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Development of LC–MS method for determination of ursolic acid: application to the analysis of ursolic acid in *Staphylea holocarpa* Hemsl.

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Abstract

Ursolic acid is a hydroxy pentacyclic triterpene, which has a chemoprotective activity in human. A reliable and reproducible liquid chromatography–mass spectrometric assay (LC–MS) was developed for the determination of ursolic acid in laboratory-made mixtures and in leaves and twigs extracts of *Staphylea holocarpa* Hemsl. The methanolic solution of the extracted ursolic acid was chromatographically analyzed using Shim Pack CLC-CN, C18 (150 × 6 mm, 5 μ) column and a mobile consisting of methanol–1% acetic acid solution (4:1) at a flow rate of 1.0 ml min^{-1}. The mass spectrometer (LCQ-Finnigan) was programmed in the positive single ion monitoring (SIM) to permit detection and quantitation of ursolic acid in MS–SIM mode at *m/z* 439.2, 411.2 and 390.9. Linear correlation ($r > 0.99$) of the peak area and the concentration of ursolic acid over the concentration range 0.25–10 μg ml^{-1} was obtained. The relative standard deviation (%R.S.D.) and percentage deviation from the nominal concentrations (%DEV) were found to be 3.03–3.59% and −4.5 to +6.2%, respectively. Analysis of laboratory-made mixtures containing known concentrations of ursolic acid, as quality control samples, gave a mean recovery percentage of 97.8%. Application of the proposed method for the analysis of leaves and twigs extracts of *S. holocarpa* Hemsl. gave mean percentage contents of ursolic acid of 0.95 and 0.25%, respectively.

Keywords: Ursolic acid; *Staphylea holocarpa* Hemsl; LC–MS

1. Introduction

Ursolic acid is a naturally occurring triterpene, which is found in many plants (Fig. 1). It possesses many important biological activities, such as anti-inflammatory, hepatoprotective, antiulcer, hypoli-

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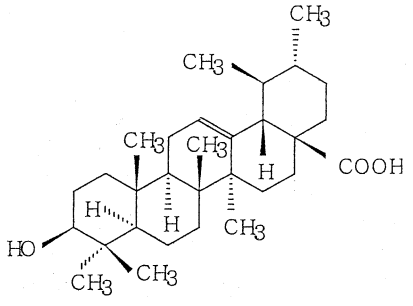

Fig. 1. Chemical structure of ursolic acid.

pidemic and antiatherosclerotic [1]. Ursolic acid was proved to have an anti-HIV activity [2] by inhibiting dimerization of HIV-1 protease [3]. A review on the various biological properties of ursolic acid has recently been reported [4]. In Chinese medicine, ursolic acid is dispensed as chemo-protective natural fatty substance in the form of pills [5] or capsules [6]. As shown in the literature, limited articles were reported on the analysis of ursolic acid. The majority of the reported articles were based on TLC analysis [7–11], whereas few articles were based on either HPLC [12–14] or GC [15] determinations. The fact that ursolic acid is a compound with almost no chemical group functionality complicates its quantitation by HPLC or GC. The described HPLC methods were lengthy and insensitive, whereas the GC method was tedious and need a derivatization reaction.

The development of a precise analytical technique for the determination of ursolic acid over a wide concentration range is becoming now more important, since the substance has been recognized as a potent chemopreventive agent of plant origin [4]. We aimed in this work to the development of a simple and reliable liquid chromatography–mass spectrometr (LC–MS) method for the determination of ursolic acid in natural plant material and formulated preparations such as capsules and pills. The validity of LC–MS method is established by determining the linearity, precision and accuracy, whereas analyzing quality control laboratory-made mixtures and natural plant samples containing ursolic acid confirm the utility of the method for sample analysis.

2. Experimental

2.1. Plant material

The leaves and twigs of *Staphylea holocarpa* Hemsl. were separately collected at the Institute of Dendrobiology, Slovak Academy of Sciences–Arboretum Mlynany. The fresh leaves and twigs were dried at room temperature (22 °C) for 3 weeks and then processed at the laboratory mill (Fritsch, Germany).

