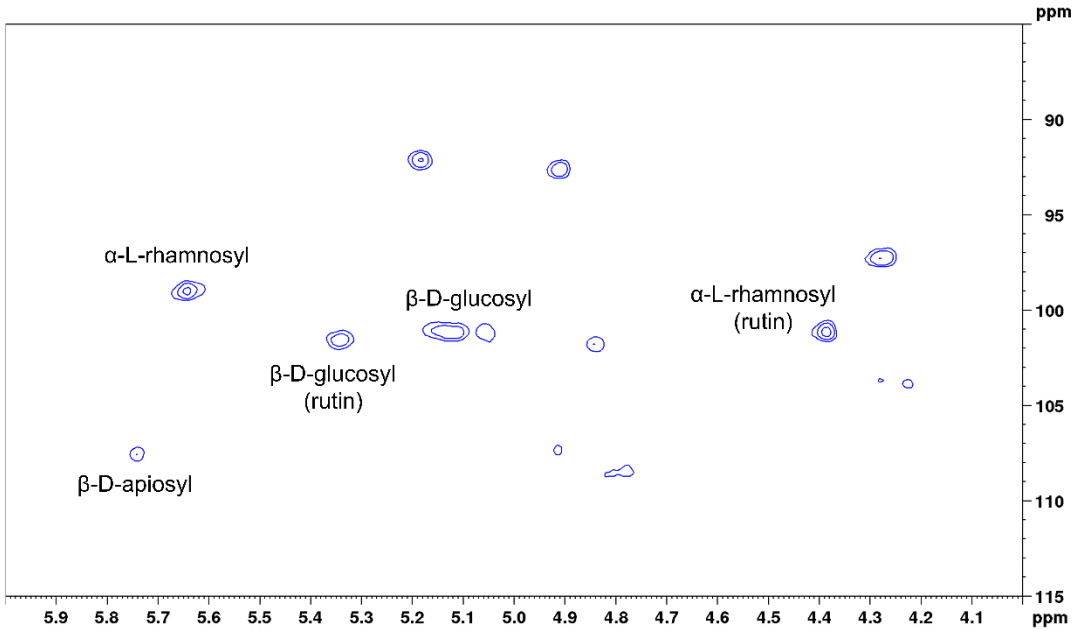


Fig. 4: HSQC diagram of buckthorn bark extract in standard solution (10.0 mmol/L rutin in DMSO-d_6) in the region of 4.0 to 6.0 ppm (^{1}H) and 85 to 115 ppm (^{13}C).

Table 1
Regression equations for frangulins and surrogate standards, coefficients of determination (R^2) and limit of quantification (LoQ). Compounds and cross correlations used for calibration are shown in parenthesis.

Cross-correlation signal	Regression equation	R^2	LoQ
methyl (frangulin A and B)	$y = 732.923\ x - 1.236.449$		
α-L-rhamnosyl (frangulin A, CH-1')	$y = 680.235\ x - 540.322$		
β-D-apiosyl (frangulin B, CH-1')	$y = 725.590\ x - 1.782.813$		
methyl (duroquinone)	$y = 961.483\ x - 1.322.030$	0.9991	1.56 mmol/L
α-L-rhamnosyl (rutin, CH-1''')	$y = 687.291\ x - 565.451$	0.9996	2.25 mmol/L
β-D-glucosyl (rutin, CH-1'')	$y = 575.704\ x - 1.990.616$	0.9995	3.38 mmol/L
β-D-apiosyl			2.60 mmol/L*

*calculated with the regression equation of β-D-glucose

Table 2
Repeatability, intra-day and inter-day precision for the relevant cross correlations. Results are given in mmol/L; standard deviations are shown in parenthesis.

