Identification of iridoid glycoside regioisomers from *Harpagophytum procumbens* using a LC-DAD-MS/SPE-NMR hyphenation as dereplication tool

Markus Godejohann,[a] Christoph Seger,[b] Li-Hong Tseng,[a] Manfred Spraul,[a] Anny Girtler,[b] Sonja Sturm,[b] Hermann Stuppner[b]

[a] *Bruker-Biospin GmbH, D-76287 Rheinstetten, Germany*
[b] *Department of Pharmacognosy, Institute of Pharmacy, Leopold-Franzens University Innsbruck, A-6020 Innsbruck, Austria*

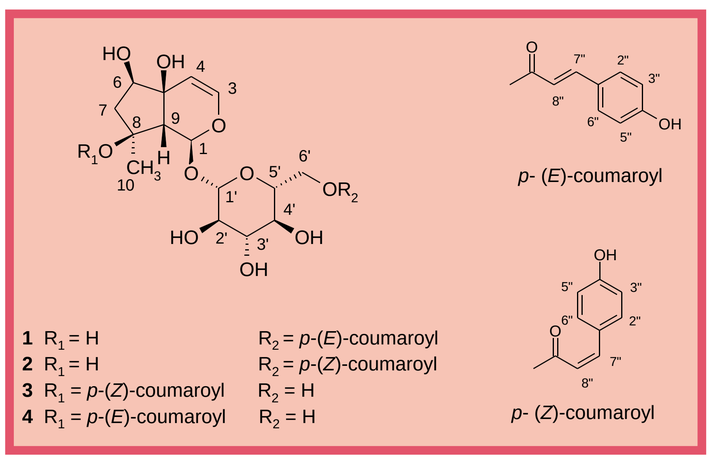
email:christoph.seger@uibk.ac.at

INTRODUCTION

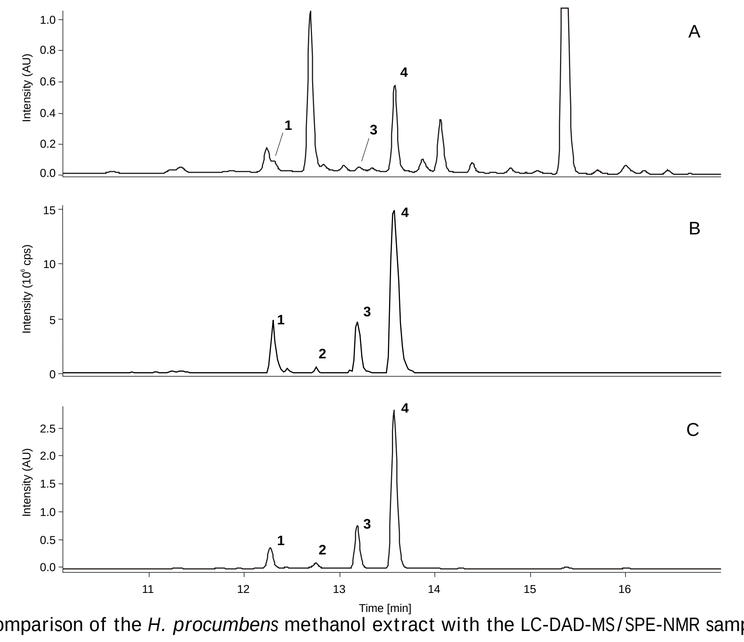
Rapid identification of already known secondary metabolites is mandatory to optimize the work flow in terms of labour and financial resources invested. This process is known as rapid dereplication [1] and combines online gathered UV, MS, and NMR data from hyphenated systems. It has become a central analytical topic in the field of phytochemical and biomedical analysis [2].

Harpagophytum procumbens D.C. (Pedaliaceae), also known as "Devils Claw", is one of the most promising phytomedicines used in the treatment of rheumatism, polyarthritis, and osteoarthritis due to its analgesic, anti-inflammatory, and antiphlogistic properties [3].

Here we demonstrate how the combination of simple chromatographic procedures with HPLC-DAD-MS/SPE-NMR hyphenation [4,5] simplifies the analytical breakdown of complex secondary metabolite matrices.