2.2. Extraction

The dried leaves or twigs ($\sim$10 g) were powdered and extracted either with petroleum ether or with chloroform for 48 h at room temperature (negative Liebermann–Burchard test). The collected extracts were filtered and evaporated under vacuum. The residues were dried, weighted and analyzed by LC–MS to determine ursolic acid content. A sequential extraction of the material with chloroform was performed after petroleum ether extraction.

2.3. LC–MS analysis

LC separations were made using LC pump Spectra System P 2000 (TSP) connected to a Shim Pack CLC-CN, C18 (150 × 6 mm, 5 μ) column (Shimadzu). The mobile phase was prepared by mixing 400 ml of methanol, 95 ml of water and 5 ml of glacial acetic acid and was pumped at a flow rate of 1.0 ml min^{-1}. Injection of the samples was done manually using a loop of 10 μl size (Cheminert^{RT}). Mass spectrometric analyses were performed using ion-trap mass spectrometer (Finnigan MAT, LCQ) operated in APCI mode. To achieve optimum mass ion detection, tuning of the mass spectrometer was done at the parent mass ion of ursolic acid using a diffusion pump directly connected to the ionization probe. The APCI conditions were selected as: vaporization temperature 430 °C, capillary temperature 150 °C, sheath gas flow 80 ml min^{-1} and corona discharge 4.38 kV. The mass spectrometer was programmed to detect the positive ions of ursolic acid in the range of m/z 300–500. Quanti-

tation was achieved using single ion monitoring (SIM). All data acquisition and processing were controlled by LCQ software.

2.4. Standard solutions

Stock solution of ursolic acid was prepared by dissolving ~10 mg of ursolic acid (Sigma grade) powder in methanol (1 μg ml^{-1}). The solution was stable for at least 1 month, when stored in glass containers at 4 °C. A working standard solution of ursolic acid at concentration 10 ng μl^{-1} in methanol was prepared. A set of six calibrators in the concentration range of 0.25–10 μg ml^{-1} was prepared in mobile phase. The solutions were measured using MS–SIM at *m/z* 439.2, 411.2 and 390.9. The peak area of each concentration was automatically calculated by the instrument build-in LCQ software.

2.5. Precision and accuracy studies

Three sets of samples at concentrations 0.25, 1.0 and 10.0 μg ml^{-1} ($n = 5$) were prepared in the mobile phase. Replicate analysis using 10 μl aliquots was performed. Peak area values were automatically measured and %R.S.D. and %DEV from the nominal concentrations were calculated.

2.6. Quality control samples

Laboratory-made mixtures, as quality control samples, were prepared by mixing and diluting ~50 mg of ursolic acid to ~200 mg with formulation excipients mixture (equal weights of lactose, starch and magnesium trisilicate). The powder was transferred to 50 ml volumetric flask, mixed with ~40 ml of methanol and sonicated for 15 min. The mixture was diluted to volume and filtered using membrane filter. A 10 μl aliquot of the filtrate was mixed and diluted to 10 ml with mobile phase and a 10 μl volume was injected into LC–MS.

2.7. Samples analysis

A weight of ~5 mg of the extract of either leaves or roots, was mixed with ~5 ml of methanol and sonicated for 30 min. The solution was filtered and an appropriate aliquot of the clear solution (10 μl) was diluted to 10 ml with mobile phase. A 10 μl aliquot was injected and analyzed under the described LC–MS conditions. The concentration of ursolic acid was determined from the linear regression equation representing the calibration curve.

3. Results

3.1. LC–MS analysis

Several trials have been made to select the proper conditions for the analysis of ursolic acid. The composition of the mobile phase was varied to achieve a rapid and maximum detection of ursolic acid. The high percentage of methanol and the appropriate acidity of the mobile phase (pH 4.6) permit optimum ionization and detection of ursolic acid molecules under APCI conditions at a vaporization temperature 430 °C. Under the selected LC–MS conditions, ursolic acid (MW 456) exhibited major peaks at *m/z* 391.3, 411.4, 439.3 and 457.1, respectively (Fig. 2). These peaks refer to the parent ion (457.1) and major fragment ions (391.3, 411.4, 439.3) of ursolic acid according to the diagram (Fig. 3). For qualitative analyses, the fragmentation pattern makes it possible to hypothesize the possible degradation or metabolic pathway of ursolic acid. For quantitative purposes, the appeared signals with the corresponding masses would be selected to determine ursolic acid. Using full mass scan (full MS) in the range *m/z* 300–500, ursolic acid showed a chromatogram at Rt < 4.0 min that referred to the parent mass ion 457.1(Fig. 4A). The peak area was increased, when SIM–MS was selected at a single mass ion 457.1 ± 2 (Fig. 4B). When SIM–MS scans using two or three mass ions (Fig. 4C–E), the peak area of ursolic acid was significantly increased. For quantitative analysis, SIM–MS at *m/z* 391.3, 411.4 and 439.3 (Fig. 4E) was recommended, because a maximum sensitivity was attained. Calibration curves ($n = 3$) were constructed by plotting the calculated peak area versus concentration over the concentration range 0.25–10.0 μg ml^{-1}. The limit

