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Comparison of Extraction Methods for Secologanin and the Quantitative Analysis of Secologanin from *Symphoricarpos albus* using ^{1}H-NMR

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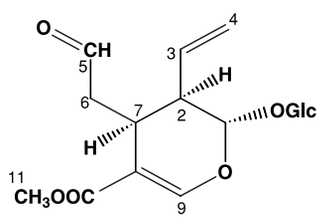
In order to develop an efficient large-scale extraction of secologanin from *Symphoricarpos albus*, different methods have been compared. Ultrasonication with organic solvents and water, microwave-assisted extraction and hot water extraction methods were evaluated for their efficiencies. Among the methods tested, ultrasonication with methanol showed the highest yield of secologanin (3.35 ± 0.24 mg/g fresh weight). For reliable quantification of secologanin a ^{1}H-NMR method was developed. The experiment was performed by the analysis of the integral of the signal of H-9, which was well separated in the range δ 7.4–7.5 in the ^{1}H-NMR spectrum. The quantity of the compound was calculated from the relative ratio of intensity of the target peak to the known amount of internal standard, 200 μg of gallic acid. This method allows rapid and simple quantification of secologanin in 5 min without any pre-purification steps. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: ^{1}H-NMR spectrometry; quantitative analysis; extraction methods; secologanin; *Symphoricarpos albus*.

INTRODUCTION

Secologanin (**1**), a monoterpene glycoside, is the precursor in the biosynthesis of more than 3000 indole and quinoline alkaloids (Hallard *et al.*, 1998). The compound is regarded as of value not only because it is important in alkaloid biosynthesis, but also because it can be used as a starting material for the chemical synthesis of pharmacologically interesting compounds (Contin *et al.,* 1998; Geerlings *et al*., 2001). This compound is exceptionally rich in functional groups and chemical reactions can be carried out at almost all carbon atoms of the aglycone skeleton. While these reactions are partially blocked by the glycosidic linkage, secologanin might be degraded by β-glucosidases present in the plant material during storage and extraction and the aglycone might undergo further breakdown reactions.

For the analysis of the content of **1** in plants, acetylation or silylation followed by GC analysis has been used (Inouye *et al.*, 1976; Naudascher *et al.*, 1989). However, the GC method demands a tedious derivatisation step. HPLC has been applied to analyse **1** in several indole alkaloids-producing plants (Dagnino *et al.*, 1996; Tikhomiroff and Jolicoeur, 2002), but the method requires a long separation time and shows peak broadening (Tikhomiroff and Jolicoeur, 2002). A method based on enzymatic conversion into strictosidine was developed by Hallard *et al.* (1998), but while the sensitivity was quite improved, it requires a source of the enzyme, i.e. strictosidine synthase.



Secologanin (1)

The reactivity of **1** prompted us to develop an efficient extraction method, avoiding breakdown of the iridoid. For developing a reliable extraction method, we compared the extraction methods of **1** from *Symphoricarpos albus* (Caprifoliaceae), which is regarded as an important source of **1** (Hallard *et al*., 1998). Ultrasonication with organic solvents, water, microwave-assisted extraction and hot water extraction were compared for their efficiencies.

For the quantification of secologanin, a ^{1}H-NMR method was developed. Quantitative ^{1}H-NMR methods for the determination of natural products, such as ginkgolides and bilobalides from *Ginkgo biloba* leaves, have been reported (Van Beek *et al.*, 1993; Choi *et al.*, 2003). An advantage of this method over HPLC and GC methods is that it requires much less sample preparation time as no pre-purification step is needed. Moreover, no calibration curves for quantification are necessary, and it takes only 4–5 min to quantify the compound by comparison with an internal standard.

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EXPERIMENTAL

Plant material. The berries of *Symphoricarpos albus* var. laevigatus (Fernald) Blake were collected at the Botanical Garden of the Gorlaeus Laboratories, Leiden University, the Netherlands, September, 2002. The voucher specimen was deposited in the Division of Pharmacognosy, Leiden University Plant material was stored at −80°C until use.