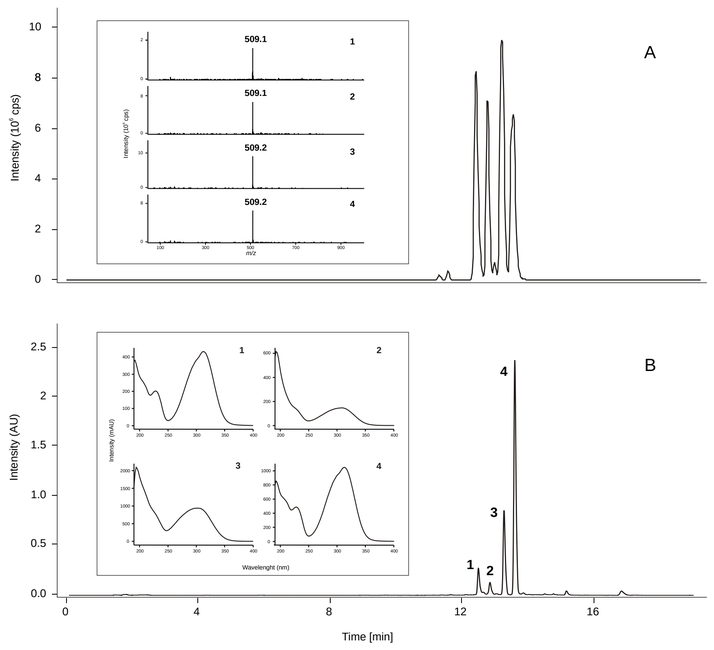


HPLC-DAD-MS



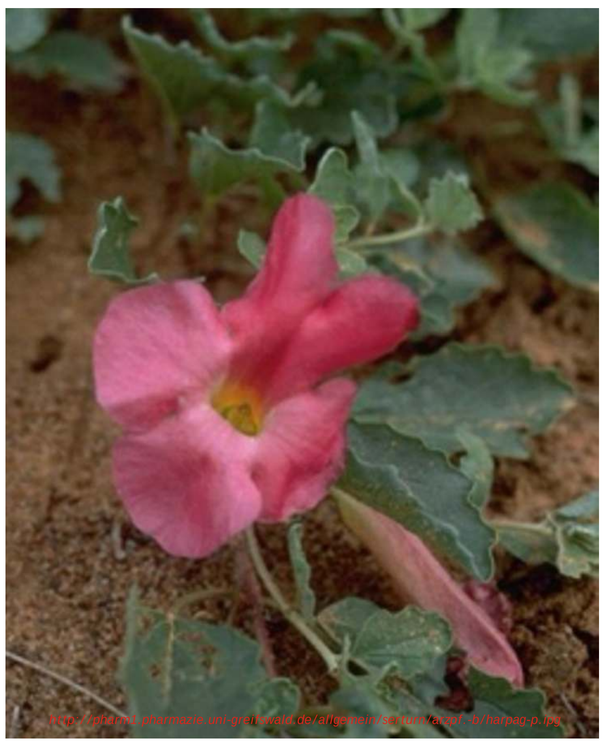
Comparison of the *H. procumbens* methanol extract with the LC-DAD-MS/SPE-NMR sample.

A and B: LC-DAD (280 nm) and LC-MS negative ESI ion trace ($[M-H]^-$ = 509) chromatograms of the crude extract. C: LC-DAD chromatogram(280 nm) of the LC-SPE/NMR sample.

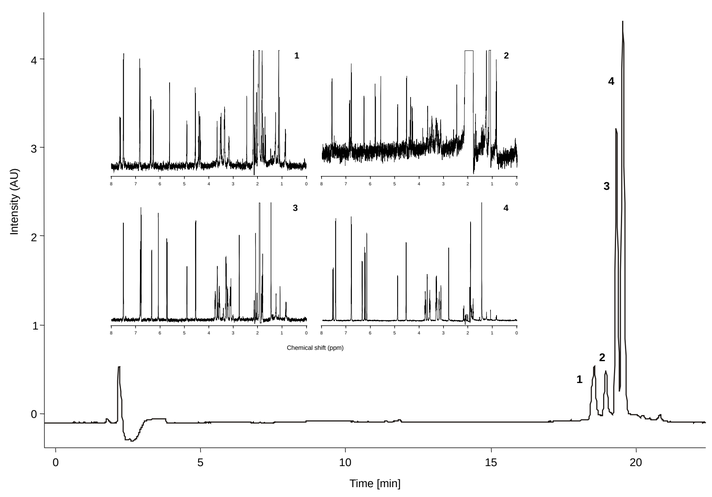


LC-DAD-MS chromatogram of the NMR sample.