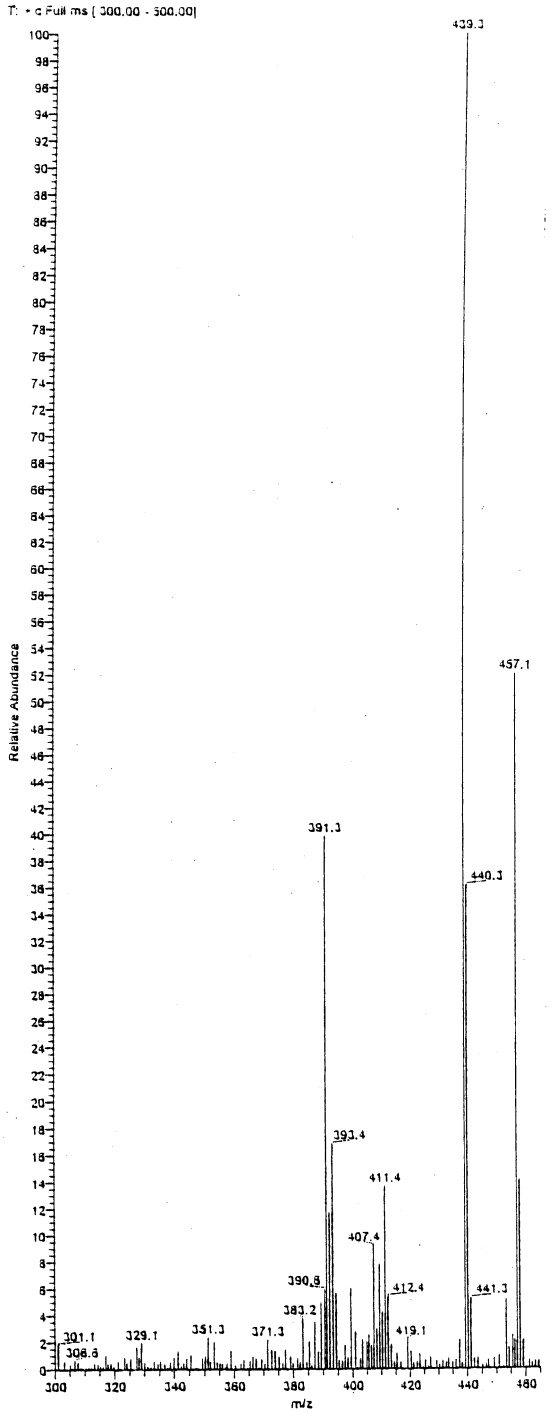

Fig. 2. Full mass spectrum of ursolic acid.

of quantitation was 0.25 μg ml^{-1}. The linear regression equation was $PA \times 10^{-6} = 9.8 + 21.06C$ ($r = 0.9999$) using a mean of three determinations. The reproducibility of the method was proven by analyzing samples of ursolic acid at three concentration levels 0.25, 2.0 and 10.0 μg ml^{-1}. The relative standard deviation (%R.S.D.) was in the range 3.03–3.59%. The accuracy of the LC–MS method was evaluated by determining the percentage deviation from the nominal concentration (%DEV) of ursolic acid in the samples. This value ranged from −4.5 to +6.2%. The precision and accuracy data (Table 1) validate the developed method and suggest the application of the method for routine analysis of ursolic acid in samples.

