Quality control of *Aloe vera (Aloe barbadensis)* and *Aloe ferox* using band-selective quantitative heteronuclear single quantum correlation spectroscopy (bs-qHSQC)

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Abstract

In the present study, band-selective quantitative heteronuclear single quantum correlation spectroscopy (bs-qHSQC) was applied for the quality control of the two *Aloe* species present in the European Pharmacopeia. After development and validation of a complete spectral range (csr-) qHSQC assay, a specific pulse program with selective excitation was applied and the measuring time was reduced from 135 to 32 minutes, while maintaining the same resolution. This bs-qHSQC method (method I) showed slightly higher RSD values compared to the csr-qHSQC method (maximum RSD of 2.80%), but better recovery rates in comparison to the assay of the Pharmacopeia (97.3% for *Aloe vera* and 96.6% for *Aloe ferox*). Apart from quantifying the total anthranoid content, the method moreover allows the quantitation of aloin among other aloin derivatives, such as 7-hydroxyaloin, as well as the differentiation of the two investigated species. Additionally, a second bs-qHSQC method (method II) for the fast (4 min) determination of the aloin A/B ratio was developed and compared to ^{13}C qNMR measurements. Showing the same results in much less analysis time, the latter approach contributes to a general problem in natural product chemistry, the co-occurrence of structurally similar compounds and their analysis in complex matrices, e.g. plant extracts.

Key words

Barbados aloes, Cape aloes, quantitative NMR, aloin, natural product, band selective HSQC

1. Introduction

In the last decades quantitative NMR spectroscopy (qNMR) became an important tool in natural product analysis, not only for the purity determination of isolated compounds but also for their quantitation in complex samples, such as plant extracts or dietary supplements [1,2]. Here, mainly one-dimensional proton NMR (^{1}H NMR) methods were applied, allowing accurate and precise quantification within short measurement times [1,2]. Furthermore, quantitative ^{1}H NMR spectroscopy is a primary analytical method showing direct proportionality between signal integral and the number of protons [3-5]. Thus, the concentration of the analyte can be obtained without the need of calibration curves [4]. However, in certain cases one-dimensional methods do not provide enough resolution, leading to signal overlap and thus inaccurate results. Another shortcoming of one-dimensional methods is their lack of specificity, which obviously can be eliminated by introducing a second dimension [2]. This increase of specificity is foremost achieved using heteronuclear techniques, such as HMQC (heteronuclear multiple quantum correlation spectroscopy) and HSQC (heteronuclear single quantum spectroscopy) experiments, of which the latter have been mostly used for quantitative purposes [6-10]. Being a proton-detected experiment, HSQC is less time-consuming and more sensitive than ^{13}C NMR methods, but still far beyond the values of one-dimensional ^{1}H NMR. However, as for qNMR in many cases only one or a few NMR signals are of interest, one way to reduce measurement times and thereby maintaining sensitivity is the use of band-selective 2D NMR experiments [11]. Band-selective heteronuclear two-dimensional experiments allow restriction of the heteronuclear shift domain without causing spectral folding, at the same time leading to improved spectral resolution [11,12]. This benefit is mainly used for the structure elucidation of complex structures, e.g. peptides or oligosaccharides [13-15], but also for

studying chlorine isotope effects in ^{13}C nuclei [16-18]. In the present study, band-selective HSQC is used for the qualitative and quantitative analysis of aloes.

The concentrated and dried juice of the secretory cells from *Aloe vera* (syn. *Aloe barbadensis*) and *Aloe ferox (syn. Aloe capensis)* is used when an easy defaecation with a soft stool is desirable, e.g. with anal fissures, haemorrhoids, or constipation, and administered as powder or dry extract for liquid and solid oral formulations [19]. Both drugs show a high content on aloin, which is a mixture of two diastereomeric anthrone C-glycosides (aloin A and B, Fig. 1), and additional anthranoids either present in *Aloe vera* (7-hydroxyaloin) or *Aloe ferox* (5-hydroxyaloin and aloinosides) [19]. In the monograph of the European Pharmacopeia, anthranoids are extracted and subsequently converted into their aglycone forms by oxidative ($FeCl_3$) cleavage of the C-glucosidic moeity, before being measured by a spectrophotometric assay (after liquid-liquid extraction) [20]. Additionally, an UPLC-MS^2 assay has been reported for the determination of aloin A and aloe-emodin in *Aloe vera* [21]. Apart from its laxative effect, aloin has also been the target of various other pharmacological studies, with already six publications investigating its biological activity this year.

Regarding the stereochemistry of aloin A and B, three studies were conducted in the 1980s, where nearly identical proton NMR spectra were reported (with the only different shift value at the 2'-OH), which is why the configurations were established using X-ray crystallography [22-24]. However, in a later study the absolute configurations were proven by two-dimensional NMR experiments and by means of circular dichroism [25]. A chemosystematic study on their occurrence in 36 aloe species found both diastereomers to be present in all of the investigated species, with a constant imbalance (less aloin B) but changing ratios between the two compounds [26]. Because the authors were only interested in the occurrence of aloin A and B (and eleven other compounds) for taxonomic/chemophenetic reasons, they did neither quantify the contents nor determine the fixed ratio of these two compounds.