Signal	Repeatability	Intra-day 1	Intra-day 2	Inter-day
methyl	22.62 (0.25)	22.08 (0.77)	22.16 (0.53)	22.19 (0.62)
β-D-glucosyl	20.11 (0.44)	18.64 (0.96)	20.00 (0.57)	19.04 (0.91)
α-L-rhamnosyl	10.57 (0.31)	10.18 (0.37)	10.26 (0.57)	10.25 (0.47)
β-D-apiosyl	5.02 (0.23)	4.85 (0.27)	4.71 (0.38)	4.81 (0.32)

Table 3
Repeatability, intra-day and inter-day precision of the calculated compound classes. Results are given in mmol/g drug material; standard deviations are shown in parenthesis. For anthraquinone glucosides and glucofrangulins, results are also given in percent expressed as glucofrangulin A as described in the European Pharmacopeia (indicated in italic font).

Compound class	Repeatability	Intra-day 1	Intra-day 2	Inter-day
total anthraquinones	0.1508 (0.0017)	0.1472 (0.0051)	0.1478 (0.0036)	0.1475 (0.0042)
anthraquinone glucosides	0.1341 (0.0029) *7.75 (0.17)*	0.1243 (0.0064) *7.19 (0.37)*	0.1300 (0.0038) *7.52 (0.22)*	0.1271 (0.0058) *7.35 (0.34)*
glucofrangulins and frangulins	0.1039 (0.0020) *6.01 (0.11)*	0.1002 (0.0033) *5.79 (0.19)*	0.0998 (0.0048) *5.77 (0.28)*	0.1000 (0.0039) *5.78 (0.23)*

Table 4
Determination of accuracy. Content of anthraquinone glucosides was compared to the content determined with the method of the European Pharmacopoiea and expressed given in percent expressed as glucofrangulin A (see above). Concentrations of glucofrangulins and frangulins are compared to the values obtained with a validated UHPLC assay.

Compound class	qNMR	Reference method	recovery rate
anthraquinone glucosides	7.35 ± 0.34%	8.38 ± 0.63%	87.7%
glucofrangulin A and frangulin A	10.25 ± 0.47 mmol/L	10.13 ± 0.06 mmol/L	101.2%
glucofrangulin B and frangulin B	4.81 ± 0.32 mmol/L	4.47 ± 0.03 mmol/L	107.6%

References

[1] S.E. Voelker, L.M. Lorenz, J.J. Litzau, Semi-quantitative determination of designer steroids by high-performance liquid chromatography with ultraviolet detection in the absence of reference material. Drug Test. Anal. 11 (2019) 428–434.

[2] Y. Xiao, S.-R. Chae, M.R. Wiesner, Quantification of fullerene (C60) in aqueous samples and use of C70 as surrogate standard. Chem. Eng. J. 170 (2011) 555–561.

[3] X. Dong, L. Li, Y. Ye, D. Zhang, L. Zheng, Y. Jian, M. Shen, Surrogate analyte-based quantification of main endocannabinoids in whole blood using liquid chromatography–tandem mass spectrometry. Biomed. Chromatogr. 33 (2019) e4439.

[4] M. Jemal, A. Schuster, D.B. Whigan, Liquid chromatography/tandem mass spectrometry methods for quantitation of mevalonic acid in human plasma and urine: method validation, demonstration of using a surrogate analyte, and demonstration of unacceptable matrix effect in spite of use of a stable isotope analog internal standard. Rapid Commun. Mass Spectrom. 17 (2003) 1723–1734.

[5] N.G. Bisset, M. Wichtl, Herbal Drugs and Pharmaceuticals, second ed., medpharm GmbH Scientific Publishers, Stuttgart, 2001.

[6] Ö. Demirezer, 1991. Glukofrangulinanthrone A/B, deren Oxidationsformen und davon abgeleitete Zuckerreste aus Rhamnus-Arten: Beiträge zur Chemisch-Analytischen und –physiologischen Kennzeichnung. Dissertation, Johann Wolfgang Goethe-Universität Frankfurt am Main.

[7] D. Frohne, Heilpflanzenlexikon, eighth ed., Deutscher Apotheker Verlag Stuttgart, 2006.

[8] I. Rosenthal, E. Wolfram, S. Peter, B. Meier, Validated method for the analysis of frangulins A and B and glucofrangulins A and B using HPLC and UHPLC, J. Nat. Prod. 77 (2014) 489–496. https://dx.doi.org/10.1021/np400736s.

