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Quantification of the total amount of black cohosh cycloartanoids by integration of one specific ^{1}H NMR signal

Serhat Sezai Çiçek[a,*], Ulrich Girreser[b], Christian Zidorn[a]

[a] Pharmazeutisches Institut, Abteilung Pharmazeutische Biologie, Christian-Albrechts-Universität zu Kiel, Gutenbergstraße 76, 24118, Kiel, Germany
[b] Pharmazeutisches Institut, Abteilung Pharmazeutische und Medizinische Chemie, Christian-Albrechts-Universität zu Kiel, Gutenbergstraße 76, 24118, Kiel, Germany

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ABSTRACT

Quantitative analysis is an important field in the quality control of medicinal plants, aiming to determine the amount of pharmacologically active constituents in complex matrices. Often biological effects of herbal drugs are not restricted to single compounds, but are rather caused by a number of often biogenetically related plant metabolites. Depending on the complexity of the analyzed plant extract, conflicts between accuracy, such as total content assays using photometric or colorimetric methods, and comprehensiveness, e.g. quantification of one or a few lead compounds can occur. In this study, we present a qHNMR approach determining the total amount of cycloartanoids in black cohosh (*Actaea racemosa*) rhizomes. Perdeuterated methanol containing 1,2,4,5-tetrachloro-3-nitrobenzene as an internal standard was used for extraction. Amounts of cycloartanoids were then measured by integrating ^{1}H NMR signals of all cycloartenoids' H-19 *exo* protons. Due to their unusually low chemical shifts, these signals are well separated from all remaining signals in crude extracts. Thus, accurate (recovery rates of 99.5–102.5%) and precise (relative standard deviations below 2.5%) quantification of cycloartanoids was accomplished. To the best of our knowledge, this is the first example of a quantification of the total amount of a pharmacologically relevant compound class by integration of one ^{1}H NMR signal characteristic for all members of this particular compound class. Additionally, we propose a new term and unit for the evaluation of medicinal plants and herbal medicinal products: the "specific partial amount of substance" of pharmacologically active constituents, indicated in mmol/g.

1. Introduction

In the last decades, quantitative ^{1}H NMR spectroscopy (qHNMR) evolved into a standard analytical technique [1,2], not only for the determination of compound purities, but also for the analysis of complex natural samples [3]. Enabled by increases in magnetic strength, qHNMR has recently been applied in various areas, such as agricultural chemistry [4], pharmaceutical science [5], forensic science [6], biochemistry and metabolomics [7], and natural product chemistry [8]. Following quantitative NMR spectroscopic studies on such prominent botanicals as ginkgo (*Ginkgo biloba* L.), licorice (*Glycyrrhiza glabra* L.), and red clover (*Trifolium pratense* L.) [9–11], the current work applies qHNMR to rhizomes and extracts of *Actaea racemosa* L. [syn.: *Cimicifuga racemosa* (L.) Nutt.].

Commonly known as black cohosh, *A. racemosa* has a long history of medical use and is represented in official monographs of many pharmacopoeias [12]. After the first medicinal product was introduced for the therapy of menopausal symptoms more than sixty years ago, the drug was subject of numerous clinical and pharmacological studies [13,14]. To this day, both the efficacy and the mechanism of action of black cohosh are still discussed controversially [12–15]. However, triterpenoids contained in the rootstock are often considered to play an important role for its beneficial effects [16–18]. So far, more than 40 triterpenoids of the 9,19-cycloartane type have been isolated from black cohosh (basic structure given in Fig. 1) [14,19].

Due to the complexity of the species' metabolite pattern and the similarity of the different structures, not only clinical trials (patients were in general receiving isopropanolic extracts) [13], but also several pharmacological studies were performed employing alcoholic extracts instead of pure compounds [20–23]. For the same reason, analytical studies on black cohosh triterpenes are challenging. Apart from fingerprint studies to discriminate black cohosh

* Corresponding author.
E-mail address: scicek@pharmazie.uni-kiel.de (S.S. Çiçek).