In this study both, the ratio between aloin A and B as well as the content of aloin and total aloe anthranoids in *Aloe vera* and *Aloe ferox* samples will be determined. The band-selective HSQC approach is thereby evaluated in terms of repeatability and accuracy and compared to a validated complete spectral range qHSQC method and furthermore to the official method of the European Pharmacopeia.

2. Materials and methods

2.1. Chemical Reagents and Material

Powdered plant material of *Aloe vera* (Lot 33669) and *Aloe ferox* (Lot 26167) was obtained from Alfred Galke GmbH (Bad Grund, Germany). Deuterated DMSO (dimethylsulfoxide-d_6, 99.80% D, Lot Q2981, Batch 0817B) for NMR spectroscopy was purchased from Eurisotop GmbH, Saarbrücken, Germany, and conventional 5 mm sample tubes were obtained from Rototec-Spintec GmbH, Griesheim, Germany. Duroquinone TraceCERT for quantitative NMR (2,3,5,6-tetramethyl-1,4-benzoquinone, 99.71%, Lot BCBR5528V) was obtained from Sigma Aldrich Co. (St. Louis, MO, USA). Analytical grade methanol, ethyl acetate and chloroform 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 and were performed at 512 nm.

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 293 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 Z5). The automatic receiver gain adjustment mode was employed, resulting always in the same maximum value for the gain. The Bruker Topspin software 3.6.0 was used and the pulse program hsqcedetgpsisp2.3 with multiplicity editing and adiabatic shaped 180° pulses of the manufacturer's pulse program library was employed. ^{13}C decoupling was performed using a GARP broadband decoupling sequence. When recording complete spectral range qHSQC spectra, for the proton (F2) channel 1024 data points were set, with a spectral width of 13.0 ppm corresponding to an acquisition time of 0.12 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 csr-qHSQC spectrum accounted to 2 h 15 min.

Band-selective HSQC measurements for the quantification of aloin and total anthranoids (bs-qHSQC method I) were performed using the shsqsctgpsi2.2 pulse program of the manufacturer's pulse program library with a band-selective shaped ^{13}C refocusing pulse. The shape form Q3.1000 was chosen and in order to achieve selective excitation over a frequency range of 3020 Hz (30 ppm) the length of the pulse was determined to 1379.2 µs with a power of 0.617 W for the used probe head. Sweep width was set to 13 ppm for ^{1}H and 30 ppm for ^{13}C (53.5 ppm ± 15 ppm). For the ^{1}H channel 1024 data points were collected, for the ^{13}C channel 52 data points were set, which were reduced to 40 by employing uniform sampling with 75 %

of the increments actually recorded. The total acquisition time for this bs-HSQC experiment with two scans per increment and an inter-scan delay of 20 s amounted to 32 minutes.

The aloin A/B ratio in the extracts was determined using the following parameters: A band-selective HSQC experiment (bs-qHSQC method II) with a frequency range of 13 ppm for ^{1}H (1024 data points) and 200 Hz (2 ppm) for ^{13}C was used, which required in this case a 17240 µs shaped pulse with the above mentioned shape form for refocusing with a power of 0.004 W with the employed probe head, 1024 × 128 increments were set, only 50 % of the increments were collected by non-uniform sampling with 2 scans per increment and an inter-scan delay of 1.5 s. After processing of the raw data to a 2D matrix with 1024 × 1024 data points, automatic baseline correction in both dimensions and automatic phase correction in the ^{1}H frequency dimension, careful manual phase evaluation and correction in both dimensions was necessary and a 1D projection of the columns for the proton shift range of 4.56-4.62 ppm (9 increments) was calculated. This 1D spectrum was finally integrated to deliver the aloin A/B ratio and the total aloin content for the stability investigations. A total measuring time of about 4 min and 14 s was achieved. For the determination of the coupling constants an HSQC pulse program without decoupling was used. Conventional ^{13}C measurements with broadband ^{1}H decoupling with collection of 65536 data points 2048 scans, an interscan-delay of 3 s, and a frequency width of 240 ppm were recorded and zero filled to 262144 data points. The measuring time was about 2 h. The corresponding 135 DEPT ^{13}C spectrum was recorded with 65536 data points, zero filled to 131072 points, 512 scans, 240 ppm frequency width and a total measuring time of 29 min.

2.3. Isolation of aloin

10.0 g of *Aloe ferox* were suspended in water and heated for 10 min over a water bath. After cooling, the mixture was filtered through a Büchner funnel and extracted five times with 200 mL of ethyl acetate. Organic phases were combined, rewashed with water and the solvent was evaporated. This procedure was repeated another four times before the combined residues were dissolved in a mixture of chloroform and methanol (6:1) under heating, and the solution was kept at -20°C over the weekend. The mixture was subsequently filtered through a Büchner funnel, yielding 335 mg of aloin crystals. Purity of the crystals was assessed with NMR using duroquinone as internal standard and resulted to be 93.20 %. An HSQC diagram of aloin is given in the supporting information (Fig. S1).