3.2. Determination of ursolic acid in laboratory-made mixtures

Quality control samples of laboratory-made mixtures containing ursolic acid at concentration of 50 mg per 200 mg of excipients mixture ($n = 5$) were prepared and analyzed by LC–MS. Recovery studies were performed and a mean percentage of 97.8% indicated the specificity of the developed method for measurement of ursolic acid content in control samples (Table 2).

3.3. Determination of ursolic acid content in plant extracts

Sequential extraction of the leaves using petroleum ether followed by chloroform permits more complete extraction of ursolic acid and other pentacyclic triterpenes compared with single extraction with chloroform. On the other hand, this sequential extraction does not increase the amount ursolic acid extracted from the twigs. Application of LC–MS for the determination of ursolic acid in the residual extracts of leaves and twigs of *S. holocarpa* Hemsl. indicated higher accumulation of ursolic acid in mature leaves compared with young twigs (Table 3).

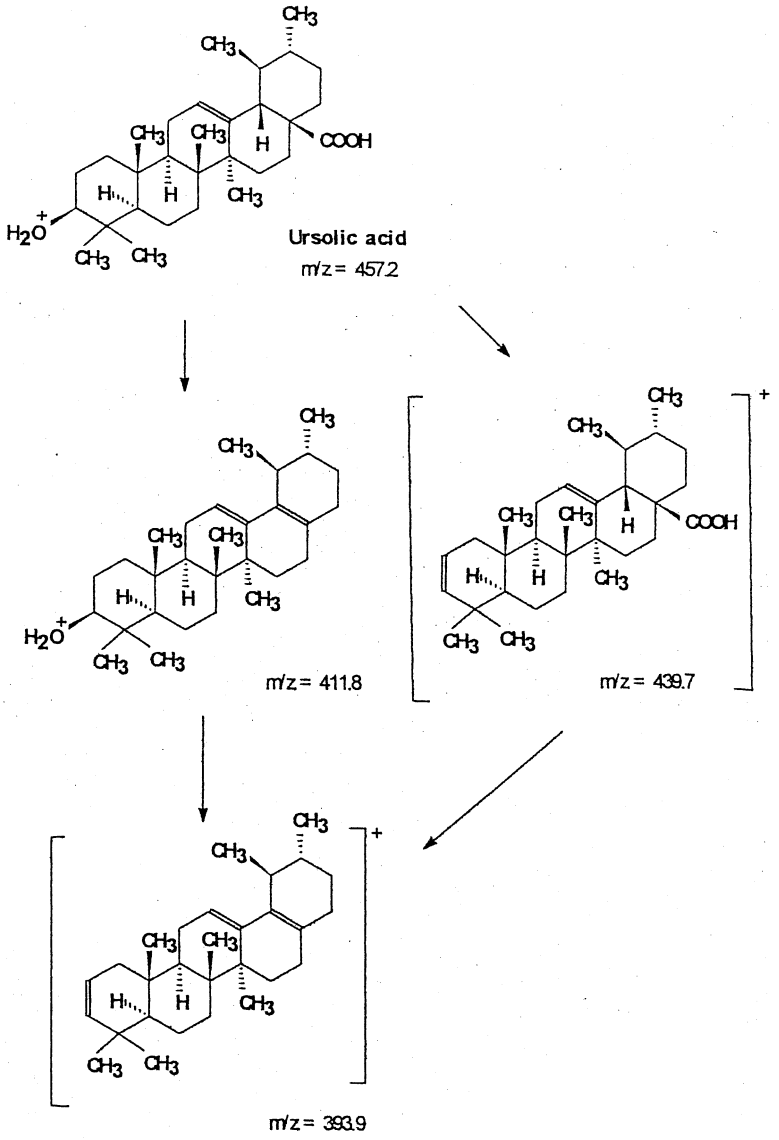

Fig. 3. Proposed fragmentation pattern of ursolic acid.

4. Discussion

The development and application of LC–MS method for the determination of ursolic acid in the plant extracts of *S. holocarpa* Hemsl. was not reported earlier. However, an attention was paid to this plant and to its constituents despite of the lack of suitable analytical method. The reason was that the twigs extract possessed a cytotoxic activity on HeLa cells [16] and on gram-positive strains (*E. faecalis*, *P. aeruginosa* and *S. aureus*) [17]. Because the plant extract contained pentacyclic triterpenes [18] including ursolic acid, it was hypothesized that these might be responsible for the observed biological activities.

