Prediction of the sweetening effect of *Siraitia grosvenorii* (luo han guo) fruits by two-dimensional quantitative NMR

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ABSTRACT

During the last decade, dried fruits of *Siratia grosvenorii* (luo han guo), also known as monk fruit, have become popular food ingredients. Luo han guo extracts, which are promoted as non-caloric natural sweeteners, are now incorporated into dietary supplements, soft drinks, and energy shakes. The compounds responsible for the sweetening effect are glycosylated cucurbitane-type triterpenoids, the so-called mogrosides. However, of the more than 40 known mogroside compounds, only 11-α-hydroxy-mogrosides exhibit a sweetening effect, whereas the other triterpenoids are non- or bitter-tasting. We have used two-dimensional quantitative NMR to determine selectively the content of 11-α-hydroxy-mogrosides in these dried fruits and thus to predict their sweetening potential. Homonuclear (H,H COSY) and heteronuclear (HSQC) methods were developed, validated, and compared. Both techniques were found suitable for the quality control of luo han guo fruits and extracts, the COSY experiment being advantageous with regard to accuracy, precision, and limit of quantification.

Keywords: *Siraitia grosvenorii*; Cucurbitaceae; luo han guo; mogrosides; HSQC; COSY; qNMR; natural sweetener

1. Introduction

Siraitia grosvenorii (Swingle) C.Jeffrey ex A.M.Lu & Zhi Y.Zhang, also referred to as *Momordica grosvenorii* or *Thladiantha grosvenorii*, is a perennial vine of the Cucurbitaceae family (Pawar, Krynitsky, & Rader, 2016). Commonly known as "monk fruit" or "luo han guo", *S. grosvenorii* is native to the southern part of China, where it has been used as a food ingredient and herbal medicine for more than 300 years (Gong et al., 2019; Soejarto, Addo, & Kinghorn, 2019). *S. grosvenorii* is applied in typical ethnomedical treatments of ailments such as coughs, lung congestion, and sore throats and was one of the first food homology species approved for medical use in China (Gong et al., 2019). The plant was listed as a medicinal and edible species by the China Ministry of Health in 1987 (Li et al., 2014). In the 1990s, the China Food and Drug Administration approved luo han guo as a food sweetener and as a substitute for sweeteners in health foods for patients with obesity and diabetes. Preparations of the dried fruits have subsequently been marketed as sugar-free food additives and used in low-calorie health-promoting juices. Since 2011, extracts and preparations of *S. grosvenorii* have become commercially available in the United States and are currently produced at large scale for utilization as table-top sweeteners (Fletcher, Pan, & Kinghorn, 2017; Pawar et al., 2016). Having been awarded the GRAS ("generally regarded as safe") status in the U.S., *S. grosvenorii* can now be considered the most popular natural sweetener after *Stevia rebaudiana* (Soejarto et al., 2019).

Like that of *S. rebaudiana*, the sweetening effect of *S. grosvenorii* constituents is reported to be up to 500 times as high as that of sucrose (Li et al., 2014; Pawar et al., 2016; Soejarto et al., 2019). However, in contrast to the sweet-tasting diterpenoids contained in stevia, the compounds responsible for the sweetening effect of luo han guo are glycosylated cucurbitane-type triterpenes. These compounds are characterized by a four-ring scaffold and various sugar

moieties at position C-3 and C-24 (Fig. 1). The major sugar present in mogrosides is D-glucose, although L-rhamnose moieties have also been reported (Qing et al., 2017). The number of attached glucose units is expressed by Roman numerals in the compound name (rhamnoses are indicated separately), with a reported maximum of seven sugar moieties (Qing et al., 2017). Depending on the nature of position C-11, which can be a carbonyl group, a methylene group, or an α-hydroxy-methine group, in the scaffold, the compounds are referred to as 11-oxo-mogrosides (**1**), 11-deoxymogrosides (**4**), or mogrosides (**2** and **3**). The last-mentioned term is also used to circumscribe all of the luo han guo triterpenoids, however, only mogrosides with the mogrol aglycone (**2** and **3**) possess the sweet taste. This means that the α-hydroxy-group is crucial for the sweetness effect, as is the presence of at least three glucose moieties (Li et al., 2014).

So far, more than 40 cucurbitane-type triterpenoids have been isolated from *S. grosvenorii*, of which 17 show the features necessary for exhibiting a sweet taste (Çiçek, 2020). With regard to the biosynthesis of mogrosides, the glucose moieties are successively attached to the aglycone during the ripening process. Mogroside V (**3**), the major triterpenoid, appears approximately 50 days after flowering and peaks after 70 days, whereas the mogroside IV class peaks as early as 40 days after flowering (Itkin et al., 2016; Pawar et al., 2016; Qing et al., 2017). Because of these variations over time and the overall complexity of the constituent pattern, quantitative (chromatographic) methods for the quality control of luo han guo have focused on one mogroside, namely mogroside V (**3**) (Deng, Liang, Yang, Liu, & Liu, 2013; Makapugay, Nanayakkara, Soejarto, & Kinghorn, 1985) or a few constituents (Li et al., 2007; Luo, Shi, Zhang, Qin, Guo, & Ma, 2016; Shen et al., 2014). In these studies, the amount of mogroside V (**3**) has been found to vary between 5.8 and 12.9 mg/g dry fruit, whereas the cumulated content of the major compounds has been reported to be between 8.8 and 18.5 mg/g (Luo et al., 2016; Makpugay et al., 1985; Shen et al., 2014). This contrasts with results obtained

from a colorimetric assay, which has determined the total content of mogrosides as being 38.2 mg/g dry fruit (Li et al., 2014). However, neither colorimetric nor chromatographic methods have so far accomplished a determination of the total amount of 11-α-hydroxy-mogrosides or an analysis of the sweetening potential of luo han guo at a molecular level.