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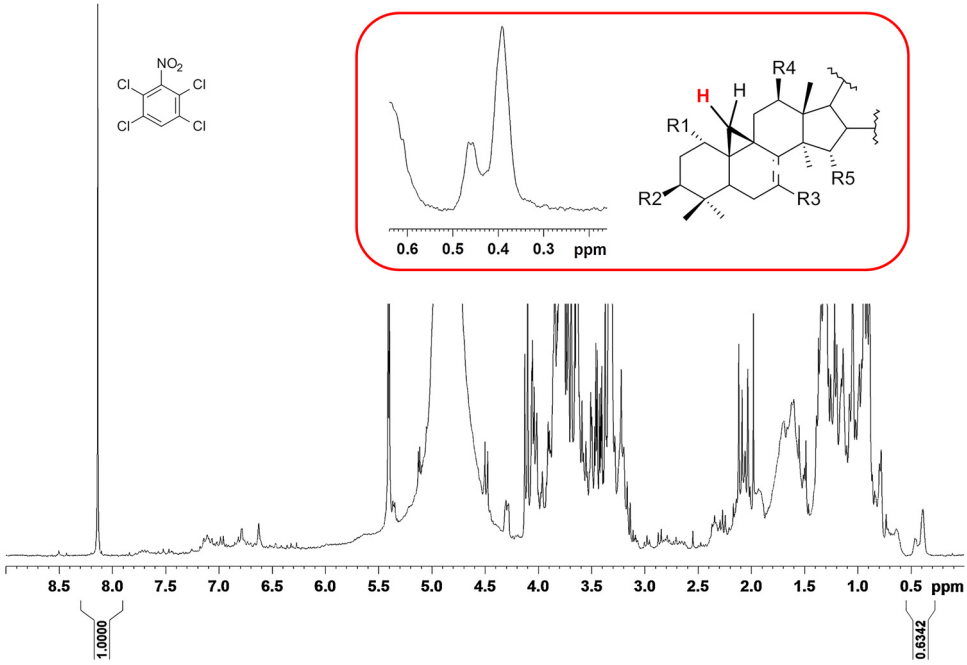

Fig. 1. ^{1}H NMR spectrum of deuteromethanolic black cohosh extract containing 2.00 mg/mL 1,2,4,5-tetrachloro-3-nitrobenzene. Left: structure and signal of 1,2,4,5-tetrachloro-3-nitrobenzene. Top right: Basic structure of cycloartanoids, proton (red) and respective signals used for integration. R1 = H or OH, R2 = α-L-arabinopyranoside or β-D-xylopyranoside or H, R3 = H or OH, R4 = H or OH or OAc, R5 = H or OH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from other *Actaea* species [24–26], HPLC has also been applied for quantification purposes. Of the four HPLC methods described in the literature [27–30], two cover the major triterpenoids occurring in black cohosh rhizomes [28,30], but assessment of the total cycloartane content still has not been accomplished.

The present manuscript describes an NMR-based approach and a new strategy in natural product analysis, quantifying a whole compound class by integration of one compound class specific signal; in the case of black cohosh, the signal of the H-19 *exo* protons of the cyclopropane rings (highlighted in red in Fig. 1).

2. Materials and methods

2.1. Chemical reagents and material

Dried and cut plant material was obtained from Alfred Galke GmbH (Bad Grund, Germany). Deuterated solvents for NMR spectroscopy were purchased from Euriso-top GmbH (Saarbrücken, Germany) and conventional 5 mm sample tubes were obtained from Rototec-Spintec GmbH (Griesheim, Germany). LC–MS grade formic acid, 1,2,4,5-tetrachloro-3-nitrobenzene (standard 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). 0.45 and 0.20 μm pore size membrane filters (Chromafil PET-45/25 and PET-20/13) were obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany).

2.2. General experimental procedures

NMR spectra were recorded using a Bruker Avance III 300 NMR spectrometer with a 5 mm PABBO broad band probe with z gradient. Extract, fractions, and pure compounds were analyzed using 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). MS analysis was carried out with a Shimadzu LCMS 8030 triple quadrupole mass spectrometer using atmospheric pressure chemical ionization. TLC was performed on silica gel 60 F254 plates using dichloromethane-methanol (85:15 v/v) as mobile phase and vanillin-sulphuric acid for detection.

2.3. Extraction and isolation

Dried and ground rhizomes (700 g) were extracted with acetone using ultrasonication, and the solvent was evaporated under reduced pressure to yield 42 g of a crude extract. The extract was then subjected to silica gel column chromatography (50 × 7.5 cm) and eluted with dichloromethane-methanol (9:1 to 5:5, followed by 100% methanol) resulting in six fractions (A–F). Fraction C (1.98 g) was chromatographed over a silica gel column (100 × 3.5 cm) using *n*-hexane-ethyl acetate-methanol (10:10:0.5 to 10:10:3 v/v/v) in a gradient manner, yielding 11 subfractions (C1–C11), of which fraction C9 gave 16.38 mg of actein after crystallization from methanol. Structures of the isolated compounds were elucidated by comparing MS and NMR spectra to literature data [31].

2.4. NMR measurements

A stock solution of 10.00 mg/mL 1,2,4,5-tetrachloro-3-nitrobenzene in methanol-d_4 was prepared and further diluted with methanol-d_4 into a standard solution of 2.00 mg/mL. For analysis of black cohosh rhizomes 100.0 mg of plant material were extracted with 1000 μL of standard solution using ultrasonication for 45 min. After 15 min of shaking at 200 rpm, the solution was directly filtered into the NMR sample tube. For quantification of commercial products, equivalents of 26 mg (four tablets of 6.5 mg each) or 25 mg (10 tablets of 2.5 mg each) plant extract were used. Tablets were crushed and sonicated for 45 min with 20.00 mL of acetone. After centrifugation at 3500 rpm, 10.00 mL