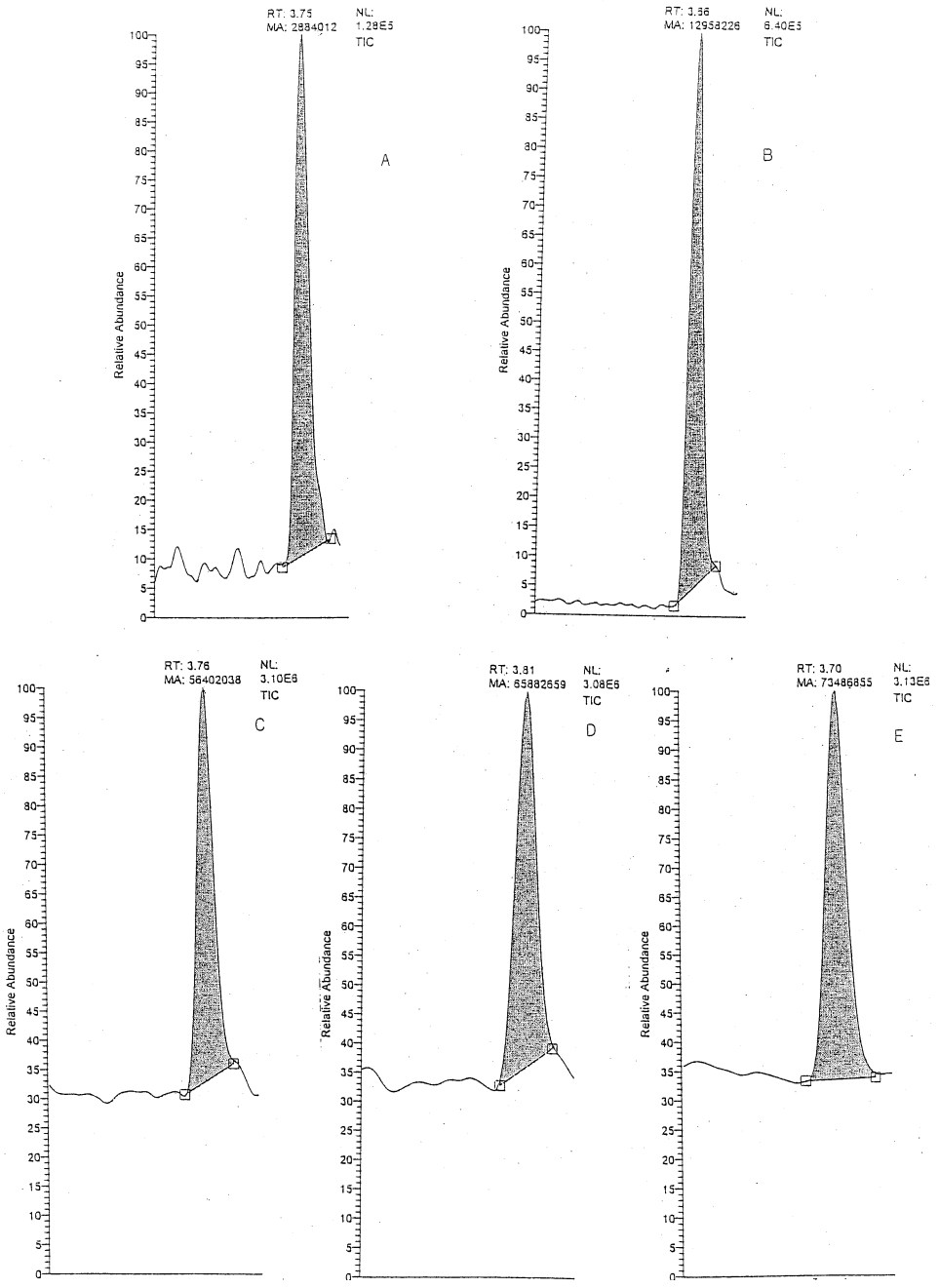

Fig. 4. LC chromatograms of ursolic acid (C: 3.0 μg ml^{-1}) using: (A) Full-SIM at *m/z* 457.1 in the range *m/z* 300–500. (B) SIM–MS at *m/z* 457.2. (C) SIM–MS at *m/z* 390.9, 439.2. (D) SIM–MS at *m/z* 391.0, 439.2, 457.1. (E) SIM–MS at *m/z* 390.9, 411.2, 439.2.