In the present work, we investigated the fruits of *S. grosvenorii* with two-dimensional NMR spectroscopy by using its characteristic measuring principle, the detection of specific structural features. We hypothesized that signals exclusively resulting from 11-α-hydroxy-mogrosides could be used for the quantification of this particular compound class. This strategy should allow the selective determination of the compounds of interest and consequently the prediction of the sweetening effect of the ripe fruits.

2. Materials and methods

2.1. Plant material and chemical reagents

Dried fruits of *Siraitia grosvenorii* (Golden Diamond, Lot B02/2549) were obtained from the Go Asia supermarket (Hamburg, Germany). 1,2,4,5-Tetrachloro-3-nitrobenzene TraceCert for quantitative NMR (Lot BCBR7216V, 99.86%), LC-MS grade formic acid, Diaion HP-20, and Sephadex LH-20 were purchased from Sigma Aldrich Co., St. Louis, MO, USA. Silica gel (40-63 µm) for column chromatography, TLC plates (silica gel 60 F_{254}), acetonitrile and water (both of LC-MS grade), gradient grade methanol, and other (analytical grade) solvents were obtained from VWR International GmbH, Darmstadt, Germany. Water used for isolation was doubly distilled in-house. Solid phase extraction (SPE) columns (Chromabond SB 3 mL/500 mg, Chromabond C18 6 ml/1000 mg) were obtained from Macherey-Nagel GmbH and Co. KG, Düren, Germany. Dimethyl sulfoxide-d_6 (99.80%, Lot S1051, Batch 0119E) and pyridine-d_5

(99.50%, Lot Q1301, Batch 1116) for NMR spectroscopy were purchased from Euriso-top GmbH, Saarbrücken, Germany, and conventional 5 mm NMR sample tubes were obtained from Rototec-Spintec GmbH, Griesheim, Germany.

2.2. General experimental procedures

Thin layer chromatography was performed using *n*-butanol–ethanol–water (40–10–15) as the eluent and vanillin sulfuric acid as the spraying reagent. Flash chromatography was carried out with a Buchi PrepChrom C-700 chromatograph and FlashPure Ecoflex (50 µm irregular, 10 g) silica gel cartridges (Büchi Labortechnik GmbH, Essen, Germany). Preparative MPLC was performed with the same instrument but by using a Buchi PrepChrom C18 column (250 × 30.0 mm, 15 m particle size). Semi-preparative HPLC was accomplished using a Waters Alliance e2695 Separations Module with an Alliance 2998 photodiode array, and a WFC III fraction collector (Waters, Milford, MA, USA) using a VP Nucleodur C18 column (250 × 10 mm, 5 µm particle size, Macherey-Nagel GmbH and Co. KG, Düren, Germany). 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 VWR 100 evaporative light scattering detector (VWR International GmbH, Darmstadt, Germany). LC-MS analyses were carried out on a Shimadzu Nexera 2 liquid chromatograph connected to an LC-MS triple quadrupole mass spectrometer using electrospray ionization (Shimadzu, Kyoto, Japan). A Phenomenex Luna Omega C18 column (100 × 2.1 mm, 1.6 µm particle size, Phenomenex, Aschaffenburg, Germany) was employed for the analysis of extracts, fractions, and pure compounds during isolation and method development. Quantification of mogroside V (**3**) was performed on a Nucleodur C18 Pyramid (250 × 4.6 mm, 5 µM, Macherey-Nagel, Düren, Germany) by triplicate injections of both, the mogroside reference and the luo han guo sample.

2.3. NMR spectroscopy

NMR spectra were recorded using a Bruker Avance III 400 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 400.33 MHz for the proton channel and at 100.66 MHz for the ^{13}C channel by means of a 5 mm PABBO broad-band probe with a *z* gradient unit. Measurements were performed at 293 K; the temperature in the probe head was calibrated with a methanol-d_4 solution and regulated within 0.1 K during the measurements. For each sample, automatic tuning and matching of the probe was performed, as was automatic shimming of the on-axis shims (*z* to z^5). The automatic receiver gain adjustment mode afforded the maximum gain value in all measurements. Bruker Topspin software 3.6.0 was employed for spectra recording.

Characterization of the purified sample in pyridine-d_5 and DMSO-d_6 was accomplished by use of conventional full spectral range routine parameters and pulse programs for structure elucidation.

2.3.1 Quantitative H,H COSY measurements

The non-phase-sensitive pulse program *cosygpmfqf* (with gradient pulses) and double quantum filter selection via the gradient ratio supplied by the manufacturer were used for correlated spectroscopy (COSY) measurements. Spectral width was set to 3.10 ± 2.40 ppm (a range of 1921 Hz) in both dimensions, with 1024 data points being collected in F2 and 128 increments in the indirect (F1) dimension. Acquisition time was calculated to be 0.267 s, and the interscan-delay was 3 s. With two scans collected per increment and 16 dummy scans, the total measuring time amounted to 14 min and 50 s (‘2-scan-COSY’); with eight scans, about 57 minutes were required to record the spectrum (‘8-scan-COSY’).

The raw data were processed as follows: zero filling to an 8k × 8k matrix, then multiplication with a sine function before two-dimensional Fourier transformation and magnitude calculation in F2, with automatic baseline correction in both dimensions and symmetrization of the resulting spectrum. Integration borders were set uniformly by using the same integration file with a range of 3.762 to 3.604 ppm for F2 and 1.792 to 1.612 ppm in F1.

2.3.2. Quantitative band-selective $^{1}H,^{13}C$ HSQC measurements

Band-selective heteronuclear single quantum correlation spectroscopy (bs-HSQC) measurements for quantification were performed using the phase-sensitive *shsqsctgpsi2.2* pulse program of the Bruker pulse program library with a band-selective shaped ^{13}C refocusing pulse and ^{13}C GARP decoupling. The ^{1}H spectral range was (4.50 ± 5.05) ppm, spectral range 4006 Hz, whereas the ^{13}C spectral range was (77.2 ± 5.0) ppm, corresponding to 1006 Hz.

