Quantification of diterpene acids in Copaiba oleoresin by UHPLC-ELSD and heteronuclear two-dimensional qNMR

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ABSTRACT

In this study, we present the quantitation of eight diterpene acids in the oleoresin of *Copaifera reticulata* Ducke by UHPLC-ELSD and quantitative HSQC (heteronuclear single quantum correlation spectroscopy). UHPLC was performed using reversed phase material and external calibration and showed RSD values of ≤ 3% (repeatability) and ≤ 4% (precision), and mean recovery rates of 91.2 to 104.8%. LOQs were determined with 10 and 20 µg/mL, and LODs with 4 and 8 µg/mL, respectively. For the qHSQC method, calibration curves of eight different NMR cross-peaks (furylic, endo- and exocyclic methine signals, exocyclic methylene and methyl signals) were established and normalized with dimethyl terephthalate, which served as internal standard. This approach allowed the direct quantification of four major and one minor diterpene, whereas simple calculation procedures led to the contents of the remaining minor compounds. Comparison with the results of the UHPLC assay showed good agreement for seven of the eight diterpene acids. In terms of precision, the qHSQC method was advantageous for the quantification of the three main compounds, whereas UHPLC-ELSD was superior in the determination of the minor components. In contrast to previous reports, kolavenic acid was identified as a major diterpene acid in the oleoresin of *Copaifera reticulata*, with amounts of 4.0 ± 0.3%.

Keywords: *Copaifera reticulata*, diterpenoids, quality control, polyalthic acid, kaurenoic acid, natural product

1. Introduction

The oleoresin of selected *Copaifera* species (Fabaceae), commonly known as Copaiba oil, is a traditional Brazilian remedy for the treatment of urinary tract infections, respiratory diseases, wound healing, rheumatism, ulcers, and tumours [1,2]. Copaiba oil is furthermore used as an antibacterial, antihelmintic, trypanocidal and leishmanicidal agent and thus plays an important role in the primary health care of the indigenous population [3,4]. Due to its wide application in folk medicine, the Copaiba oleoresin was subjected to various studies examining its chemical composition and the correlation with its health benefits [1,5]. In the course of these studies, *in vitro* and *in vivo* activities were reported for the crude oleoresin, its fractions, and several isolated compounds [6-10].

According to literature, sesquiterpenes constitute the major compound class in Copaiba oil, with shares of up to 90% by weight, whereas diterpenes, and here mainly diterpene acids, account for the remainder of the oleoresin [5]. Interestingly, the sesquiterpene composition shows only slight variation between the medicinally used *Copaifera* species (35 sesquiterpenes were detected in all of these species) [5]. In contrast, the diterpenes (kaurane-, clerodane-, and labdane-type diterpenes) show high interspecific variability [1], and therefore became of particular interest in recent years, even more so, as different biological activities were observed among the investigated *Copaifera* species [5-10].

This interest not only concerned the pharmacology of the diterpene acids, but also had an impact on the analytical works, which focussed on the characterization and differentiation of *Copaifera* species by LC-MS [10-12]. The first work by Santiago et al. compares three *Copaifera* species, *C. duckey*, *C. mulitjuga*, and *C. reticulata*, with regards to the abundance of six different diterpene acids and one sesquiterpene acid. However, one of the two main peaks in the chromatograms of *C. duckey* and *C. reticulata* is not identified in this study. Additionally, the chromatograms show varying retention times for *ent*-copalic acid (**6**) among the three species

and an asymmetric peak shape in the chromatogram of *C. duckey*, indicating the presence of a co-eluting compound. The second study by Bardají et al., which investigated only *C. reticulata*, accepts these findings, whereas the third study introduces *ent*-kaurenoic (**5**) acid as another major diterpene acid, but still does not address the unidentified main compound. The compound was finally identified as (13*E*)-*ent*-labda-7,13-dien-15-oic (**8**) acid by Carneiro et al. in a study on the quantification of six diterpene acids by HPLC-PDA [13]. Unfortunately, the presented method shows deficits in separation, as *ent*-copalic acid (**6**) and (13*E*)-*ent*-labda-7,13-dien-15-oic (**8**) are co-eluting, and as it does not take *ent*-kaurenoic acid (**5**) into account, which was before declared as one of the most abundant diterpenes. To conclude, no clear-cut qualitative statement and therefore no sound quantitative statement can be made on the diterpene acids in the Copaiba oleoresin, even though all four analytical studies were published by the same institution.

In order to clarify the question on the kinds of diterpene acids and their content in Copaiba oleoresin, an SPE-UHPLC-ELSD assay was developed. Applying evaporative light scattering as detection method eliminates the risk of not detecting compounds due to weak UV-absorption or ionization and thus allows more meaningful analysis of the diterpene acids, which have been enriched in a previous step. As alternative approach, a quantitative NMR method, based on heteronuclear single quantum correlation spectroscopy (HSQC), a two-dimensional (2D) NMR technique with indirect detection of carbon signals via the more sensitive proton shifts, will be presented. Quantitative one-dimensional (1D) 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. The influence of experimental NMR parameters has been investigated in detail and are well understood [14-16]. However, one-dimensional quantitative NMR spectroscopy requires separated signals for single compounds or groups of compounds. In the case of complex multicomponent mixtures with

severe signal overlap, for example in metabolomics studies, two-dimensional NMR techniques have been employed and proved applicable, when using specialized pulse programs and measuring schemes or employing suitable internal standard compounds [17-20]. The results of such a qHSQC method and the UHPLC-ELSD method will be discussed in terms of precision and accuracy, and in comparison to previous publications.