2.4. Spectroscopic analysis

Powdered plant material of *Aloe vera* (100 mg) and *Aloe ferox* (150 mg) were extracted according to the European Pharmacopoeia. Therefore, the drug was put in a 100 mL Erlenmeyer flask before adding 1000 µL of methanol and 1000 µL of warm (60°C) dd water. The mixture was shaken and further 25 mL of warm water were added. After 30 minutes of shaking at constant temperature, the mixture was cooled and filtered into a 100 mL round bottom flask. Erlenmeyer flask and filter were washed with water and the solvent was then evaporated to a volume of about 10 mL before transferring the solution into a 20 mL scintillation vial. After solvent evaporation in the vacuum centrifuge, the dry extract was dissolved in 1000 µL of DMSO-d_6; 500 µL of this solution were transferred to an NMR tube, and both, ^{1}H NMR as well as HSQC spectra were recorded.

To quantify the anthraquinones by quantitative HSQC, calibration curves for the methylene group (3-CH_2OH, total anthraquinone content) and the glycoside (H-10, aloin content) signals were established using external calibration with solutions of 6.525, 13.05, 19.58, 26.10, 39.15

and 52.2 mg/mL of aloin in DMSO-d_6 (Table 1). 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 and setting the threshold to 0.05.

2.5. Method validation

The method was validated for linearity, repeatability, precision, accuracy, and limit of quantification. 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. Limit of quantification was determined by serial dilution of standard solution and accuracy of the qHSQC method was assessed by comparison of the obtained values with 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. Validation of the band selective qHSQC (method I) was accomplished by measuring *Aloe vera* and *Aloe ferox* samples six-fold and comparison of both, mean concentrations and relative standard deviations to the values obtained with the csr-qHSQC assay.

3. Results and discussion

3.1. Method development

The aim of the present study was the direct quantification of aloin and the total content of aloe anthranoids in crude extracts of *Aloe vera* and *Aloe ferox* by quantitative NMR; thus, providing an alternative and more comprehensive method for the quality control of these two medicinal plants. The assay of the Pharmacopeia does not separate aglycones from glycosides before cleavage of the sugar moiety, as it does for other anthranoid drugs such as Sennae folium or Frangulae cortex. Thus, quantification of glycosides gives additional information on possible degradation processes. This is of even more importance, as the low stability of aloin has been described before [27].

Regarding the structures of aloin A and B (Fig. 1), the only characteristic signal for one-dimensional proton NMR (H-10, 4.56 ppm) was overlapped by the signal of the methylene group linked to position 3 of the anthranoid scaffold (3-CH_2OH, 4.58 ppm). Other signals were either not specific enough (glucose signals) or could not be unambiguously quantified (due to similar co-occurring compounds in the extract). Thus, extracts were measured with HSQC and the CH-10 cross correlation of both aloins was chosen for quantification. With a ^{13}C shift of 44 ppm (44.2 ppm for aloin A and 43.9 ppm for aloin B), the cross peak was well separated from the cross peak of the 3-CH_2OH methylene group, which shows a carbon shift of 62.4/62.5 ppm, as well as from all other signals in the extracts (Fig. 2 and 3). The latter cross peak was additionally elected in order to quantify the total amount of aloe anthranoids. Aloe anthranoids consist of an aloe-emodin scaffold, which is characterized by a methylene group in position 3. In contrast, frangula-emodin and chrysophanol show a methyl group in this position and rhein

a carboxylic acid. *Aloe ferox* was also reported to contain small amounts of chrysophanol [19]. However, in the sample used in this study, chrysophanol was neither detected by HSQC nor by 1D HNMR measurements.

Determination of the coupling constants using an HSQC pulse program without decoupling revealed ^{1}J(CH) coupling constants of 132 Hz for the methine group (CH-10) and 139 Hz for the methylene group (3-CH_2OH). Due to the strong intensities of both signals in the aloe extracts, adaption of delay times was not considered necessary and the method was validated with a standard parameter set optimized for 145 Hz. Using cross peaks obtained from isolated aloin for calibration (Fig. S1), the method was evaluated for linearity and limit of quantitation, precision and repeatability, specificity and selectivity, as well as accuracy with regard to the method of the European Pharmacopeia.

3.2. Method validation

3.2.1. Linearity and quantitation limit

For validation of linearity, calibration curves for the signals of the CH-10 cross peak and the cross peak of the 3-CH_2OH methylene group of aloin were established over a calibration range of 6.525 to 52.2 mg/mL. In Table 1 regression equations and coefficients of determination for both signals are shown, as well as the limits of quantification of the aloin content and the total amount of anthranoids, which is thus additionally expressed in mmol/L. Due to the wide calibration range of the method, amounts of 50 to 200 mg of *Aloe vera* and 75 to 300 mg of *Aloe ferox* could be quantified with this method. However, application of 100 mg of *Aloe vera* and 150 mg of *Aloe ferox* showed the best repeatability and still very good solubility of the extract.