Solvents and chemicals. Highest grade methanol, ethanol and acetone were purchased from Merck Biosolve (Valkenswaard, The Netherlands); d_6-dimethylsulphoxide (DMSO-d_6, 99.9%) and DCl HCl-d_1 were purchased from Cortec (Paris, France); gallic acid (1,3,5-trihydroxybenzene) was obtained from Sigma (St Louis, MO, USA). Secologanin was purified as described by Stevens (1994) and identified by ^{1}H-NMR.

^{1}H-NMR apparatus and parameters. ^{1}H-NMR spectra were recorded in 600 µL DMSO-d_6 to which 10 µL DCl were added using a Bruker (Karlsruhe, Germany) AV 400 spectrometer (equipped with an Indy Silicon graphics computer). For each sample, 64 scans were recorded with the following parameters: 0.17 Hz/point, pulse width = 4.0 µs, and relaxation delay = 10.0 s. Free Decay Induction (FIDs) were Fourier transformed with Line Broadening Factor (LB) = 0.12 Hz, Gaussian Broadening Factor (GB) = 0 Hz. For quantitative analysis, peak area was used and the start and end points of the integration of each peak were selected manually.

Extraction. A sample (**1** ***g***) of fresh *S. albus* berries was ground using a mortar and pestle under liquid nitrogen and extracted with 20 mL of each solvent (methanol, ethanol, acetone and water, respectively) for 30 min using ultrasonication (Sonicor, Copiague, NY, USA). For microwave extraction, the samples were irradiated using a domestic microwave apparatus‡ (SMC, China; full power 700 W) for 5 s after which the power was switched off for a cooling period of 10 s so that the desired temperature (ca. 85–90°C) could be achieved. This procedure was repeated until the net irradiation time was around 2 min. To extract with hot water, *S. albus* berries (**1** ***g***) were extracted with 20 mL boiling water for 2 min. All extractions were repeated twice. After extraction, samples were filtered using Whatman (clittort, NJ, USA) no. 2 filter paper and evaporated *in vacuo*. After addition of internal standard, the ^{1}H-NMR spectra of the samples were measured. All experiments were carried out in triplicate.

HPLC analysis. The HPLC instrument consisted of a Waters (Milford, MA, USA) model 600E pump, 991 photodiode array detector and 5200 integrator. Aliquots (50 µL) of sample solutions were injected using a Waters 717 plus auto-sampler. Detection was carried out between 210 and 350 nm. A Vydac (Hesperia, CA, USA) C_{18} reverse-phase column (250 × 4.6 mm i.d.; 5 µM) was used. Gradient elution was performed using solvent A (water:acetonitrile:trifluoroacetic acid, 95:5:0.01, v/v/v) and solvent B (water:acetonitrile:trifluoroacetic acid, 5:95:0.01, v/v/v) according to the following scheme: solvent B 0–23%, 0–27 min; solvent B 23% isocratic 27–34 min; solvent B 23–100%, 34–40 min. The flow rate was 1.0 mL/min.

‡ Use in the laboratory of equipment designed for domestic purposes is not recommended and represents a potential danger of which users should be made fully aware.