2. Materials and methods

2.1. Chemical Reagents and Material

Copaiba oleoresin was obtained through perforation of the trunk of a *C. reticulata* tree located in Rondon do Pará, Brazil, in August 2016. The plant species was determined by the Brazilian Agricultural Research Corporation – Ministry of Agriculture, Livestock, and Food Supply. Deuterated methanol (Lot P3021, 99.80%) and chloroform (Lot D007F, 99.80%) for NMR spectroscopy were obtained from Euriso-top GmbH, Saarbrücken, Germany, and conventional 5 mm sample tubes were purchased from Rototec-Spintec GmbH, Griesheim, Germany. LC-MS grade formic acid, dimethyl terephthalate (Lot BCBT9974, TraceCERT, with a purity of 99.95% for quantitative NMR) were obtained from Sigma Aldrich Co., St. Louis, MO, USA. Acetonitrile, methanol, water (all of LC-MS grade), and other (analytical grade) solvents were purchased from VWR International GmbH, Darmstadt, Germany. SPE columns (Chromabond SB 3 mL/500 mg) were obtained from Macherey-Nagel GmbH & Co. KG, Düren, Germany.

2.2. General experimental procedures

UHPLC analyses were performed on a VWR-Hitachi Chromaster Ultra RS equipped with a 6170 binary pump, 6270 autosampler, 6310 column oven, 6430 DAD, and a Sederé 100

evaporative light scattering detector using a Phenomenex Luna Omega C18 column (100 × 2.1 mm, 1.6 µm particle size). LC-MS analyses were carried out on a Shimadzu Nexera 2 Liquid chromatography connected to a LC-MS 8030 triple quadrupole mass spectrometer using electrospray ionization in the negative mode. Preparative MPLC was accomplished with a Buchi PrepChrom C-700 using a Buchi PrepChrom C18 column (250 × 30.0 mm, 15 µm particle size).

NMR spectra were recorded using a Bruker Avance III 300 NMR spectrometer operating at 300 MHz for the proton channel and 75.5 MHz for the ^{13}C channel with a 5 mm PABBO broad band probe with a z gradient unit. Measurements were performed at 298 K, the temperature was calibrated with a methanol-d_4 solution. For each sample automatic tuning and matching of the probe was performed and additionally automatic shimming of the on-axis shims (Z to Z5). The automatic receiver gain adjustment mode was employed. The Bruker Topspin software 2.1 pl 6 was used and the pulse program *hsqcedetgpsisp2.3* with multiplicity editing and shaped 180° pulses of the pulse program library was employed with a setting of the relevant delay for the ^{1}JCH coupling to 3.45 ms, which corresponds to 1/(2 × 145 Hz). ^{13}C decoupling was performed by the GARP sequence. For the proton (F2) channel 640 data points were recorded, with a spectral width of 8.9 ppm corresponding to an acquisition time of 0.12 s; for the ^{13}C (F1) channel 512 increments with a spectral width of 165 ppm and thus an incremented delay of 80.3 µs were selected. 2 Scans were collected per increment with an optimized inter-scan delay of 10 s. Total measurement time for each HSQC diagram was 3 h. Settings for quantitative one-dimensional ^{1}H NMR experiments are described in Cicek et al. 2018 [21].

2.3. Isolation of standard compounds

4.50 g oleoresin were dissolved in 300 mL diethyl ether and extracted five times with 5% potassium hydroxide aqueous solution. The combined aqueous layers were acidified to pH 2

using 25% hydrochloric acid resulting in precipitation of the diterpene acids. The aqueous layers were then extracted with diethyl ether yielding 1.56 g of diterpenoids after evaporation of the solvent. The diterpene fraction was subjected to preparative MPLC using isocratic elution for 60 minutes with methanol-water (80:20 v/v) followed by methanol-water (85:15 v/v) for another 60 minutes. The resulting 168 fractions yielded *ent*-agathic acid (**1**, fractions 20-22, 9.3 mg), (13*E*)-*ent*-labd-8(17)-en-15,18-dioic acid (**2**, fractions 24-29, 19.1 mg), *ent*-agathic acid 15-*O*-methyl ester (**3**, fractions 34-36, 6.0 mg), *ent*-polyalthic acid (**4**, fractions 38-85, 276 mg) and *ent*-kaurenoic acid (**5**, fractions 96-106, 133 mg), *ent*-copalic acid (**6**, fractions 109-112, 14.1 mg), kolavenic acid (**7**, fractions 116-126, 18.5 mg), and (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**8**, fractions 137- 146, 39.3 mg) (Fig. 1). Structures of the isolated compounds were elucidated by comparing MS and NMR spectra to literature data [15-20].

2.4. Chromatographic analysis

Solvents used for the experiments were 0.1% formic acid in water and methanol applying the following gradient: 50% methanol to 77% methanol in 3 minutes, to 82% methanol in 28 minutes, to 90% methanol in 30 minutes. Post-run 5 minutes. Flow rate was 0.2 mL/min, injection volume was 2 µL, and temperature was set to 25°C. Drift tube temperature of the ELSD was 50°C and nitrogen gas flow was set to 3.5 L/min. Gain was at level 5, filter at 6 s and sampling rate at 100 ms (10 Hz). Additionally, UV traces were recorded at 205 and 210 nm wavelength. For quantification of the diterpenes calibration curves of the isolated compounds were established in concentration ranges of 80 to 1600 µg/mL for *ent*-polyalthic acid (**4**), 40 to 800 µg/mL for *ent*-kaurenoic acid (**5**) and 20 to 400 µg/mL for all other compounds. Extraction of diterpene acids from oleoresin was accomplished by anion exchange solid phase extraction. Therefore, SPE columns were equilibrated with two column volumes of petroleum ether (boiling point range 40-60°C). 50.0 mg of Copaiba oleoresin was dissolved in 500 µL of

dichloromethane, subjected to the SPE column and washed with three column volumes of petroleum ether. After drying of the SPE column, diterpene acids were eluted with 2000 µL 5% formic acid in methanol and further diluted to a final volume of 10.00 mL with pure methanol.