The shape form Q3.1000 was chosen for the selective refocusing pulse and, in order to achieve selective excitation over the frequency range of 1000 Hz (10 ppm), the length of the pulse was determined to 3448 µs with a power of 0.0986 W for the probe head in use. For the ^{1}H channel, 1024 data points were collected, whereas for the ^{13}C channel, 64 data points were set. The acquisition time was 0.128 s, and the inter-scan delay was set to 3.15 s. The relevant delays in the pulse program were set corresponding to a coupling constant of $^{1}J_{CH}$ = 135 Hz. With 16 dummy scans and 16 scans per increment, the total acquisition time for this HSQC experiment was about 57 minutes.

The raw data were processed as follows: zero filling to a 4k × 4k matrix and then multiplication of both dimensions with a squared sine function before two-dimensional Fourier transformation and phase correction. Phase correction parameters of the previous experiment were used and were carefully visually controlled and manually adopted for the relevant cross peak in both dimensions for each measurement. After automatic baseline correction in both

dimensions, integration borders were set uniformly by using the same integration file with a range of 3.77 to 3.60 ppm in F2 (^{1}H) and 78.10 to 76.26 ppm in F1 (^{13}C, bs-HSQC$_{wide}$). Additionally, an alternate integration range (bs-HSQC$_{small}$) was used with much narrower ranges of 3.77 to 3.60 ppm in F2 (^{1}H) and 77.37 to 76.76 ppm in F1 (^{13}C).

2.4. Extraction and isolation

A total of 600 g powdered dried fruits was exhaustively extracted with methanol by maceration, and the solvent was evaporated under reduced pressure to yield 114.3 g crude extract. The crude extract was dissolved in 300 mL water and chromatographed over Diaion HP-20, with subsequently elution with water, methanol 30%, methanol 80%, and methanol 100%. The methanol 80% fraction (17.5 g) was subjected to silica gel column chromatography by using dichloromethane–methanol–water in three portions of 815 mL with the following ratios: 8:2:0.2, 7:3:0.5, and 6:4:1. Of the resulting eight fractions (A–H), fraction G (8.70 g) was chromatographed with Sephadex LH-20 to give another five fractions (G1–G5). Fraction G3 (5.70 g) was subjected to preparative MPLC by applying a solvent mixture of 0.1% formic acid in water (solvent A) and methanol (solvent B) with the following gradient: 38% B to 45% B in 60 min, and to 95% B in 30 min. Of the resulting five fractions (G3A–G3E), fraction G3B (716 mg) was chromatographed using silica gel flash chromatography and a gradient of dichloromethane–methanol–water (8:2:0.2 to 7:3:0.5) to give six fractions. The third fraction (211 mg) was separated by semi-preparative HPLC by using 0.025% formic acid in water (solvent A) and acetonitrile (solvent B) with a gradient of 25% B to 30% B in 30 minutes, a flow rate of 2 mL/min, and an oven temperature of 35°C, to give 26.9 mg compound **1** and 27.5 mg compound **3**. Fraction G3C (371 mg) was purified using normal phase solid phase extraction to give another 113.8 mg compound **3**. Fraction G3D (242 mg) was subjected to semi-

preparative HPLC by using 0.025% formic acid in water (solvent A) and acetonitrile (solvent B) with a gradient of 20% B to 25% B in 30 minutes, a flow rate of 3 mL/min, and an oven temperature of 35°C, to give 27.5 mg compound **2** and 32.3 mg compound **4**.

2.5. Quantitative analysis

A total of 1.00 g dried and ground fruits was extracted three times with 50 mL methanol-water 80% (v/v) by using ultra-sonication for 15 min each. After filtration, the solution was evaporated to dryness. The residue was re-dissolved in 3 mL methanol-water 20% (v/v) and subjected to an equilibrated SPE C-18 column (1 g, 6 mL). After the column had been washed with three column volumes of methanol-water 20% (v/v), the desired compounds were eluted with three column volumes of methanol (100%). The combined methanol fractions were evaporated to dryness, re-dissolved in 600 µL DMSO-d_6, and transferred to NMR sample tubes. For calibration, 26.4 mg mogroside V (**3**) were dissolved in 1000 µL DMSO-d_6 and diluted to the desired concentrations with the same solvent. Purity of the compound was assessed using 1,2,4,5-tetrachloro-3-nitrobenzene (6.20 mg) as the internal standard.

For the quantification of 11-α-hydroxy-mogrosides, calibration solutions 1–5 with concentrations of 20.34, 15.26, 8.48, 3.39, and 1.70 mmol/L were prepared and analyzed with bs-HSQC and H,H COSY measurements. For the latter experiment, two different parameter sets were investigated by using either 2 or 8 scans and the resulting acquisition times of 15 min or 57 min, respectively.

2.6. Method validation

The developed 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. Therefore, 5-point calibration curves were created and expressed as linear functions. In order to define a limit of quantitation, a solution of 1.00 mmol/L mogroside V (**3**) was prepared. Precision and repeatability were assessed using samples of luo han guo. Repeatability measurements were conducted six times on the same sample. For the determination of precision, six different samples were prepared and measured once within one day (intra-day precision) or on two different days (inter-day precision). Determination of accuracy was accomplished by spiking the samples with various amounts of standard solution as follows:

- high spike (120%): 150 µL sample + 450 µL calibration solution 2
- medium spike (100%): 150 µL sample + 225 µL calibration solution 2 + 225 µL calibration solution 3
- low spike (80%): 150 µL sample + 450 µL calibration solution 3

All obtained values were expressed as % recovery by using the mean value of the inter-day measurements (N=12) for the sample concentration. Additionally, an overall mean value was calculated from the inter-day measurements of all four experimental results (N=48), and the deviations of the results obtained from the four methods were compared.