RESULTS AND DISCUSSION

Quantitative analysis using ^{1}H-NMR

Quantification of **1** by ^{1}H-NMR is possible by means of the integral or intensity of a well-separated specific proton signal of the compound. Secologanin has a characteristic signal around δ 7.4–7.5 (dependent on the NMR solvent) in the ^{1}H-NMR spectrum, which is due to the H-9 proton in the pyran ring. However, pure **1** dissolved in methanol-d_4 and in acetone-d_6 exhibited a mixture of **1** and a derivative formed with the solvents by an enolisation of the aldehyde group (Stevens, 1994; Tomassini *et al.*, 1995). To avoid this reaction, secologanin was dissolved in d_6-dimethylsulphoxide (DMSO-d_6). The H-9 proton of secologanin (δ 7.48) was well separated and could be integrated in this condition, even though this peak appeared as a doublet (J = 1.6 Hz) coupled with the H-7 proton, because it is in a region where no interference from other signals occurred. To determine the true content of **1**, it was necessary also to quantify the hemiacetal and acetal derivatives. The **1** isolated from *S. albus* showed a mixture of **1** and its derivatives at a ratio of 6:4, which was calculated by the integration of H-9 peak in δ 7.48 and δ 7.34, respectively (Fig. 1). Addition of 10 µL of DCl transformed all of these derivatives to **1**. As shown in Fig 2, the amount of secologanin, which was calculated from the peak area compared to internal standard, correlated well and the addition of DCl (10 µL in 600 µL DMSO-d_6) increased the sharpness of the peak. This condition had one more advantage; the peak area of

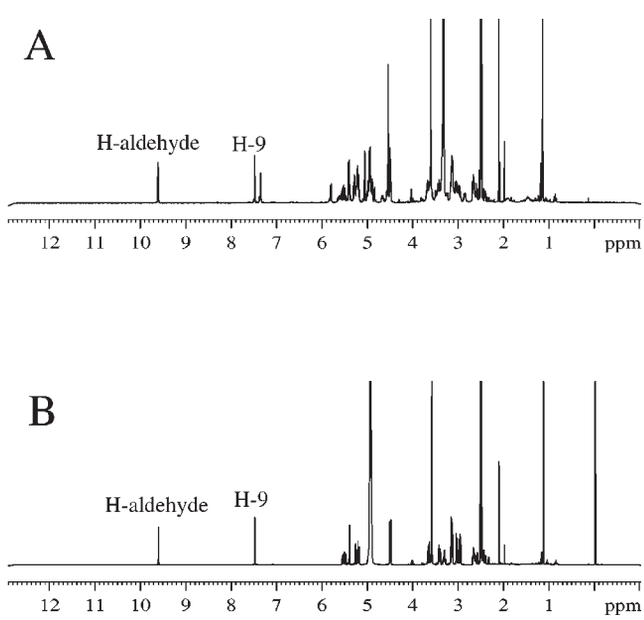

Figure 1. ^{1}H-NMR spectra of secologanin in (A) DMSO-d_6 and (B) DMSO-d_6+DCl, 400 MHz.

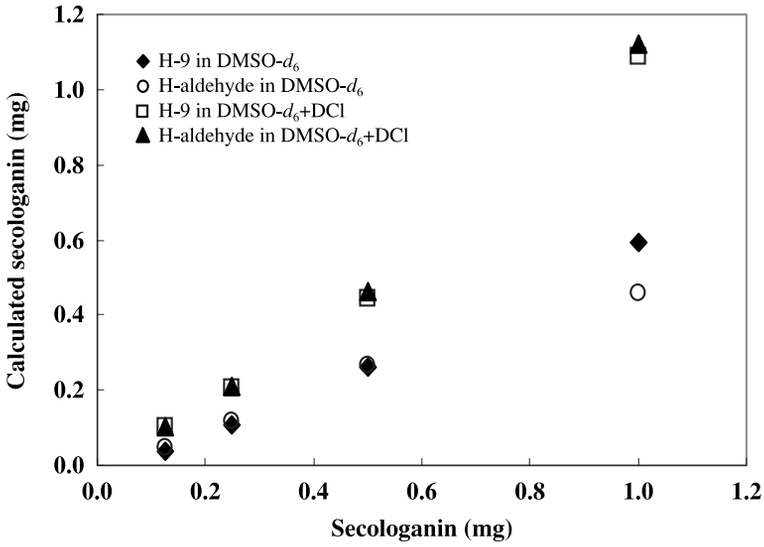

Figure 2. Calibration for secologanin calculated from the integral of H-9 and H-aldehyde compared with internal standard. (◆) Calculated from H-9 signal in DMSO-d_6; (○) calculated from H-aldehyde in DMSO-d_6; (□) calculated from H-9 signal in DMSO-d_6+DCl; and (▲) calculated from H-aldehyde signal in DMSO-d_6+DCl.

the aldehyde proton could also be used as a target peak for the quantitative analysis because the exchange rate of aldehyde proton to deuterated proton in this solvent was very slow. Therefore, in all experiments one drop of DCl was added before the measurement of ^{1}H-NMR. The two signals showed a highly linear correlation ($\gamma^2 = 0.9920$ of H-9 and $\gamma^2 = 0.9939$ of aldehyde peak).