Structurally, ursolic acid belongs to pentacyclic triterpenic substances. The chemical structure of compound lacks the presence of UV absorbing chromophores, which limits the chromatographic determination of ursolic acid by HPLC using UV or fluorescence detection. Furthermore, a preli-

Table 1
Precision and accuracy data for the determination of ursolic acid by LC–MS

Nominal concentration (μg ml^{-1})	Calculated concentration (μg ml^{-1})	% DEV[a]	%R.S.D.[b]
0.25	0.258	+3.2	
	0.245	−2.0	
	0.244	−2.4	
	0.263	+5.2	
	0.245	−2.0	
Mean ± S.D.	0.251 ± 0.009		3.59
2.0	1.91	−4.5	
	2.02	+1.0	
	2.04	+2.0	
	1.93	−3.5	
	1.99	−0.5	
Mean ± S.D.	1.98 ± 0.06		3.03
10.0	9.98	−0.2	
	9.77	−2.3	
	10.3	+3.0	
	10.62	+6.2	
	9.95	−0.5	
Mean ± S.D.	10.12 ± 0.34		3.36

[a] %DEV, % deviation from the nominal concentration.
[b] %R.S.D., % relative standard deviation.

Table 2
Recovery percentages of ursolic acid form laboratory-made mixtures using LC–MS

Nominal concentration (μg ml^{-1})	Calculated concentration (μg ml^{-1})	Recovery %
1.0	1.014	101.4
	1.017	101.7
	0.971	97.1
	0.969	96.9
	0.943	94.3
	0.956	95.6
Mean ± S.D.		97.8 ± 3.05

minary derivatization process is necessary for the analysis of ursolic acid by GC. Application of LC–MS technique is appropriate because the method does not depend on the presence of a particular chromophore in the molecule. Furthermore, the method is fast, straightforward and quantitation of ursolic acid at concentration up to 0.25 μg ml^{-1} can be achieved. Laboratory-made mixtures of ursolic acid were selected for validation studies as they provide better indication of the accuracy and precision criteria of the method compared with the validation performed using plant extracts. This is due to variation of the extract's composition, which is highly influenced by plant species and extraction procedures. Moreover, dosage forms containing ursolic acid such as pills and capsules are commonly dispensed in Chinese medicine [5–7]. The results of the validation studies undertaken indicate that the method developed for determination of ursolic acid is accurate and reproducible and is suitable for determination of ursolic acid (Tables 1 and 2).

The validated LC–MS method was further used for the determination of ursolic acid in various plant extracts of the powdered leaves or twigs using either petroleum ether or chloroform. Additionally, consequent extraction of the material, previously extracted with petroleum ether, using chloroform was performed. Such sequential extraction was used in order to ensure a complete extraction of ursolic acid from the plant material. The obtained data indicated that the accumulation of ursolic acid, as a secondary metabolite, was

Table 3
Determination of ursolic acid in extracts of leaves and twigs of *S. holocarpa* Hemsl. by LC–MS

Sample number	Material	Extracting solvent	Extracted from 10.0 g of the raw dry material (g)	UA in extracts (%)	UA extracted from the plant material (%)	The overall maximum content of UA in dry plant material (%)
1	Leaves	PE[a]	0.69	12.3	0.85	0.95
2	Leaves	CH (following PE)[b]	0.162	6.2	0.10	(content of samples 1+2; sequential extraction)
3	Leaves	CH	0.803	9.6	0.77	
4	Twigs	PE	0.098	15.9	0.16	
5	Twigs	CH (following PE)	0.07	9.7	0.07	
6	Twigs	CH	0.193	13.0	0.25	0.25

[a] Petroleum ether.
[b] Chloroform.

almost four times higher in mature leaves (0.95%) compared with the young twigs (0.25%). The higher content of ursolic acid in the leaves compared with twigs points towards their suitability as a source of ursolic acid for commercial preparations containing the powdered leaves of *S. holocarpa* Hemsl. in form of pills or capsules.

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