2.5. Spectroscopic analysis and data processing

To quantify the diterpene acids by quantitative HSQC, calibration curves for eight different cross correlations were established using solutions of 9.00, 18.0 and 36.0 mg/mL of compound **4** and 3.00, 6.00 and 9.00 mg/mL of compound **8**, respectively. As these calibration curves were used for determination of additional compounds, regression equations were calculated in mol/L. All solutions were prepared with methanol-d_4 containing 1.50 mg/mL dimethyl terephthalate, which served as internal standard and for normalization purposes. For analysis of Copaiba oleoresin, the diterpene acids were removed as described above, but after eluting the acids with 5% formic acid in methanol the solvent was evaporated to dryness and the residue was re-dissolved in 500 µL internal standard solution. Data processing was performed using the Topspin software. The raw data matrix was zero-filled to 2048 data points in both dimensions before multiplication with squared sine function with an SSB value of 2. Automatic phase correction was carefully manually checked and if necessary adjusted to achieve pure positive or negative absorption signals. 2D HSQC data was processed and evaluated using the manufacturer's software topspin 2.1 pl6. Thus, the automatic peak detection routine ("peak picking") in the 2D mode was employed in the spectral region of interest including the internal standard, with the following parameters: mi 0.03; maxi 1; ppdiag 1; ppresol 5; ppmpnum 50; ppiptyp parabolic; psign both. All thereby detected peaks were evaluated for signal intensities and integrated using the automatic peak integration of the topspin software.

2.6. Method validation

Both methods were validated for linearity, repeatability, precision and accuracy. The UHPLC method was additionally validated for limit of detection and limit of quantification. Limit of detection (S/N ratio of 3) and limit of quantification (S/N ratio of 10) were determined by serial dilution of standard solutions. Accuracy of the UHPLC method was determined by spiking samples with different amounts of standard compounds (low, medium and high spike). This was accomplished by mixing 750, 500 and 250 µL of sample solution with 250, 500 and 750 µL of standard solution 4, resulting in increasing concentrations of compounds **1** and **3** and decreasing concentrations of the other compounds in the final solutions.

The theoretically present amounts in relation to the determined ones were expressed as percent of recovery. Accuracy of the qHSQC method was assessed by comparison of the results with the results obtained from the UHPLC experiments. Precision measurements included intra- and inter-day precision and repeatability and were accomplished in the same manner for both assays. 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. For UHPLC analysis, 5-point calibration curves were created and expressed as quadratic functions, due to the exponential response of the detector. Calibration curves of cross correlations for the qHSQC experiments were linear and created by 3-point measurements.

3. Results

3.1. UHPLC-ELSD

As the contents of the desired compounds in the oleoresin vary between less than 0.5% and more than 20%, calibration curves had to cover different concentration ranges. Thus, calibration curves from 80 to 1600 µg/mL for compound **4**, from 40 to 800 µg/mL for compound **5**, and from 20 to 400 µg/mL for all other compounds were established. Regression equations and the respective coefficients of determination are shown in Table 1. Limits of quantitation (LOQ) and detection (LOD) for each compound were determined by subsequent dilution of standard compounds and are also given in Table 1. Due to broader peak shapes of compounds **5** to **8** (Fig. 2), LOQ and LOD of these compounds were higher (20 and 8 µg/mL) than those of the remaining compounds (10 and 4 µg/mL).

Investigation of repeatability led to relative standard deviations of 1.2% for compound **4** to 2.7% for compound **6** (Table 2). The higher RSD value of compound **6** might be due to its imperfect separation from compound **7**. Precision measurements included both, intra-day and inter-day variation. Intra-day precision resulted in relative standard deviations of 1.3% to 3.7%, whereas inter-day variability was found to range from 1.6% to 4.0%. Here also the more polar (and better separated) compounds showed lower deviations.

Accuracy was determined by dividing the measured concentration of the spiked sample through the estimated concentration (un-spiked sample concentrations plus spiked amount of standard compounds). Overall recovery rates were found between 86.5 and 111.1% and are depicted in Table 3. Mean recovery rates were calculated as 104.8% (**1**), 103.0% (**2**), 94.0% (**3**), 98.3% (**4**), 103.8% (**5**), 92.8% (**6**), 96.0% (**7**), and 91.2% (**8**).

3.2. Quantitative NMR

The quantitative NMR method consisted of a standard 2D HSQC NMR experiment of the spectrometer manufacturer's pulse program library, which is a gradient selected and sensitivity enhanced $^{1}H - ^{13}C$ HSQC experiment with carbon decoupling during acquisition of the free

induction decay (FID), having the advantage that it can be employed by all spectrometers equipped with probe heads with gradient selection and does not require especially written pulse-programs. Thereby the signals are dispersed into the second (indirect ^{13}C) dimension. As the NMR experiments in this investigation were performed at a moderate field strength of about 7.1 T, corresponding to a proton resonance frequency of 300 MHz, two scans (FIDs) were collected per increment to achieve sufficient intensity of the cross peaks. The intensities of these cross peaks are not directly proportional to the analytes' concentrations but depend on several parameters, especially the heteronuclear C,H coupling constant over one bond (^1J), homonuclear H,H coupling constants mainly over two and three bonds (^2J, ^3J), uniform excitation of all signals , and the T_1 and T_2 relaxation rates. For the relaxation time T_1 a value of 10 s was experimentally determined to be sufficiently long, all the other factors were accounted for by using dimethyl terephthalate as internal standard with similar structural motifs, that is an aromatic methine group and a methoxy group. The total experimental time can be considered to be reduced drastically when working either at higher magnetic fields or using cryogenic probes (reduction of the number of scans per increment) or using nonuniform sampling (reducing the number of increments [20]) which was not possible with the employed hard- and software. The parameters for data acquisition and processing are given in sections 2.2 und 2.5, respectively.