3. Results and discussion

3.1. Isolation of standard compounds and revision of NMR data

As a first step, standard compounds were isolated for method development and eventual validation. At least one compound with an α-hydroxy-group in position C-11 and one compound without this structural feature were required. Therefore, we focused on the isolation of mogroside V (**3**) and 11-oxo-mogroside V (**1**), the two most abundant mogrosides in the ripe fruits of *S. grosvenorii* (Shen et al., 2014). The isolation steps followed in principle the procedure described by Li et al. (2006) for the isolation of mogroside V (**3**). The isolation process was monitored by LC-DAD-ELSD and LC-MS using the method described by Qing et al. (2017).

All in all, four cucurbitane glycosides were obtained and identified as 11-oxo-mogroside V (**1**), mogroside IVe (**2**), mogroside V (**3**), and 11-deoxymogroside V (**4**) by using MS and NMR experiments and comparisons with literature data (Akihisa et al., 2007; Chaturvedula, & Prakash, 2011; Kasai et al., 1989; Niu et al., 2017; Prakash, & Chaturvedula, 2014; Qing et al., 2017). However, the reported NMR shift values for mogroside V (**3**) and 11-oxo-mogroside V (**1**) (Chaturvedula, & Prakash, 2011) differed significantly from our own NMR measurements and from the NMR data of similar compounds (Akihisa et al., 2007; Niu et al., 2017). Revised NMR data for the two major components mogroside V (**3**) and 11-oxo-mogroside V (**1**) are given in Tables S1 and S2 together with those of the other two isolated compounds. Moreover, we reassigned shift values for five signals of mogroside IVe (**2**), which had not been unambiguously assigned by the time of its structure elucidation (Kasai et al., 1989). Additionally, we added missing values for the sugar moieties of the compound also referred to as mogroside IV in older references. The NMR spectra of all four compounds are depicted in Figs. S1–S30.

3.2. Method development

285 3.2.1. Selection of NMR solvents and signals for quantification

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287 In our quest to identify and quantify all 11-α-hydroxy-mogrosides, we first analyzed
288 significant signals of the terpenoid moiety by comparing mogroside V (**3**) and 11-oxo-
289 mogroside V (**1**). Several prerequisites had to be fulfilled for the selective quantification of the
290 11-α-hydroxylated species, in addition to structures with either a methylene group or a carbonyl
291 function in the respective position (see Fig. 1). Because of the severe overlap of many aliphatic
292 signals of the terpenoid ring system, the high frequency shifted methine proton next to the
293 hydroxyl group (H-11) displays a unique feature of sweet-tasting mogrosides. In the one-
294 dimensional ^{1}H NMR spectrum recorded in pyridine-d_5, this signal unfortunately overlapped
295 several methine signals of the glucose moiety. Thus, direct integration was not possible. HSQC
296 experiments showed similar results, because the shift value of C-11 was also within the bulk of
297 glucose signals. However, selectivity was achieved when the nuclei in the neighbourhood of
298 this proton were considered. H-11 showed coupling with the two protons in position 12, both
299 having a chemical shift of 2.19 ppm (Table S2). This coupling appeared as a clearly separated
300 cross peak in the two-dimensional H,H COSY spectrum for the 11-α-hydroxy-mogrosides and
301 showed no overlap with any sugar signals (Fig. S15, 4.18 ppm/2.19 ppm).

302 NMR spectra for compounds **1** and **3** were additionally recorded in DMSO-d_6, because of its
303 potent solvency for both terpene glycosides and the crude extracts. Moreover, dimethyl
304 sulfoxide is a far more economic NMR solvent. Again, 1D proton NMR spectra did not provide
305 sufficient resolution for the H-11 methine signal (Fig. S19), which now overlapped with three
306 other diasterotopic protons of the terminal glucose methylene groups GII, GIV, and GV (Fig.
307 1). However, this time not only H,H COSY measurements showed good resolution (Fig. S21),
308 but also HSQC experiments clearly separated the CH-11 cross peak from all other glucose
309 signals (Fig. S22). Consequently, DMSO-d_6 was chosen as the NMR solvent, and the cross

peak of the CH-11 correlation at 3.67 ppm/76.7 ppm (HSQC) and the cross coupling of the H-11 methine signal with the H-12 methylene protons at 3.67 ppm/1.67 ppm (H,H COSY) were selected for further method development.

3.2.2. Extraction and sample preparation

For the extraction of the dried fruits, pure methanol (Shen et al., 2014) or methanol-water (80% v/v) (Qing et al., 2017) have been described as suitable solvents for analytical purposes, with a ratio of 1 g powdered plant material to 100 mL of solvent being applied. In our study, the use of methanol resulted in a yield of approximately 24% (w/w) of crude extract calculated based on the amount of dried fruits, whereas the methanol-water mixture delivered 36% (w/w) of crude extract. The higher mass fraction of the methanol-water extract was expected, and a higher concentration of α-hydroxy-mogrosides was observed. LC-MS analysis of the crude extracts revealed that both solvents efficiently extracted mogrosides with two, three, four, or five sugar moieties, but only the methanol-water mixture yielded hexaglycosidic mogrosides. Thus, methanol-water 80% (v/v) was used for subsequent extractions.

In order to increase the concentration of α-hydroxy-mogrosides and to reduce NMR measurement time, the crude extract was further purified by C-18 solid phase extraction. Therefore, various methanol-water mixtures (0, 20, 50, and 80%) were evaluated for their ability to wash polar analytes off the column without eluting the compounds of interest. LC-MS analysis confirmed that, with methanol (20%), a large number of unwanted constituents were removed before elution of the α-hydroxy-mogrosides with pure methanol. This procedure significantly increased the concentration of the α-hydroxy-mogrosides, leading to intense signals in both the H,H COSY and HSQC experiments.