A: LC-MS negative ESI ion trace ($[M/H]^-$ = 509) chromatogram with peak mass spectra as inserts. B: LC-DAD (280 nm) chromatogram with peak UV spectra as inserts.



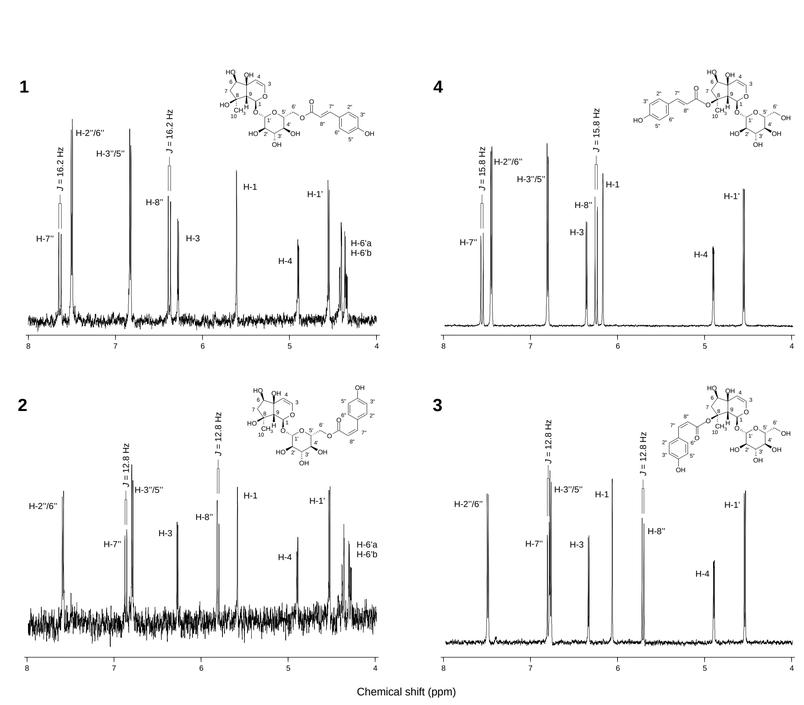
HPLC-DAD-MS/SPE-NMR



Above: LC-DAD derived chromatogram (205 nm) of the LC-DAD-MS/SPE-NMR experiment. The inserted NMR spectra (CD_3CN) were obtained by single trapping of analytes 1-4 on RP-18 SPE cartridges.

Below: Comparison of signal to noise (S/N) ratios using different sample transfer conditions. Relative S/N ratios are given in brackets.
Experiment 1: Off-line LC-DAD-NMR system.
Experiment 2-4: LC-DAD-MS/SPE-NMR hyphenation.

	Sampling conditions	NMR solvent	1	2	3	4	Sum 1-4
# 1	Loop collection	D_2O/CH_3CN gradient	18.9 (7)	6.7 (3)	69.2 (25)	177.8 (65)	272.6
# 2	Hysphere C18	CD_3CN	37.4 (10)	12.6 (3)	81.0 (21)	260.1 (66)	391.1
# 3	Hysphere GP	CD_3CN	42.4 (23)	14.0 (8)	9.7 (5)	119.6 (64)	185.7
# 4	Hysphere GP	CD_3OD	2.2 (1)	9.2 (6)	93.1 (55)	63.9 (38)	168.4



Down-field region of the ^{1}H-NMR spectra of analyte 1-4 with compete signal assignments and chemical structures.

SUMMARY

Extract breakdown and sample enrichment was facilitated by simple chromatographic separation steps.

The LC-DAD-MS/SPE-NMR hyphenation allowed obtaining NMR spectra of all four analytes within one working day.

Even for the least analyte present (2, 5 µg on column) a proton NMR spectrum with sufficient S/N (14.0) was achievable within less than 10 minutes.

The four analytes were identified as (*E*/*Z*) pairs of 8-O-(p-coumaryl)-harpagide (8-PCHG) and 6'-O-(p-coumaryl)-harpagide.