The quantitative NMR method was validated for linearity, precision, repeatability, and accuracy. For validation of linearity, calibration curves of eight different cross correlations (three furylic methine signals, two excocylic methylene signals, one exocyclic and one endocyclic methine signal, as well as one methyl group signal) were established using compounds **4** and **8** (Fig. 3). Regression equations are given in Table 4 and show coefficients of determination between 0.9978 and 1.0000. As mentioned above, calibration curves were normalized using dimethyl terephthalate. Here, the compound's aromatic methine signal was

chosen for normalization of the diterpenes' furylic and olefinic correlations, whereas the methyl group of the diterpenes was normalized with the methyl ester signals of the internal standard. These methyl ester signals were furthermore used for direct quantification of compound **3**. The content of diterpene acids in the Copaiba oleoresin was calculated using the following procedure based on the determination of the intensities of selected cross peaks:

4: The summed intensities of the three furylic signals (CH-14, CH-15 and CH-16) were divided by 3.

5: The summed intensities of the two kaurenoic exocyclic methylene signals (CH_2-17) were divided by 2.

8: The intensity of the endocyclic methine signal of CH-7 was used.

7: The intensity of the endocyclic methine signal of CH-3 was used.

6: The intensity of the methyl group (CH_3-18) signal minus the endocyclic methine signal of **8** was calculated.

3: Direct quantification of the methyl ester signal was performed using the methyl ester signal of the IS.

1: The intensity of the exocyclic methine (CH-14) signal minus the contents of **3**, **6**, **7**, and **8** was calculated.

2: The intensities of the exocyclic methylene (CH_2-17) signal divided by 2 were calculated and the contents of **1**, **3**, **4**, and **6** were subtracted.

These calculated concentrations of diterpene acids in the Copaiba oleoresin were used to determine the methods' repeatability, precision and accuracy (Table 5). Regarding the methods' repeatability, major differences between the individual compounds can be observed. While the main compounds (**4**, **5**, **7**, and **8**) show relative standard deviations of 1.9% to 2.4%, RSD values

increase to 3.9% for compound **6**, over to 9.9% and 13.7% for compounds **2** and **3**, respectively, and to 54.7% for compound **1**. These strongly differing values can be explained in two ways, the compounds' amounts in the Copaiba oleoresin and the error propagation due to the mode of calculation, as e.g. the standard deviation of compound **6** also includes the deviation of compound **8**, and the deviations of compounds **1** and **2** even include the deviations of four other components. A fact, that can also be observed within the precision measurements. Here, RSD values of compounds **1** to **4** were ranging from 1.0 % to 4.5% (intra-day) and from 1.7% to 4.3% (inter-day), while compounds **3** and **6** showed intra-day deviations from 3.7 to 7.4 and inter-day deviations of 6.8% (**3**) and 10.9% (**6**). Relative standard deviations of compounds **1** and **2** were 67.3% (intra-day) and 76.8% (inter-day), and 33.3% and 27.9%, respectively.

To investigate the methods' accuracy, results of the inter-day precision were compared to the inter-day precision results of the UHPLC method (Fig. 4). Correlations of the qHSQC results with those were very good for compounds **4**, **5** and **8** (± 5%), and still acceptable for compounds **1**, **3**, **6** and **7** (± 15%). Only for compound **2** much higher results in the qHSQC assay were obtained, indicating that another (not isolated) minor component might have been co-quantified.

4. Discussion

4.1. Solid phase extraction

In this study, two methods for the quantitative determination of eight diterpene acids in the oleoresin of *Copaifera reticulata* were developed. For both methods, strong anion exchange solid phase extraction was applied to separate the acids from the crude oleoresin. This SPE procedure, which was adapted from a method for the separation of fatty acids from cholesterol [28], had several advantages. First of all, it was important to know that all signals in the

respective spectra and chromatograms originated from the desired compounds, even more for the qHSQC approach, where subsequent signal separation is hardly accomplished. Secondly, the acidic fraction showed good solubility in methanol, whereas the crude oleoresin was only entirely soluble in chloroform or dichloromethane. Similar to the acidic fraction, also the pure diterpene acids were much better soluble in methanol, which was crucial for the calibration of the cross peak signals, with concentrations of up to 36 mg/mL. And last but not least, for validation purposes methanol is much more reliable than chloroform or dichloromethane, due to its higher boiling point and its miscibility with water. An overlay of the HSQC spectra of both the acidic and the remaining neutral fraction after solid phase extraction is shown in Fig. S1.

4.2. UHPLC-ELSD

For the development of the UHPLC method, different stationary phases and solvent systems were tested, and the overall best resolution was achieved using a Luna Omega C18 column (100 × 2.10 mm, 1.6 µm) and a solvent system consisting of 0.1% (v/v) formic acid in water and methanol. Other tested stationary phases did not show the necessary separating capacity for compounds **6**, **7**, and **8**. Acetonitrile instead of methanol led to co-elution of compounds **6** and **8**, and the use of ammonium formate (5 and 10 mM) instead of formic acid led to peak fronting. As increasing flow rates and temperatures resulted in decreasing resolutions for **6**, **7**, and **8**, separation was carried out at 25°C and 0.2 mL/min. Injection volumes of 2 µL and an ELSD gain of 5 (out of 7) allowed both, good repeatability and sufficient sensitivity at the same time, and thus enabled the quantification of both major (**4**, **5**, **7**, and **8**) as well as minor components (**1**-**3**, and **6**) in the oleoresin of *Copaifera reticulata*.