335 3.2.3. Homonuclear two-dimensional NMR (H,H COSY)

336

337 Utilizing the standard pulse programs delivered by the spectrometer manufacturer, we
338 developed a method for the measurement and integration of several H,H COSY spectra. H,H
339 COSY measurements provide the option of the selective detection of one special coupling via
340 the corresponding cross peak with proton excitation and proton detection at 3.67 ppm/1.67 ppm.
341 Among several H,H COSY variants, double quantum filtered experiments involving the
342 application of gradient pulses and thereby the suppression of singlets and artefacts are
343 considered extremely robust (Shaw, Salaun, Dauphin, & Ancian, 1996). We employed an
344 established simple non-phase-sensitive measurement allowing easy integration of parts of the
345 2D plot. Because only one signal region was of interest, we used a small spectral width,
346 observing the aliphatic region in the range of 0.7 to 5.5 ppm and thus reducing measurement
347 time (Fig. 2). The crucial experimental parameter to be investigated for quantitative analysis is
348 the scan repetition time, as this parameter is responsible for the length of the NMR experiment.
349 It has to be sufficiently long to allow complete relaxation of the protons. We investigated this
350 parameter by running H,H COSY experiments with various delays between 0.5 and 20 s for the
351 standard compound (**3**) and the extract after sample preparation. No significant differences of
352 the integrals of the cross peak of H-11/H-12 in the two cases were observed in the scan
353 repetition time range of 2 s to 20 s (data not shown). Thus, a setting of 3 s was used for all H,H
354 COSY experiments in this study. Two measurements were made: a fast 2-scan-COSY (15 min)
355 and an 8-scan-COSY (57 min) with improved signal to noise ratio. During the evaluation of the
356 processing and integration parameters, we obtained most reproducible results by improving the
357 resolution by zero filling and, after automatic baseline correction on both dimensions, by
358 symmetrizing the H,H COSY spectrum (see section 2.3.1.).

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360 3.2.4. Heteronuclear two-dimensional NMR (HSQC)

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362 The other nucleus in the neighbourhood of the H-11 methine proton is the carbon atom at
363 position 11. This one-bond coupling is responsible for the cross signal in the HSQC spectrum
364 at 3.67 ppm/76.7 ppm (Fig. 3). Here, protons are excited, the polarization is transferred to the
365 carbon atom and back to the proton again, which are then detected in a sensitive manner
366 (Schleucher et al., 1994). We used this approach as a second quantitative method and observed
367 that no actual overlap occurs with any glucose signals. For the optimization of this HSQC
368 experiment, we investigated once more the effect of the relaxation time and found no significant
369 difference between delays of 2 to 20 s (data not shown). Therefore, a value of about 3 s was
370 also chosen for this experiment. We drastically reduced the number of increments for the carbon
371 frequency by working with band-selective excitation of only a range of 10 ppm (Girreser,
372 Ugolini, & Çiçek, 2019). Furthermore, delays in the pulse program were adapted to the coupling
373 constant of this methine unit (Çiçek, Ugolini, & Girreser, 2019), which we determined at 135
374 Hz. Finally, we compared the traditional recording of each increment with non-uniform
375 sampling (Jaravine, Ibraghimov, & Orekhov, 2006) and obtained more reproducible results
376 with traditional sampling (data not shown). In this manner, we created a method requiring a
377 measuring time of about 57 min. Again, we paid attention to the data processing by zero filling
378 of the 2D matrix, automatic baseline correction, and the selection of a proper integration range.
379 In this case, we selected a small and a wide integration range and compared the results of the
380 different ranges (see section 2.3.2).

381

382 *3.3. Method validation*

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384 3.3.1. Linearity and quantitation limit

For the validation of linearity, calibration curves in four different modes were established over a concentration range of 1.70 to 20.34 mmol/L, with mogroside V (**3**) as an external calibration standard (Table 1). Purity of the compound was checked by using a certified reference standard (1,2,4,5-tetrachloro-3-nitrobenzene) and integration of the H-6 in the cucurbitane scaffold, plus the H-6b of the G_I glucose moiety, by 1D proton NMR. The purity of mogroside V (**3**) was determined to be 99.09%.

All four calibration curves showed acceptable coefficients of determination, with values between 0.9965 and 0.9986 (Table 1). In the HSQC experiments, both modes of integration yielded almost the same slopes and only differed in their offsets, which were naturally higher for the wide integration range. The differences between the two COSY experiments were characterized by a significantly higher slope for the 8-scan-COSY, which furthermore showed a negligible offset.

Because of the use of integration fields instead of the threshold integration, a limit of quantitation had to be defined. Therefore, a solution of 1.00 mmol/L was prepared and determined under the four measurement/integration modes. Whereas, with the 8-scan-COSY, this concentration was determined within 50% of the actual value, the other three modes did not show sufficient accuracy at this level. For these three modes, the limit of quantitation was defined as 1.70 mmol/L (Table 1). When these values were calculated as mogroside V (**3**), as we did in the quantitative assays (Table 2), they corresponded to 1.3 mg/mL (1.00 mM) or 2.2 mg/mL (1.70 mM), respectively. Because 1.00 g luo han guo was applied in the assays, the same concentrations are present per gram of dried fruit.

3.3.2. Precision and repeatability

HSQC measurements showed similar repeatability, with a relative standard deviation of 3.38% for the wide integration mode and 3.25% for the small integration parameters (Table 2). Moreover, in terms of precision, both variants were comparable, with RSD values between 5.45% and 6.35%. H,H COSY measurements showed better repeatability than the HSQC experiment, with RSD values of 1.95% for the 2-scan-COSY and 1.89% for the 8-scan-experiment. The latter experiment additionally displayed excellent values during the precision measurements, with relative standard deviations of 1.66 to 1.73%. The 2-scan COSY, in contrast, showed RSD values similar to those of the HSQC experiment, with relative standard deviations of 4.43 to 5.37%. Interestingly, no significant differences were observed between the intra-day and inter-day measurements, indicating the robustness of the developed NMR methods (Table 2).