3.2.2. Precision and repeatability

Repeatability for both, the amount of aloin as well as the total content of anthranoids was assessed for each of the two plant species, showing relative standard deviations of 2.06% and 1.18% for *Aloe vera* and 0.71% and 0.66% for *Aloe ferox*, respectively (Table 2). Thereby, the amount of aloin is expressed in % weight (which is also the unit of the European Pharmacopeia) and the total amount anthranoids is given in mmol/g, a unit that has been suggested for the quantification of (pharmacologically active) compound classes in one of our previous studies [28]. Regarding the RSD values observed for the two plant species, the method showed a much better repeatability for *Aloe ferox* than for *Aloe vera*, which is why subsequent precision studies were conducted with the latter species. One possible explanation for the differing RSD values is the higher amounts of plant material applied for the analysis of *Aloe ferox* and the thus higher concentrations of aloin in the sample.

Precision measurements of *Aloe vera* samples on two different days revealed intra-day RSD values of 2.98% and 1.58% for the aloin content and 1.62% and 2.33% for the total anthranoid content, respectively (Table 3). In contrast, inter-day RSD values were significantly higher, with 6.77% for the aloin content and 4.36% for the total anthranoid content. However, with intra-day RSD values of less than 3% and inter-day values of less than 7%, the precision is comparable to other methods applied in natural product analysis and still far below the values obtained with the method of the Pharmacopeia, which were found to be 15.8% (*Aloe vera*) and 13.0% (*Aloe ferox*), respectively (inter-day, N = 10).

3.2.3. Specificity and selectivity

Regarding the specificity of the method, the heteronuclear 2D NMR assay offers a great advantage compared to 1D approaches, by using the characteristic cross correlation of the C-glucosidic bond of the anthrone ring or the hydroxymethylene group attached to the aromatic ring, respectively. In terms of selectivity, further anthranoids have been reported to be present in the investigated species [19]. One such compound is 7-hydroxyaloin [29,30], which is a marker compound for *Aloe vera* (and not present in *Aloe ferox*) and shows a cross peak in position 4.51 ppm/43.8 ppm, that is detectable (and quantifiable) in the samples (Fig. 2). 5-hydroxyaloin and aloinosides A and B are both characteristic for *Aloe ferox*. Aloinosides, which are known to be only present in one of two chemotypes [26] show a higher ^{13}C shift of the methylene group due to the attached rhamnose moiety and could therefore be clearly distinguished form aloins A and B [31]. However, they were not present in the plant material used for this study. 5-Hydroxyaloin, instead, is considered a marker compound for *Aloe ferox*. Because NMR data for this compound were only retrieved in acetone-d_6 [32], no clear identification of the relevant cross peak is possible. However, the lower shift of the ^{13}C values and the higher shift of the ^{1}H values of the methylene group, as well as the fact that the signal of 7-hydroxyaloin was distinguishable, let assume no co-quantification of the compound. Thus, the developed method delivers both, specific determination of aloe anthranoids by quantification of the methylene signal as well as selective quantification of aloin among other anthranoids by integration of the CH-10 cross peak. Additionally, *Aloe vera* can be distinguished from *Aloe ferox* by the presence of the CH-10 cross peak of 7-hydroxyaloin.

3.2.4. Accuracy

For the determination of accuracy both samples were measured ten times with the official method of the European Pharmacopeia, which uses spectrophotometric measurements of the

anthraquinone forms after oxidative cleavage of the sugar moieties. Thereby, a minimum of 28% hydroxyanthracene derivatives (calculated as aloin) is required for *Aloe vera* samples and a minimum of 18% for samples of *Aloe ferox*. Quantification of the samples investigated in the present study with the method of the Pharmacopeia revealed contents of 37.33 ± 5.90% for *Aloe vera* and 25.11 ± 3.26% for *Aloe ferox* (Table 4). Thus, both samples fulfilled the requirements of the Pharmacopeia. Regarding the results obtained with the quantitative HSQC approach, lesser values were obtained for both plant species. In the case of *Aloe vera*, the content of aloin was determined with 27.04%, whereas the total anthranoid content was calculated with 34.40% and thus was much closer to the content of the Pharmacopeia (92.2% recovery rate). As mentioned above, *Aloe vera* also contains 7-hydroxyaloin as additional anthranoid, which was subsequently quantified using the calibration curve of aloin. The value of 7-hydroxyaloin was determined with 4.74 ± 0.12%, and, after the amount of aloin, is the main contribution to the total anthranoid content. For *Aloe ferox*, the differences between the amount of aloin and the total anthranoid content were less significant (21.57% versus 22.48%), indicating that additional anthranoids are less abundant in this species, or at least in this chemotype.