Next, a suitable internal standard was selected. Preferably this should be a stable, non-volatile compound with one sharp singlet. Phloroglucinol and maleic acid have been used for the ^{1}H-NMR analysis of ginkgolides (Van Beek *et al*., 1993; Choi *et al*., 2003), so these compounds, among others such as anthracene and gallic acid, were tested. Gallic acid proved to be most suitable for the analysis of **1**. The signal showed only one sharp singlet at δ 6.91, which was well separated from the signals of **1** and other compounds in the extracts, and a good linearity between the amount and peak area was found. Thus the amount of **1** in plant extracts was calculated by comparing the peak area of gallic acid signal with the signals of **1**. For the quantification to be optimal, full relaxation of the protons of secologanin and the internal standard had to be achieved. For this purpose a rather large acquisition time of 10 s was used.

Extraction of secologanin from *Symphoricarpos albus* berries

Using the ^{1}H-NMR analysis, the yields of several extraction methods were evaluated to find the most efficient and reliable one. Results obtained with ultrasonication with organic solvents and water, microwave-assisted extraction in water and hot water extraction were compared. Table 1 shows the content of **1** calculated from the integral of H-9 and the aldehyde proton and by HPLC analysis. Using the ^{1}H-NMR analysis method, the content obtained by measuring H-9 was generally higher than the content calculated from the aldehyde signal.

Table 1. Secologanin yields from different extraction methods by the integral of H-9 and H-aldehyde in ^{1}H-NMR spectrum and HPLC analysis[a]

	Secologanin content calculated from (mg/g fresh weight ± SD)		
	^{1}H-NMR		
Method	H-9	Aldehyde	HPLC analysis
Sonication with methanol	3.35 (± 0.24)	2.95 (± 0.35)	3.13 (± 0.07)
Sonication with ethanol	3.32 (± 0.23)	3.10 (± 0.49)	3.30 (± 0.17)
Sonication with acetone	2.73 (± 0.20)[b]	2.82 (± 0.30)	2.36 (± 0.32)[c]
Sonication with water	0.84 (± 0.15)[c]	0.82 (± 0.20)[c]	0.82 (± 0.12)[c]
Microwave-assisted extraction	2.10 (± 0.19)[c]	1.74 (± 0.32)[c]	1.96 (± 0.56)[c]
Hot water extraction	2.39 (± 0.16)[c]	2.11 (± 0.17)[c]	2.28 (± 0.20)[c]

[a] All experiments are based on triplicate measurements.
[b] Significantly different from the methanol extraction, $p < 0.01$.
[c] Significantly different from the methanol extraction, $p < 0.001$.

This was probably due to the H-9 peak being in a more crowded region compared with the aldehyde signal, causing overlap with other signals.