3.3.3. Accuracy and comparability

Spiking experiments were conducted for the determination of accuracy (Table 3). Various calibration solutions were used to achieve concentrations of 80, 100, and 120% of the assay concentration (mean values of the precision measurements). With regard to the HSQC experiments, both integration methods revealed good recovery rates and showed slightly better recoveries for the small integration range. H,H COSY measurements also resulted in excellent recovery rates, with maximum deviations of 4.9% for the 2-scan-experiment and 2.8% for the 8-scan-COSY. In addition, the mean values obtained from the precision measurements were compared with each other and showed usual deviations of not more than 3% (Table 3).

3.3.4. Specificity and selectivity

The total content of 11-α-hydroxy-mogrosides can be determined from both the HSQC experiment and the H,H COSY measurements. Moreover, these value-determining compounds can be quantified among additional non-sweet-tasting derivatives, e.g., 11-oxo-mogrosides or 11-deoxy-mogrosides. Therefore, the present methods can be rendered specific and selective at the same time. The selective determination of the sweetening principle can be achieved in two ways: either by direct observation of the CH-11 methine unit (HSQC) or, alternatively, through indirect (H,H COSY) measurement via the coupling to the methylene group in the neighbourhood (CH_2-12). This highlights the advantage of NMR spectroscopy over other analytical methods, namely its characteristic measuring principle and the possibility of quantifying the structural features necessary for the biological effect.

3.4. Quality control

In the above, we have presented two experiments for the quality control of *S. grosvenorii*. The amount obtained for the 11-α-hydroxy-mogrosides was determined as being 15.2 to 16.2 mg/g dried fruit (calculated as mogroside V). In comparison, the amount of mogroside V (**3**) was determined as 9.2 mg/g dried fruit by using the method of the Food Chemicals Codex (Figs. S31 and S32) (FCC 11, 2018). This value is in accordance with the average amounts described in the literature for mogroside V (**3**), namely 9.5 mg/g (Li et al., 2014) or 10.5 mg/g (Pawar et al., 2013), respectively. However, the significantly lower value compared with our assays indicates the large amounts of additional constituents that contribute to the sweetening effect of luo han guo and that are not quantified with the chromatographic methods reported to date.

We investigated two variants in the two experiments. For the HSQC measurements, we used different integration ranges (bs-$HSQC_{wide}$ and bs-$HSQC_{small}$), whereas for the H,H COSY experiments different scan numbers were applied (2-scan-COSY and 8-scan-COSY). For the

HSQC experiment, a selective pulse program decreased the ^{13}C spectral range to 10 ppm and thus significantly reduced the measurement time. Validation of the four resulting methods according to the ICH guideline Q 2 R(1) have revealed similar values for the two HSQC variants thus indicating the applicability of the two integration methods. For the H,H COSY measurements, a 2-scan experiment was investigated for the fast quantification (15 min) of 11-α-hydroxy-mogrosides, in addition to an 8-scan variant, resulting in the same measurement time as in the HSQC experiments (57 min). The 2-scan-COSY revealed similar validation results as the HSQC experiments, whereas the 8-scan-COSY was superior in terms of accuracy, precision, and limit of quantitation.

Furthermore, all methods were able selectively to quantify the total content of the value-determining 11-α-hydroxy-mogrosides. Because the assay is based on molarity (and the content is subsequently referenced to mogroside V), the method can also be applied to unripe fruits, and the sweetening potential can be predicted and estimated at an early stage of cultivation. Another issue when dealing with luo han guo is the possible dilution of extracts with extracts of unripe fruits (Pawar et al., 2013). Although not being intended for the detection of adulterations, our method can also be performed to address this problem by using the cross correlations of the H-6 protons of the non-terminal glucose moieties G_I and G_{III} (Fig. 1). These cross peaks can be found in the H,H COSY spectrum at 3.57/3.90 ppm for G_I and at 3.40/4.11 ppm for G_{III} and have a ratio of 2:1 with regard to the H-11/H-12 cross peak of pure mogroside V (**3**) (Fig. 2). In luo han guo samples, this ratio is approximately 2.75:1. The higher ratio results from the remaining mogrosides, which obviously show similar grades of glycosylation. However, in the case of dilution with unripe fruits, the ratio would be far below 2:1, because of the increased presence of mogrosides with only two terminal sugar moieties (one at C-3 and one at C-24) (Itkin et al., 2016). Thus, the characteristic signals of the non-terminal glucose moieties would be significantly reduced (or absent in the case of complete falsification). The

detection of adulteration would also be feasible with the HSQC experiment by using the respective C-H correlations. Here, a ratio of 0.6:1 was observed in our experiments for mogroside V (**3**) and 0.85:1 for luo han guo samples. However, in the band-selective mode, the ^{13}C spectral width would have to be increased resulting in longer measurement times.