3.3. Band-selective qHSQC (bs-qHSQC)

3.3.1. bs-qHSQC method I

In order to overcome the long acquisition time of the complete spectral range HSQC, a specific pulse program (shsqsctgpsi2.2) was applied, reducing the spectral width of the ^{13}C channel from 165 to 30 ppm and the measuring time from 135 to 32 minutes (Fig. 4 and Fig. S2-S4). Thus, the experiment was in the time range of conventional HPLC methods. However, by recording 52 increments for the measured spectral width, the resolution was kept at the same

level as the validated csr-qHSQC approach, still obtaining one cross peak signal for the aloin A and B mixture. The band-selective qHSQC method was subsequently evaluated in terms of repeatability (Table 2) and accuracy (Table 4), with regard to the csr-qHSQC method and the assay of the European Pharmacopeia. Repeatability measurements using bs-qHSQC, revealed RSD values of 2.80% and 1.65% (aloin and total anthranoids) for *Aloe vera*, as well as 1.86% and 1.34% for *Aloe ferox*, respectively, and thus were somewhat higher than with the csr-qHSQC approach. However, in terms of accuracy, the bs-qHSQC method revealed higher recovery rates for the total anthranoid content (97.3% for *Aloe vera* and 96.6% for *Aloe ferox*) than the csr-qHSQC approach when compared to the method of the Pharmacopeia.

3.3.2. bs-qHSQC method II

After the method was successfully tested for its use in the quantification of aloin and the total anthranoid content, band-selective HSQC was additionally evaluated for the separation of the CH-10 cross peaks of aloin A and B and the determination of their ratio in the respective extracts. It was shown that much stronger improvements of the resolution are possible for the samples under discussion by choosing e.g. 400 increments for the ^{13}C spectral range and collecting only 10% of those by non-uniform sampling, thereby using the same acquisition time and allowing the separation and independent quantification of aloin A and aloin B in the aloin standard, but not in the extracts. Therefore, the spectral width of the ^{13}C channel was further reduced to 2 ppm and the number of increments was set to 128, only collecting half of the increments via non-uniform sampling. Additionally, delay times were optimized for the coupling constant of the CH-10 methine group (132 Hz). The inter-scan delay was set to 1.5 s, because similar relaxation rates were assumed for the methine unit in the diastereoisomeric aloins. No significant differences in the determined aloin A/B ratio were observed when running

this experiment with a delay of 20 s (data not shown). Thus, cross peaks of both diastereomers were separated in 4 minutes measuring time (Fig. 5a and b). For quantification, 1D projections of the proton range were created and the respective signals were integrated (Fig. 5c), giving an aloin A/B ratio of 1 to 0.80 for the isolated compound. This ratio was also observed when $^{13}C\{^{1}H\}$ NMR, 135 DEPT ^{13}C NMR and 2D sHSQC methods were applied (Fig. S5-S7). Thus, the bs-qHSQC method II was used to investigate the aloin A/B ratio in the two plant species. However, the extract of *Aloe vera* and that of *Aloe ferox* showed the same ratio for the two diastereomers.

3.3.3. Stability of aloin

After samples of aloe extracts were found to lack the CH-10 cross peak after storage of one month, extracts of both species as well as the standard compound were evaluated for their stability in solution. Additionally, the aloin A/B ratio was investigated, in order to find different degradation dynamics between the two diastereomers. Thereby, the content of aloin and the ratio of the two diastereomers was obtained using the bs-qHSQC method II and subsequent integration of the 1D projections. Whereas the aloin content in the extracts was reduced to approximately 50% within 10-20 days (Fig. S8), the aloin standard compound did only show a degradation of a few percent during that time. Regarding the two diastereomers, both aloin A as well as aloin B decomposed to the same extent. However, considering the use of aloe in liquid preparations, e.g. Swedish bitters, the stability of aloin and its derivatives is at least questionable, even more so as such preparations contain other herbs that might contribute to the degradation of these rather labile components.

4. Conclusion

In this study, a band-selective quantitative HSQC method for the quality control of *Aloe vera* and *Aloe ferox* was established. The method allows the determination of the total anthranoid content, and thus the value required in the European Pharmacopeia, as well as the selective quantification of aloin (and 7-hydroxyaloin) among other anthranoids. Moreover, the presence of the 7-hydroxyaloin signal allows the differentiation of the two species. Validation according to ICH guidelines revealed acceptable values in terms of precision and accuracy, which are comparable to standard methods. The use of specific pulse programs furthermore allows quantification in time ranges, which are comparable to e.g. HPLC methods. As a second outcome of this study, a fast band-selective HSQC method for the separation of proton-bearing carbons with close shift values was established and used to determine the ratio of aloin A and B. The co-occurrence of diastereomers or other chemically similar and thus not easily separated compounds is a general phenomenon in phytochemistry. Therefore, fast analysis of their ratios might facilitate their investigation.

Supporting information

The supporting information contains complete spectral range and band-selective (bs-) HSQC diagrams of aloin, bs-HSQC diagrams of *Aloe vera* and *Aloe ferox*, ^{13}C {^{1}H} NMR and ^{13}C (135 DEPT) NMR spectra and an HSQC diagram for the determination of the aloin A/B ratios as well as graphs for the degradation of aloin in aloe extracts.

Conflict of interest

The authors declare no conflict of interest.