[9] G.W. Francis, D.W. Aksnes, Ø. Holt, Assignment of the ^{1}H and ^{13}C NMR spectra of anthrquinone glycosides from *Rhamnus frangula*, Magn. Reson. Chem. 36 (1998) 769–772.

[10] European Pharmacopoeia, ninth ed., European Directorate for the Quality of Medicines & Health Care (EDQM), Strasbourg, 2017.

[11] N. Meier, B. Meier, S. Peter, E. Wolfram, In-silico UHPLC method optimization for aglycones in the herbal laxatives *Aloe barbadensis* Mill., *Cassia angustifolia* Vahl pods, *Rhamnus frangula* L. bark, *Rhamnus purshianus* DC. bark, and *Rheum palmatum* L. roots, Molecules 22 (2017) 1838.

[12] U. Holzgrabe, Quantitative NMR spectroscopy in pharmaceutical applications, Prog. NMR Spectroscopy 57 (2010) 229–240.

[13] T. Gödecke, J.G. Napolitano, M.F. Rodriguez-Brasco, S.-N. Chen, B.U. Jaki, D.C. Lankin, G.F. Pauli, Validation of a Generic Quantitative ^{1}H NMR Method for Natural Products Analysis, Phytochem. Anal. 24 (2013) 581–597.

[14] S.K. Baharti, R. Roy, Quantitative ^{1}H NMR spectroscopy, Trends Anal. Chem. 35 (2012) 5–26.

[15] I.A. Lewis, S.C. Schommer, B. Hodis, K.A. Robb, M. Tonelli, W.M. Westler M.R. Sussman, J.L. Markley, Method for determining molar concentrations of metabolites in complex solutions from two-dimensional $^{1}H - ^{13}C$ NMR spectra, Anal. Chem. 79 (2007) 9385–9390.

[16] F. Fardus-Reid, J. Warren, A. Le Gresley, Validating heteronuclear 2D quantitative NMR, Anal. Methods 8 (2016) 2013–2019.

[17] L. Zhang, G. Gellerstedt, Quantitative 2D HSQC NMR determination of polymer structures by selecting suitable internal standard references, Magn. Res. Chem. 45 (2007) 37–45.

[18] R.K. Rai, N. Sinha, Fast and accurate metabolic profiling of body fluids by nonlinear sampling of $^{1}H - ^{13}C$ two-dimensional nuclear magnetic resonance spectroscopy, Anal. Chem. 84 (2012) 10005–10011.

[19] S.S. Cicek, A.L. Pfeifer Barbosa, U. Girreser, Quantification of diterpene acids in Copaiba oleoresin by UHPLC-ELSD and heteronuclear two-dimensional qNMR. J. Pharm. Biomed. Anal. 160 (2018) 126–134.

[20] M.S.M. Rawat, D.S. Negi, M.S. Panwar, G. Pant, S. Shibata, Y. Okada, Y. Oshida, T. Okuyama, Anthraquinone glycosides from *Rheum moorcroftianum* Royle, Pharmazie 44 (1989) 509–510.

[21] K. Bock, C. Pedersen, Determination of one-bond carbon-proton coupling constants through ^{13}C-satellites in ^{1}H-n.m.r. spectra, Carbohyd. Res. 145 (1985) 135–140.

[22] J.R. Snyder, A.S. Serianni, DL-apiose substituted with stable isotopes: synthesis, n.m.r.-spectral analysis, and furanose anomerization, Carbohyd. Res. 166 (1987) 85–99.

[23] J.G. Napolitano, D.C. Lankin, S.-N. Chen, G.F. Pauli, Complete ^{1}H NMR spectral analysis of ten chemical markers of *Ginkgo biloba*, Magn. Reson. Chem. 50 (2012) 569–575.

[24] https://www.sigmaaldrich.com/analytical-chromatography/analytical-standards/application-area-technique/organiccrm.html. (Accessed 26 October 2018).

[25] S.S. Cicek, U. Girreser, C. Zidorn, Quantification of the total amount of black cohosh cycloartanoids by integration of one specific ^{1}H NMR signal. J. Pharm. Biomed. Anal. 155 (2018) 109–115.