Comparison of the obtained ELSD chromatograms to chromatograms of the first two LC-MS studies reveals three major differences. The presence of *ent*-kaurenoic acid (**5**) as a major component, the significantly decreased content of (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**8**), and the occurrence of kolavenic acid (**7**). The latter has probably been confused with *ent*-copalic acid (**6**) in the mentioned publications and has most definitely been co-quantified with **6** and **8** in the study of Carneiro et al. [13]. Regarding the MS/MS approach of da Silva et al. [12], it is presumable that co-analysis also happened here, as all three compounds show masses of 304.474 Da and only SIR (instead of MRM) mode was applied for compounds of this molecular weight.

4.3. Quantitative NMR

To accomplish quantification of the same eight compounds by quantitative NMR, nine cross correlations were selected (highlighted in red in Fig. 1). These correlations comprised furylic methine signals CH-14, CH-15, and CH-16 of compound **4**, both cross peaks for the exocyclic methylene group in position 17 (compounds **1**-**6**), the endocyclic methine signals of compounds **7** and **8** (cross peaks for CH-3 and CH-7, respectively), the exocyclic methine signal of CH-14 in the side chain of compounds **1**, **3**, **6**-**8**, the methyl group in position 18 of compounds **6** and **8**, and the methyl ester of compound **3**.

According to literature, signal intensities as well as integrals are used for heteronuclear two-dimensional qHSQC [17–20]. Additionally, an internal standard can be applied for normalization purposes. Thus, in the next step four different quantification modes (intensities, normalized intensities, integrals and normalized integrals) were tested by comparison with quantitative ^{1}H NMR (qHNMR) using the acidic Copaiba fraction (Fig. S2, Table S1). For qHNMR four signals of compound **4** were chosen (highlighted in red in Fig. S2) and directly quantified with the internal standard. Quantitative two-dimensional NMR was accomplished

by external calibration with compound **4**. Therefore, calibration curves of the furylic CH-correlations and the exocyclic methylene group were established using either intensities, normalized intensities, integrals or normalized integrals. Dimethyl terephthalate was used for normalization and also as internal standard for qHNMR. As shown in Table S2, quantitative ^{1}H NMR results varied between 22 and 24%. When looking at the results of the two-dimensional measurements, both absolute intensities and absolute integrals showed very low recovery rates of less than 64 and 78%, respectively, indicating that normalization is essential to obtain accurate results. Of the normalized quantification modes, the difference between intensities and integration were negligible when applied for the furylic methine signals (recovery rates from 100.9 to 102.7% for intensities versus 96.6 to 99.8% for integrals), but evident for the methylene signal, where a recovery rate of 115.6% was observed for the integration method (in contrast to 100.5% for intensities). Thus, all further calculations were performed with signal intensities and normalization with dimethyl terephthalate.

The ^{1}H NMR spectrum moreover reveals the necessity of a two-dimensional qNMR approach. Even though several signals from neutral substances were separated by the SPE procedure (Fig. S1), only a few signals showed good resolution and peak shape, such as the furylic protons of compound **4**, the methoxy group of compound **3** and one of the exocyclic methylene protons. The remaining methylene protons showed signal overlay with residual water. However, water suppressing pulse programs would cause saturation effects of analyte signals near the water resonance and thus lead to false negative quantifications. Another problem appears for the endocyclic methine protons of compounds **7** and **8**, which show vicinal coupling (^{3}J) and aliphatic coupling (^{4}J) with neighbouring protons, resulting in very broad multiplets and hampering precise integration. Finally yet importantly, the quantification of the methyl group in position 18 of compounds **6** and **8** is hardly feasible with respect to the high number of interfering aliphatic signals.

Summarizing, the four main compounds (**4**, **5**, **7**, and **8**) and one minor compound (**3**) can, with some limitations, also be quantified with ^{1}H NMR. However, for the quantification of the remaining compounds, determination of the mentioned methyl-group in position 18 is crucial and thus a two-dimensional qNMR approach becomes necessary. Not only for the quantitation of *ent*-copalic acid (**6**), but also for the subsequent determination of compounds **1** and **2**. As these 3 compounds were quantified by a calculation procedure based on content subtraction of before measured compounds, higher RSD values occur. But still, in terms of accuracy, only compound **2** significantly deviates from the value measured by UHPLC-ELSD (Fig. 4).

5. Conclusion

In this study, two methods for the quantitative determination of diterpene acids in the oleoresin of *Copaifera reticulata* have been established. The first method applies UHPLC-ELSD after a solid phase extraction procedure with strong anion ion exchange material. The obtained results differ strongly from the previous investigations on Copaiba, as *ent*-kaurenoic acid and kolavenic acid were both identified as major components in the acidic fraction of the oleoresin. Due to the use of evaporative light scattering detection, accurate and precise, as well as meaningful results were obtained, rendering the method a valuable tool for the quality control of commercial Copaiba oil. As the additional compounds reported for other *Copaifera* species show shorter retention times than *ent*-copalic acid (**6**), the method should (at least after slight modifications) also be suitable for comparison studies.