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Figures

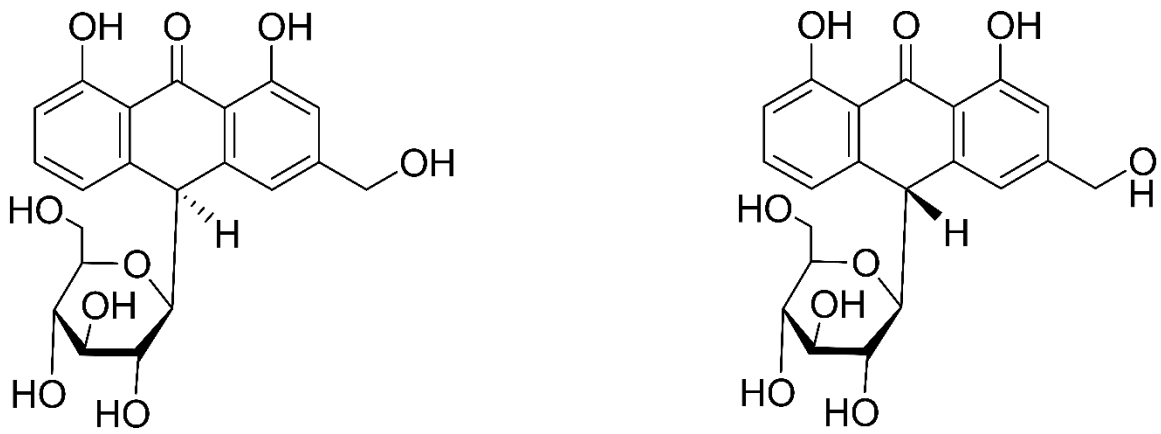


Fig. 1: Chemical structures of aloin A (left) and B (right).

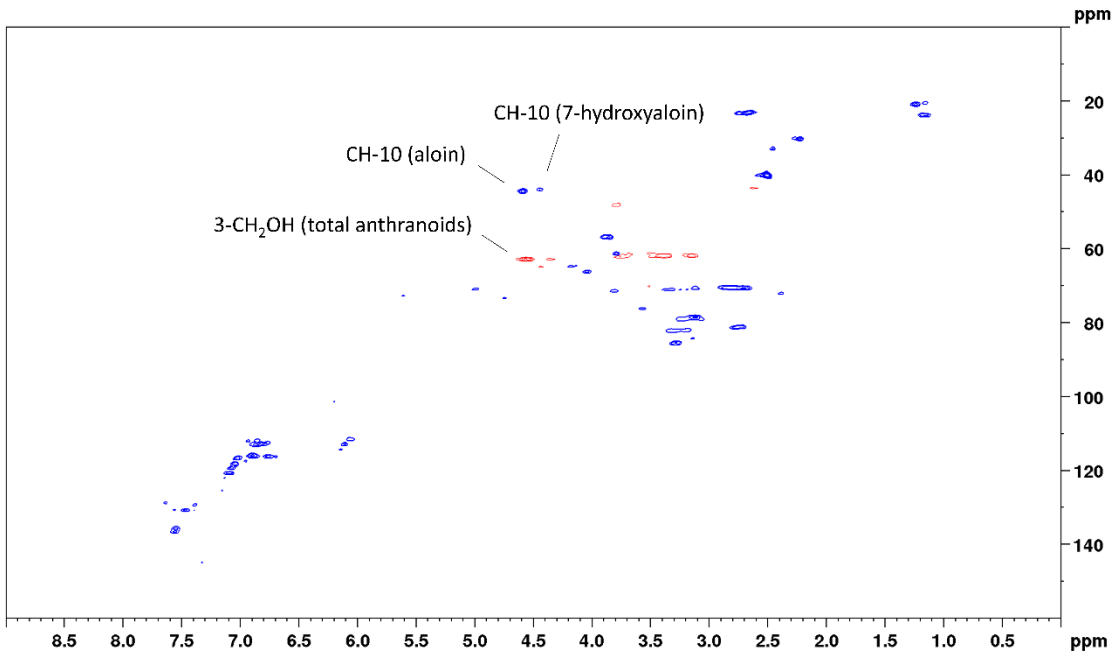


Fig. 2: Complete spectral range HSQC diagram of *Aloe vera* extract in DMSO-d_6 (100 mg drug per mL, positive contour lines are shown in blue colour, negative contour lines in red colour).