Analysis time is an important issue when dealing with quality control methods and is one of the reasons that two-dimensional NMR methods are readily overlooked. However, in the present study and with regard to the application of band-selective approaches for the HSQC experiment, we have demonstrated that even heteronuclear techniques deliver precise and accurate results within reasonable measurement times. For example, in the HPLC assay for the mogroside V (**3**) content of the Food Chemicals Codex, the last peak in the extract eluted after more than 50 minutes (Fig. S32). Thus, a measurement time of 60 minutes was chosen, which corresponds to the measurement times of three of our methods. Chromatographic separation of extracts will always need several minutes of elution time, especially for more complex extracts such as the extracts of luo han guo. Elution times can (to some extent) be decreased using smaller particle sizes, selective extraction, or mass spectrometric detection methods. Deng et al. (2013) have used micelle-mediated cloud-point extraction and quantified mogroside V (**3**) in 20 minutes. However, the relatively low accuracy and precision values and the extraction yield of only 80% in their investigation are major shortcomings for the quality control of luo han guo. In two other studies, LC-MS was applied in the multiple reaction monitoring (MRM) mode and quantified five and eight mogrosides, respectively (Shen et al., 2014; Luo et al., 2016). The analysis time of the two methods was 12 and 10 minutes and thus in the range of the 2-scan-COSY experiment. Moreover, in terms of accuracy and precision, the two LC-MS methods are comparable with the 2-scan-COSY, showing slightly better precision values and somewhat lower recovery rates. The linear range of MRM methods usually is in the nanomolar scale and obviously results in lower limits of quantification. These low LoQs render MRM

methods an ideal tool for trace analysis. For quality control assays, however, the LoQ is not a crucial parameter and is not even requested in the respective ICH guideline, because the assay concentration is determined during method development. Nevertheless, broader quantitation ranges paired with selectivity allow easier method adaption to other purposes, e.g., analysis of dietary supplements. Both characteristics are given for the NMR methods described herein. In contrast to all previously reported assays, all (rather than just a few) of the sweet-tasting 11-α-hydroxy-mogrosides have been quantified in the ripe fruits of *Siraitia grosvenorii*.

4. Conclusion

In this study, two-dimensional quantitative NMR was successfully applied for the analysis of *S. grosvenorii* fruits. The determination of the total content of sweet-tasting mogrosides was accomplished by using NMR signals resulting from the 11-α-hydroxy-group that occurs in this particular compound class and that is the crucial structural feature for the sweetening effect. In contrast to our previous work in which we quantified the total amount of a compound class by one specific NMR signal (Çiçek, Girreser, & Zidorn, 2018), only the compounds relevant for the biological effect were determined this time. The selective quantification of bioactive compound (sub)classes is not only a valuable tool for the quality control of food ingredients or herbal medicines but can also be applied to the screening of extracts during, for example, drug discovery. Furthermore, the present study provides another example for the advantage of quantitative NMR spectroscopy in natural products analysis in which several constituents contribute to a biological effect.

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Figures

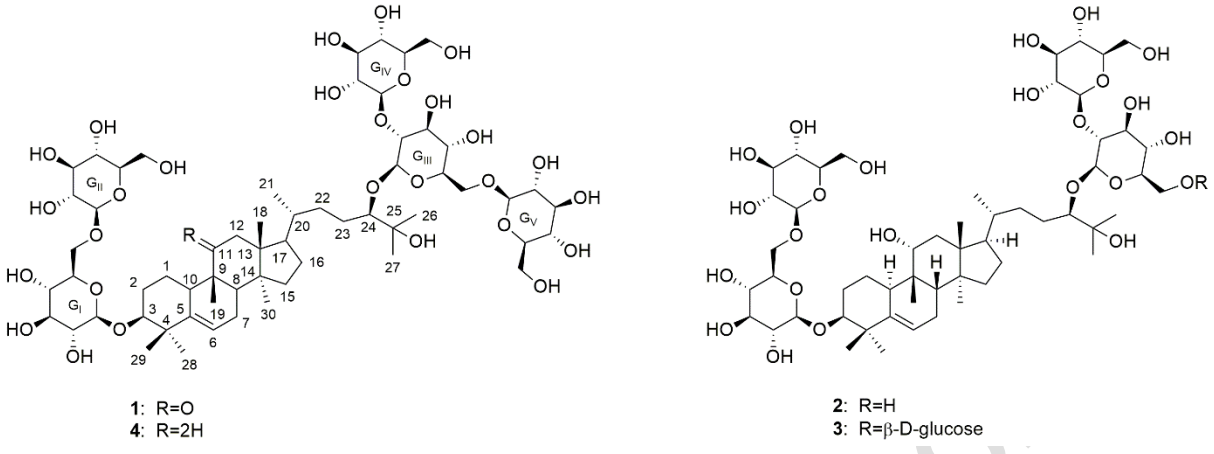


Fig. 1: Chemical structures of 11-oxomogroside V (**1**), mogroside IVe (**2**), mogroside V (**3**), and 11-deoxymogroside V (**4**). Exact stereochemistry can be retrieved from compounds **2** and **3**.