Additionally, a two-dimensional quantitative NMR approach, qHSQC, is presented for the quantitation of the same eight compounds. Compared to the UHPLC assay, the qNMR method shows better precision for the three most abundant compounds (**4**, **5** and **8**), whereas standard

deviations for the minor compounds are significantly higher. Nevertheless, the method is suited for analysis of *Copaifera reticulata* oleoresin, needing only two major components (**4** and **8**) to determine the contents of eight diterpene acids. For investigation of other *Copaifera* species, calculation procedures must be adapted and additional signals (e.g. CH signal in position 3 of *ent*-hardwikiic acid) may have to be taken into account. However, as diterpene acids are neither restricted to the genus *Copaifera* nor to the Fabaceae family, the presented qHSQC method displays a useful alternative for their determination.

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Figure Captions

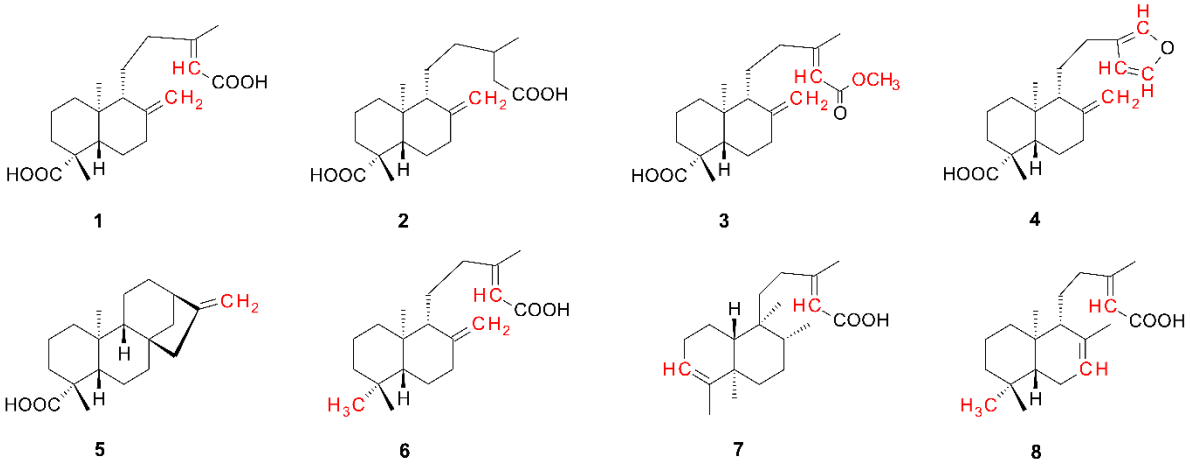

Figure 1: Chemical structures of isolated compounds: *ent*-agathic acid (**1**), (13*E*)-*ent*-labd-8(17)-en-15,18-dioic acid (**2**), *ent*-agathic acid 15-*O*-methyl ester (**3**), *ent*-polyalthic acid (**4**), *ent*-kaurenoic acid (**5**), *ent*-copalic acid (**6**), kolavenic acid (**7**), and (13*E*)-*ent*-labda-7,13-dien-15-oic acid (**8**). Signals used for quantitative NMR are highlighted in red.