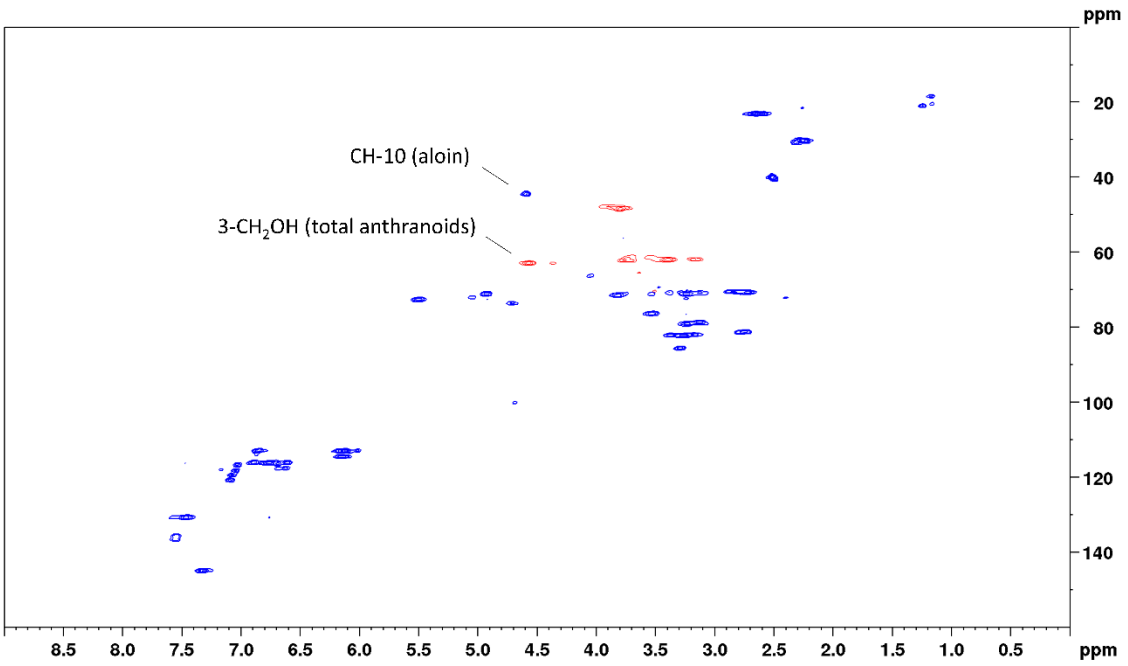


Fig. 3: Complete spectral range HSQC diagram of *Aloe ferox* extract in DMSO-d_6 (150 mg drug per mL, positive contour lines are shown in blue colour, negative contour lines in red colour).

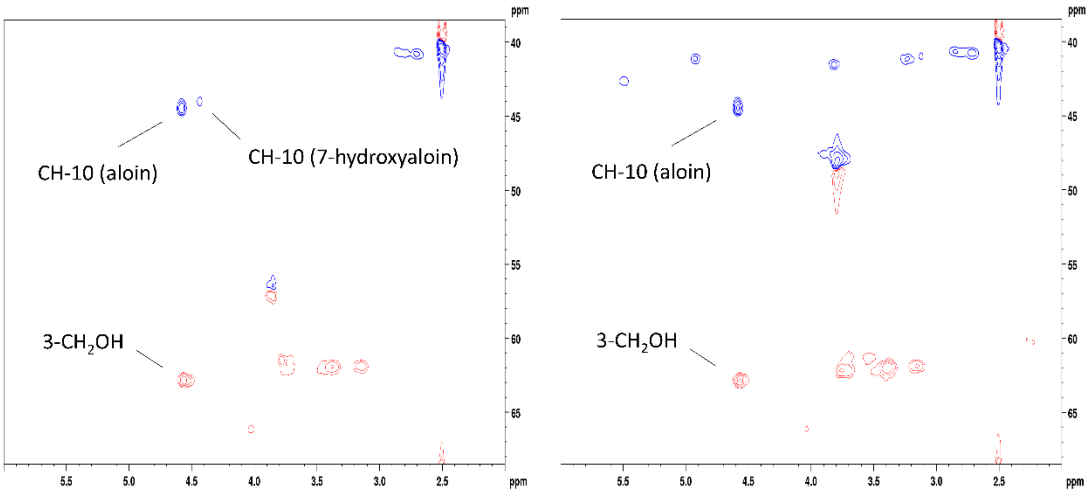
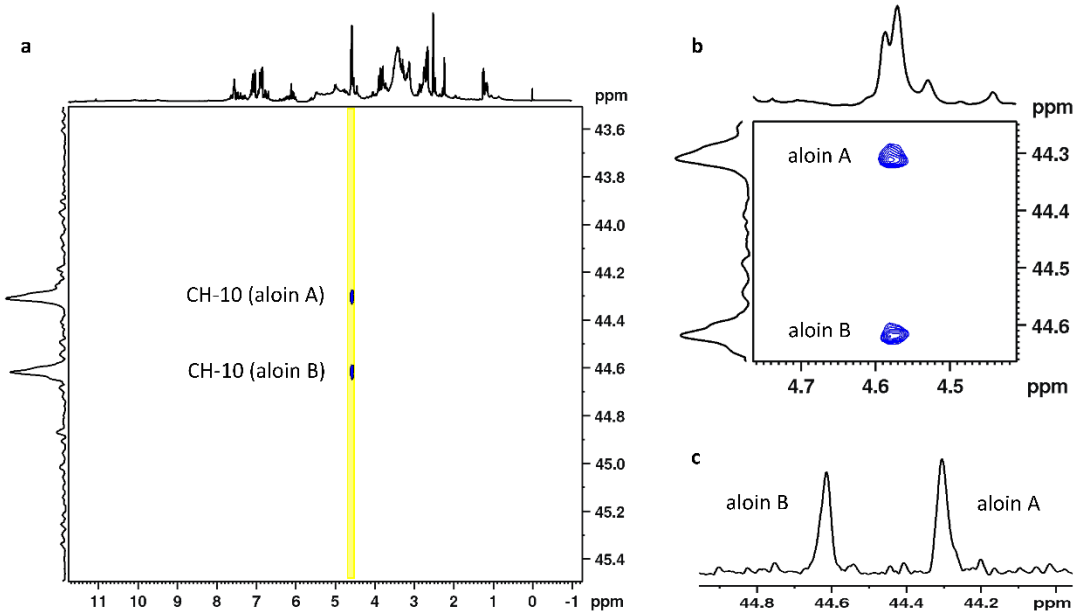