Among the methods evaluated, ultrasonication with methanol and ethanol showed higher efficiency than any other method. Ultrasonication with acetone was less efficient than with methanol and ethanol. Secologanin content in the water sonication extract showed the lowest yield. This was probably due to *β*-glucosidase not acting in the organic solvents such as methanol and acetone, while in the aqueous medium it hydrolysed **1** to its aglycone and glucose. The lower amount of **1** present was concluded from ^{1}H-NMR spectrum (Fig. 3). It was reported that the hydrolysis of **1** by *β*-glucosidase yielded a bicyclic compound (Tietze, 1983). The breakdown of **1** by *β*-glucosidase could also be concluded from the colour change of *S. albus* berries during extraction with water. However, hot water extraction and microwave extraction could be alternative methods to extract **1** from the plant material because enzymes were denatured at higher temperature, and a high yield of **1** could be obtained (Vera Rocha, 2000). In a recent report, hot water extraction was found to be a more efficient technique than conventional organic acid extraction to extract iridoid glycosides such as catapol and aucubin from *Veronica lonifolia* leaves (Suomi *et al.*, 2000). Microwave-assisted extraction was reported to be efficient for extracting tanshinones from *Salvia miltorrhiza* (Pan *et al.*, 2002). However, in the present study, sonication using alcohols proved to be the most efficient method for the extraction of secologanin and its repeatability was excellent. In more than seven individual experiments, we obtained the same results.

In the case of highly reactive compounds such as secologanin, a rapid and simple extraction method was vital to reduce the risk of decomposition. The use of ^{1}H-NMR as a quantitative method reduced sample preparation procedures and additionally provided qualitative information in the products of degradation or derivatisation if this eventually occurred.

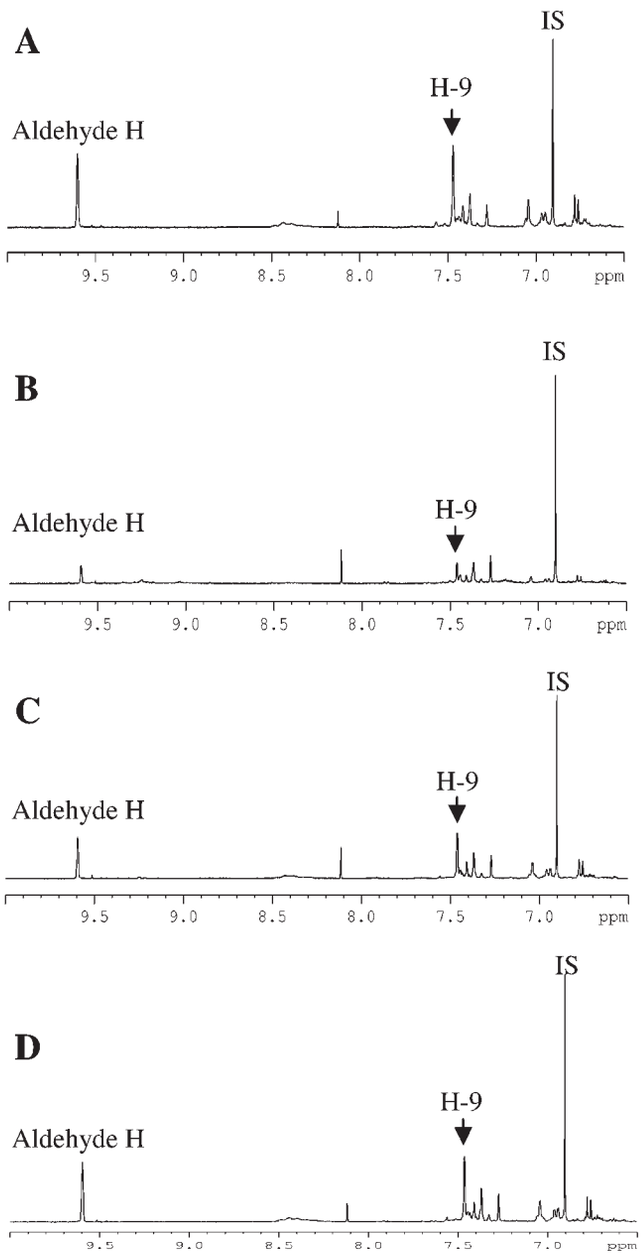

Figure 3. ^{1}H-NMR spectra of *Symphoricarpos albus* extracts in different extraction methods (δ 10.0–6.5, 400 MHz). (A) Sonicated with MeOH; (B) sonicated with water; (C) microwave-assisted extraction with water; (D) hot water extraction, IS, internal standard (200 μg of gallic acid).

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