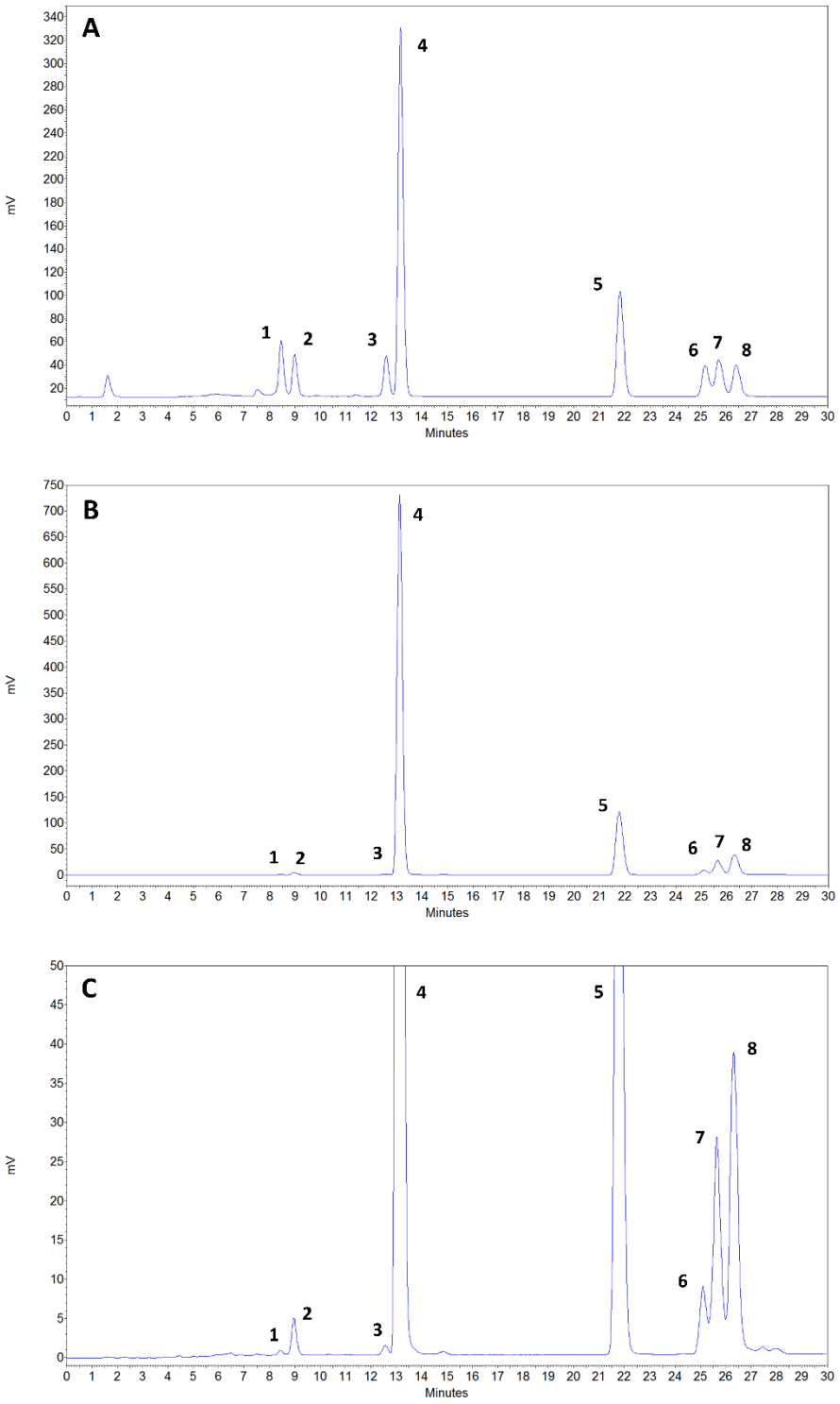

Figure 2: UHPLC-ELSD chromatograms of standards (A) and acidic Copaiba fraction (B and C).

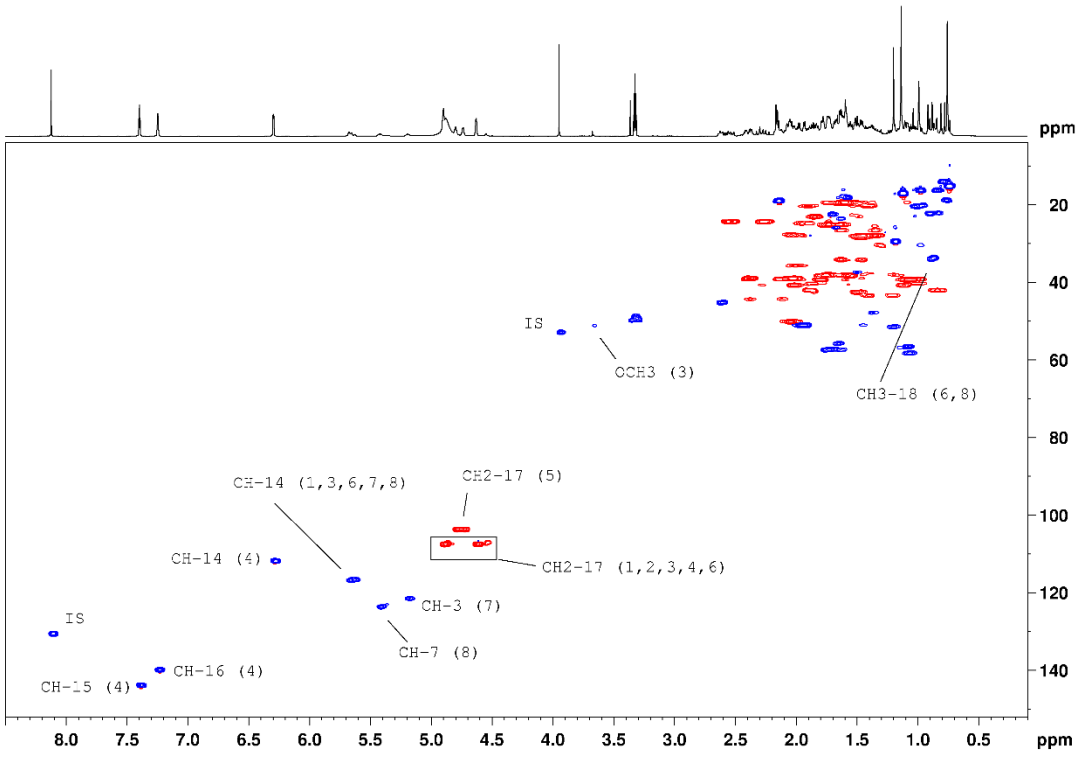

Figure 3: HSQC diagram of the acidic Copaiba fraction in methanol-d_4 containing 1.50 mg/mL dimethyl terephthalate. Signals used for integration are marked with corresponding correlations and the respective compounds (in parenthesis).

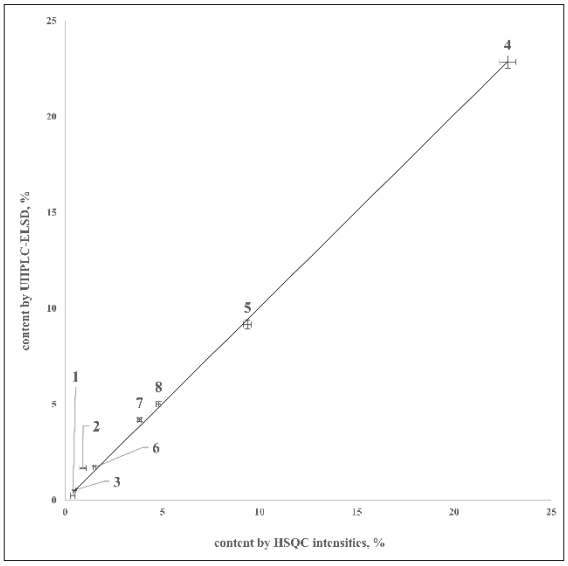

Figure 4: Comparison of UHPLC-ELSD and HSQC quantitation methods for compounds **1-8** in Copaiba oleoresin.

Table 1

Regression equations, correlation coefficients (R^2), LOD and LOQ (both in μg/mL) of compounds **1-8**.