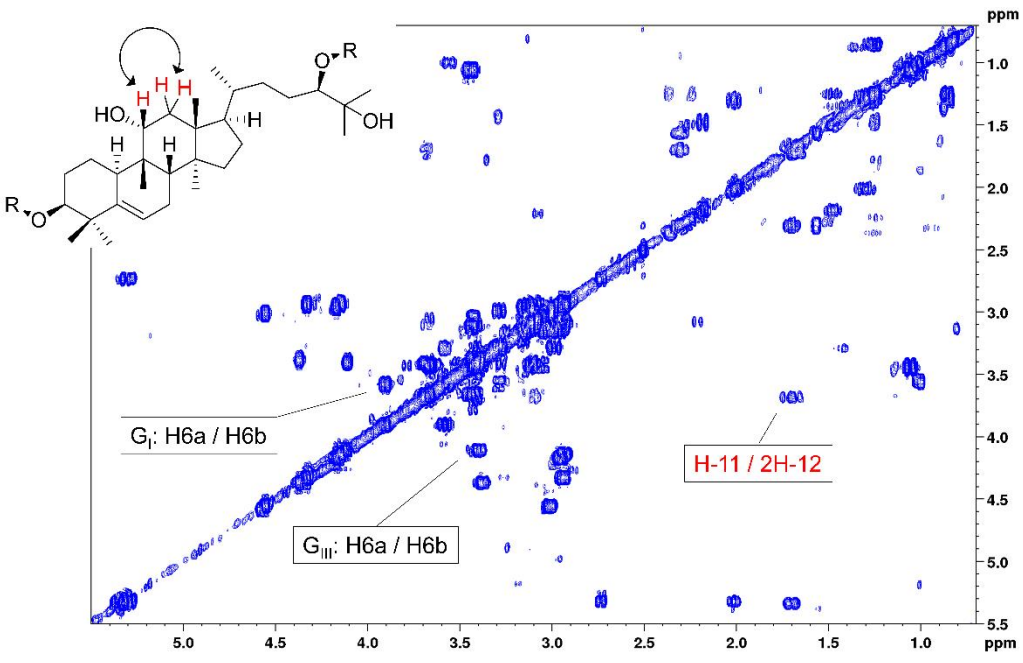
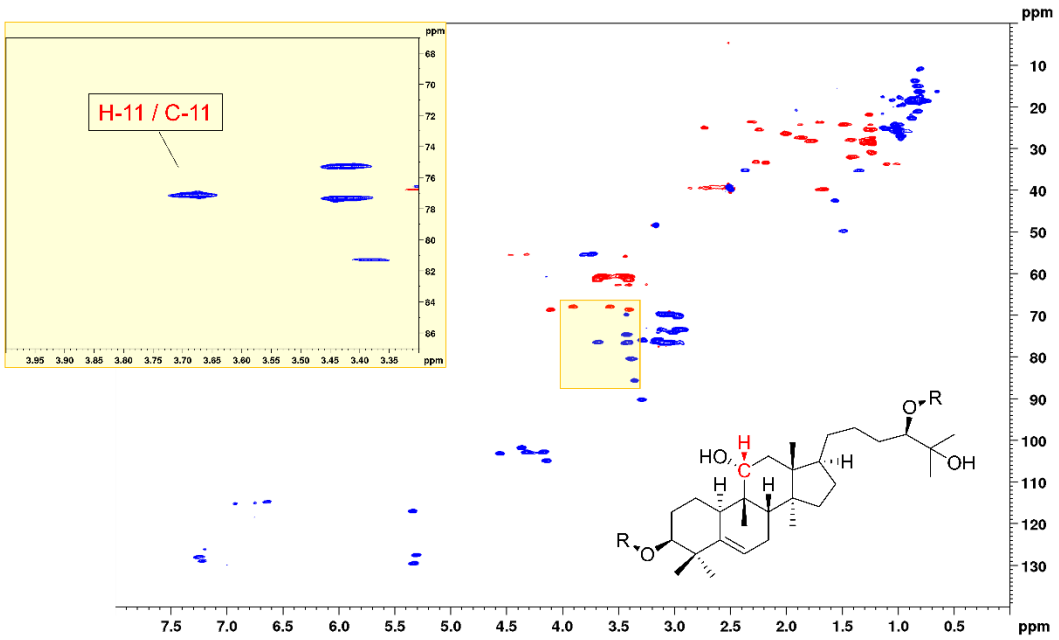


Fig. 2: H,H COSY diagram of luo han guo sample recorded in DMSO-d_6 in the region of 0.7 to 5.5 ppm. The signal used for quantitative NMR is highlighted in red.



550

551 Fig. 3: Complete spectral range HSQC diagram of luo han guo sample recorded in DMSO-d_6

552 and band-selective HSQC diagram in the region of 3.3 to 4.0 ppm (^{1}H) and 67 to 87 ppm (^{13}C)

553 (top left, only signals in the ^{13}C-range from 72.2 to 82.2 ppm are detected). Signal used for

554 quantitative NMR is highlighted in red.

555
556
557

Table 1

Regression equations, coefficients of determination (R^2), and limits of quantitation (LoQ) obtained from various NMR measurements (y: 2D NMR integral in arbitrary units, x: concentration in mmol/L)

Measurement	Regression equation	R^2	LoQ
bs-HSQC (wide integration)	$y = 44.04\times10^6 x + 11.78\times10^6$	0.9965	1.70 mM
bs-HSQC (small integration)	$y = 45.86\times10^6 x - 20.80\times10^6$	0.9981	1.70 mM
H,H COSY (2 scans, 15 min)	$y = 40.58\times10^6 x - 59.16\times10^6$	0.9970	1.70 mM
H,H COSY (8 scans, 57 min)	$y = 174.67\times10^6 x - 6.60\times10^6$	0.9986	1.00 mM

Table 2

Repeatability and intra-day and inter-day precision obtained from various NMR measurements. Results are given in mg/g drug material; RSD values are shown in parenthesis.

Measurement	Repeatability	Intra-day 1	Intra-day 2	Inter-day
bs-HSQC$_{wide}$	15.57 (3.38%)	16.21 (6.35%)	16.13 (5.45%)	16.17 (5.65%)
bs-HSQC$_{small}$	15.55 (3.25%)	15.87 (5.73%)	16.02 (6.00%)	15.95 (5.61%)
H,H COSY$_{2\ scans}$	14.94 (1.95%)	15.00 (5.10%)	15.85 (4.43%)	15.43 (5.37%)
H,H COSY$_{8\ scans}$	14.98 (1.89%)	15.20 (1.73%)	15.28 (1.71%)	15.24 (1.66%)

Table 3

Accuracy of various NMR measurements. Values are expressed as the percentage of recovery (spiking experiments) and deviation from the mean value of all four experimental variants.

Measurement	High spike (120%)	Medium spike (100%)	Low spike (80%)	Deviation from the mean value
bs-HSQC$_{wide}$	101.2%	94.1%	89.3%	+3.0%
bs-HSQC$_{small}$	95.2%	96.2%	93.8%	+1.6%
H,H COSY$_{2\ scans}$	95.1%	103.2%	97.6%	-1.7%
H,H COSY$_{8\ scans}$	97.2%	98.8%	101.3%	-2.9%

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