One of these metabolites is a new natural product (3) and two more (1,2) have not been described from *Harpagophytum procumbens* yet. The fourth analyte, 8-(*E*)-PCHG (4), is a prominent chemotaxonomical discriminator within the genus *Harpagophytum* [6,7].

RESULTS SPE-NMR HYPHENATION

- SIMPLIFY: Preparative chromatographic extract breakdown yielded mixtures with only few analytes. This matrix simplification allowed accelerating the HPLC-SPE-NMR assay.
- CONCENTRATE: Matrix simplification is paralleled by analyte enrichment. This helps cutting down the NMR analysis time.
- OPTIMIZE: Careful optimization of the SPE transfer step is mandatory on a case to case basis but not time consuming owing to the rapid HPLC-SPE-NMR assay.
- COMPARE: Changing from partially deuterated LC solvents to defined deuterated NMR solvents allows direct data comparison with NMR literature.

Experimental parameters ISOLATION

Dried Harpagophytum procumbens secondary root tubers (400 g) of European Pharmacopoeia quality were ground and extracted exhaustively with methanol.

The LC-DAD-MS/SPE-NMR sample was obtained by crude fractionation of an aliquot (30 g) of this extract (150 g) over a silica gel column (dichloromethane/methanol step gradient) followed by HSCCC (ethyl acetate/n-propanol/water, lower phase = mobile phase) of a subfraction of 1.4 g. Final enrichment was facilitated by methanolic Sephadex-LH20 column chromatography of an 0.36 g HSCCC fraction. Sample preparation for the LC-NMR hyphenation experiments was carried out by dissolving an aliquot (7 mg) of the final fraction (50 mg) in 0.5 ml methanol.

Experimental parameters HPLC-SPE-NMR

HPLC:
Stationary phase: Trentec Supersphere C-18 (250 mm x 2 mm) (Gerlingen, Germany).
Mobile phase: 0.1% (v/v) formic acid (A) and 0.1% (v/v) formic acid in acetonitrile (B)
Gradient: 0 min, 2% B; 8 min, 18% B; 18 min, 45% B; 25 min, 98% B at 0.2 mL/min.
Injection volume: 10 µl.
Make-up solvent: 0.1% (v/v) formic acid at a flow rate of 0.8 ml/min.
SPE stationary phase: Hysphere GP or Hysphere C18 cartridges (10 x 2mm).
Transfer solvent: 255 µl deuterated acetonitrile or deuterated methanol.
NMR: 1D ^{1}H NMR spectra were recorded using multiple solvent suppression. All spectra were recorded at 300 K and referenced to residual solvent peaks.

INSTRUMENTATION

LC-DAD-MS (Innsbruck) and LC-DAD-MS/SPE-NMR (Bruker BioSpin, Rheinstetten) measurements were carried out using Agilent 1100 Liquid Chromatographs from Agilent (Waldbronn, Germany). MS measurements were performed on ESQUIRE-3000 ion trap mass spectrometers (Bruker Daltonik, Bremen, Germany) with electrospray ion sources. For the LC-DAD-MS/SPE-NMR hyphenation 5% of the eluent were split into the MS using a BNMI (Bruker BioSpin Rheinstetten NMMS-Interface). The Bruker/Spark Prospekt 2 Solid Phase Extraction Unit (Bruker BioSpin, Rheinstetten, Germany & Spark, Emmen, the Netherlands) was used for automatic peak trapping. After trapping SPE cartridges were dried with nitrogen gas to reduce residual solvents. Finally, trapped peaks were eluted into an AVANCE 600 MHz NMR spectrometer equipped with a LC SEI 13C 1H probe head with an active volume of 30 µl from Bruker BioSpin (Rheinstetten, Germany).

REFERENCES

[1]. Cordell, GA et al. (1999) Pure Appl Chem 71: 1089-1094.
[2]. Wolfender JL et al. (2003) J Chrom A 1000: 437-455.
[3]. Chrubasik, S et al. (2003) Reumatology 42: 141-148.
[4]. Nyberg, NT et al. (2001) Magn Reson Chem 39: 236-240.
[5]. Exarchou, V et al. (2003) Anal Chem 75: 6288-6294.
[6]. Potterat, O et al. (1991) Phytochem 30: 889-892.
[7]. Kikuchi, T et al. (1983) Chem Pharm Bull 31: 2296-2301.