	Regression equation	R^2	LOD	LOQ
1	$y = 1.675\times10^7 x^2 + 9.801\times10^5 x - 9.136\times10^3$	1.0000	4	10
2	$y = 1.154\times10^7 x^2 + 8.081\times10^5 x - 8.522\times10^3$	0.9999	4	10
3	$y = 1.556\times10^7 x^2 + 2.968\times10^5 x - 1.928\times10^3$	1.0000	4	10
4	$y = 5.548\times10^6 x^2 + 2.175\times10^6 x - 6.030\times10^2$	1.0000	4	10
5	$y = 1.440\times10^7 x^2 + 4.251\times10^5 x + 8.642\times10^2$	1.0000	8	20
6	$y = 2.105\times10^7 x^2 + 3.838\times10^5 x + 2.458\times10^4$	1.0000	8	20
7	$y = 2.515\times10^7 x^2 + 3.269\times10^5 x + 2.206\times10^4$	1.0000	8	20
8	$y = 2.347\times10^7 x^2 + 6.286\times10^5 x + 2.029\times10^4$	0.9999	8	20

Table 2

Repeatability, intra-day and inter-day precision of the UHPLC method. Results are given in percent; standard deviations are shown in parenthesis.

Compound	Repeatability	Intra-day 1	Intra-day 2	Inter-day
1	0.40 (0.01)	0.40 (0.01)	0.41 (0.01)	0.41 (0.01)
2	0.92 (0.02)	0.86 (0.02)	0.88 (0.02)	0.87 (0.02)
3	0.49 (0.01)	0.49 (0.01)	0.50 (0.01)	0.50 (0.01)
4	22.76 (0.33)	22.60 (0.43)	23.35 (0.39)	22.98 (0.55)
5	9.38 (0.23)	9.40 (0.30)	9.83 (0.36)	9.62 (0.36)
6	1.52 (0.05)	1.48 (0.04)	1.56 (0.04)	1.52 (0.06)
7	3.83 (0.09)	3.70 (0.11)	3.87 (0.06)	3.79 (0.13)
8	4.81 (0.12)	4.67 (0.09)	4.95 (0.13)	4.81 (0.18)

Table 3

Accuracy of the UHPLC method. Concentrations are given in mg/mL, amounts are given in mg, and recovery is expressed in percent.

Compound	Sample concentration	Spiked amount	Total concentration	Recovery
1	0.0152	0.0100	0.0280	111.1
	0.0101	0.0200	0.0310	102.9
	0.0051	0.0300	0.0352	100.4
2	0.0326	0.0100	0.0440	103.4
	0.0217	0.0200	0.0462	110.6
	0.0109	0.0300	0.0388	95.1
3	0.0186	0.0100	0.0297	103.8
	0.0124	0.0200	0.0290	89.7
	0.0062	0.0300	0.0320	88.4
4	0.8616	0.0400	0.8932	99.1
	0.5744	0.0800	0.6369	97.3
	0.2872	0.1200	0.4007	98.4
5	0.3606	0.0200	0.4157	109.2
	0.2404	0.0400	0.2879	102.7
	0.1202	0.0600	0.1792	99.4
6	0.0569	0.0100	0.0678	101.3
	0.0380	0.0200	0.0505	87.2
	0.0190	0.0300	0.0440	89.9
7	0.1419	0.0100	0.1600	105.3
	0.0946	0.0200	0.1054	91.9
	0.0473	0.0300	0.0701	90.6
8	0.1802	0.0100	0.1895	99.6
	0.1202	0.0200	0.1212	86.5
	0.0601	0.0300	0.0788	87.5

Table 4

HSQC cross correlations, regression equations and coefficients of determination (R^2). Compounds used for calibration are shown in parenthesis.

Cross correlation signal	Regression equation	R^2
Furylic methine (CH-14) (**4**)	$y = 55.037\ x + 0.0150$	0.9999
Furylic methine (CH-15) (**4**)	$y = 46.659\ x + 0.0108$	0.9995
Furylic methine (CH-16) (**4**)	$y = 47.903\ x + 0.0335$	0.9997
Exocyclic methylene CH-17a (**4**)	$y = -42.877\ x + 0.0230$	1.0000
Exocyclic methylene CH-17b (**4**)	$y = -39.743\ x + 0.0043$	1.0000
Exocyclic methine CH-14 (**8**)	$y = 27.291\ x + 0.0353$	0.9978
Endocyclic methine CH-7 (**8**)	$y = 35.896\ x + 0.0252$	0.9990
Methyl group CH_3-18 (**8**)	$y = 119.07\ x + 0.0256$	0.9999

Table 5

Repeatability, intra-day and inter-day precision, and accuracy of the qHSQC method. Results are given in percent; standard deviations are shown in parenthesis.

Compound	Repeatability	Intra-day 1	Intra-day 2	Inter-day	Accuracy
1	0.22 (0.12)	0.52 (0.28)	0.22 (0.15)	0.37 (0.28)	89.6
2	1.67 (0.17)	1.12 (0.37)	1.40 (0.22)	1.26 (0.35)	144.9
3	0.52 (0.07)	0.59 (0.03)	0.57 (0.04)	0.58 (0.04)	115.5
4	22.85 (0.42)	22.19 (0.23)	22.35 (0.45)	22.27 (0.38)	96.9
5	9.15 (0.20)	9.28 (0.16)	9.11 (0.15)	9.20 (0.18)	95.6
6	1.71 (0.07)	1.52 (0.11)	1.82 (0.07)	1.67 (0.18)	109.7
7	4.19 (0.09)	4.33 (0.20)	4.17 (0.09)	4.25 (0.18)	112.1
8	5.01 (0.12)	5.15 (0.11)	4.97 (0.08)	5.06 (0.14)	105.1

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