Fig. 4: Band-selective HSQC diagrams (method I) of *Aloe vera* (100 mg drug per mL, left) and *Aloe ferox* (150 mg drug per mL, right) extracts in DMSO-d_6 (positive contour lines are shown in blue colour, negative contour lines in red colour).



1

2 Fig. 5: Determination of the aloin A/B ratio in the *Aloe vera* extract using the band-selective

3 qHSQC method II (a and b) and the calculated 1D projection (c).

Table 1

Regression equations for the calibrated cross correlations of aloin, coefficients of determination (R^2) and limits of quantification (LoQ). Determined compounds are shown in parenthesis.

Cross-correlation signal	Regression equation	R^2	LoQ
H-10 (content of aloin A and B)	$y = 1.183.115\ x - 5.487.028$	0.9986	1.63 mg/mL
3-CH_2OH (total anthranoids)	$y = -2.058.685\ x + 5.603.050$	0.9988	0.815 mg/mL 1.95 mmol/L*

*for quantification of total anthranoids molar concentrations were used

Table 2

Repeatability for *Aloe vera* and *Aloe ferox* samples using complete spectral range (csr-) and band-selective qHSQC. Results are given in % weight for the content of aloin and in mmol/g for the total anthraquinone content (AQs).

	csr-qHSQC				band-selective qHSQC			
	Aloe vera		*Aloe ferox*		*Aloe vera*		*Aloe ferox*	
N	aloin	AQs	aloin	AQs	aloin	AQs	aloin	AQs
1	27.56	0.8310	21.66	0.5419	30.34	0.8733	24.87	0.5711
2	27.80	0.8344	21.72	0.5411	30.70	0.8706	24.70	0.5793
3	26.93	0.8254	21.71	0.5359	29.95	0.8697	24.70	0.5776
4	27.03	0.8141	21.53	0.5350	30.59	0.8844	23.85	0.5737
5	26.60	0.8177	21.38	0.5329	28.43	0.8730	24.56	0.5924
6	26.34	0.8100	21.4	0.5365	30.42	0.8415	23.84	0.5848
mean	27.04	0.8221	21.57	0.5372	30.07	0.8687	24.42	0.5798
SD	0.56	0.0097	0.15	0.0035	0.84	0.0144	0.45	0.0078
RSD	2.06%	1.18%	0.71%	0.66%	2.80%	1.65%	1.86%	1.34%

Table 3

Intra- and inter-day precision for *Aloe vera* samples using csr-qHSQC. Results are given in % weight for the content of aloin and in mmol/g for the total anthraquinone content (AQs).

	Intra-day (day 1)		Intra-day (day 2)		Inter-day (N=12)	
N	aloin	AQs	aloin	AQs		
1	33.49	0.9214	29.78	0.8467		
2	33.45	0.9364	29.71	0.8430		
3	32.62	0.9271	29.03	0.8432		
4	34.13	0.9253	28.91	0.8798		
5	31.62	0.9059	29.18	0.8604		
6	34.24	0.9507	30.05	0.8894		
mean	33.26	0.9278	29.444	0.8604	31.35	0.8941
SD	0.99	0.0150	0.465	0.0200	2.12	0.0390
RSD	2.98%	1.62%	1.58%	2.33%	6.77%	4.36%

Table 4

Determination of accuracy. Content of aloin and total anthraquinone content obtained from complete spectral range (csr-) and band-selective (bs-) qHSQC were compared to the content determined with the method of the European Pharmacopoeia.

		qHSQC	Ph.Eur.	recovery rate
Aloe vera (csr)	aloin	27.04 ± 0.56%	37.33 ± 5.90%	72.4%
	total AQs*	34.40 ± 0.41%		92.2%
Aloe vera (bs)	aloin	30.07 ± 0.84%		80.6%
	total AQs*	36.34 ± 0.60%		97.3%
Aloe ferox (csr)	aloin	21.57 ± 0.15%	25.11 ± 3.26%	85.9%
	total AQs*	22.48 ± 0.15%		89.5%
Aloe ferox (bs)	aloin	24.42 ± 0.45%		97.3%
	total AQs*	24.26 ± 0.33%		96.6%

* calculated as aloin