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Quantitative analysis of sesquiterpene lactones in extract of *Arnica montana* L. by ^{1}H NMR spectroscopy

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ABSTRACT

^{1}H NMR spectroscopy was used as a method for quantitative analysis of sesquiterpene lactones present in a crude lactone fraction isolated from *Arnica montana*. Eight main components – tigloyl-, methacryloyl-, isobutyryl- and 2-methylbutyryl-esters of helenalin (H) and 11α,13-dihydrohelenalin (DH) were identified in the studied sample. The method allows the determination of the total amount of sesquiterpene lactones and the quantity of both type helenalin and 11α,13-dihydrohelenalin esters separately. Furthermore, 6-O-tigloylhelenalin (HT, **1**), 6-O-methacryloylhelenalin (HM, **2**), 6-O-tigloyl-11α,13-dihydrohelenalin (DHT, **5**), and 6-O-methacryloyl-11α,13-dihydrohelenalin (DHM, **6**) were quantified as individual components.

1. Introduction

Arnica montana L. (Asteraceae) is a well known medicinal plant. Various arnica preparations are applied externally for the treatment of hematomas, contusions, sprains, rheumatic diseases, and superficial inflammation of skin [1]. The main components responsible for pharmacological properties of this plant are sesquiterpene lactones of the 10α-methylpseudoguaianolide type like helenalin, 11α,13-dihydrohelenalin, and their short chain esters [2]. According to the European Pharmacopoeia the total lactone content should not be less than 0.40% calculated as helenalintyglate with reference to the dried drug [3]. Several analytical methods – spectrophotometric [4], RPLC [2], GC [5], and GC–MSD [6] have been published for quantification of lactones in *Arnica* species.

Application of ^{1}H NMR techniques for the determination of the amount of biologically active compounds in plant extracts [7–9] increased in the past years. The main advantages of the ^{1}H NMR method, in comparison with the above mentioned are: the non-destructive nature of the method; no need of chromatographic purification of individual compounds; authentic samples are not necessary for calibration curves; short duration of the time needed for measurement. It should be noted that the method allows the determination of the total lactones without the need of their preliminary identification using common proton signals. The literature survey revealed that up to now the NMR analysis has been applied only for qualitative characterization of the lactone composition of arnica extract [10]. In this paper ^{1}H NMR was used for the quantitative evaluation of the sesquiterpene lactones in extracts of *A. montana*.

2. Experimental

2.1. Plant material

The studied sample was cultivated in the experimental field (Beglica, The Rhodopes) and collected in full flowering stage. A voucher specimen (SOM-Co 1255) was deposited in the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences.

2.2. Preparation of a crude lactone fraction

Air dried flowers of *A. montana* (10 g) were extracted with chloroform (4 × 200 ml) for 10 min at 25 °C in an ultrasonic bath. The solvent was removed and the obtained total extract (610 mg) was worked up as it has been previously described [11] to give the crude lactone fraction (123.50 mg).

The crude lactone mixture was further separated into four fractions (F1–F4) by prep.TLC on silica alufolien using n-pentane–diethylether (35:65, v/v) as the solvent system.

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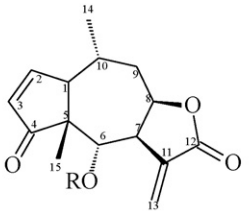

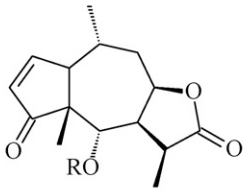

	Helenanolides R			Dihydrohelenanolides R	
1	HT	$-CO(CH_3)C{=}CHCH_3$	**5**	DHT	$-CO(CH_3)C{=}CHCH_3$
2	HM	$-CO(CH_3)C{=}CH_2$	**6**	DHM	$-CO(CH_3)C{=}CH_2$
3	HIB	$-COCH(CH_3)_2$	**7**	DHIB	$-COCH(CH_3)_2$
4	HMB	$-CO(CH_3)CHCH_2CH_3$	**8**	DHMB	$-CO(CH_3)CHCH_2CH_3$

1. 6-O-tigloylhelenalin
2. 6-O-methacryloylhelenalin
3. 6-O-isobutyrylhelenalin
4. 6-O-(2-methylbutyryl)-helenalin
5. 6-O-tigloyl-11α,13-dihydrohelenalin
6. 6-O-methacryloyl-11α,13-dihydrohelenalin
7. 6-O-isobutyryl-11α,13-dihydrohelenalin
8. 6-O-(2-methylbutyryl)-11α,13-dihydrohelenalin

Fig. 1. Structures of compounds **1**–**8**.

2.3. Chemicals and reagents

Chloroform-d_1 (99.8%) was purchased from Deutero GmbH. 3,4-Dimethoxybenzaldehyde (99%) was purchased from Aldrich, ALUGRAM® SIL G/UV_{254} for TLC (0.20 mm layer) were supplied from MACHEREY-NAGEL. Santonin and xerantholide were available from our previous study. Their structures and purity were determined by ^{1}H NMR.

2.4. Quantitative experiments

2.4.1. Sample

- *For quantitative determination*. 19.70 mg of the crude lactone fraction and 1.19 mg of the standard (3,4-dimethoxybenzaldehyde) were mixed and dissolved in 0.6 ml $CDCl_3$. The signal at 9.79 ppm from the CHO group of the standard was normalized to 100% and used as an internal integral reference.
- *For validation of the results*. Three mixtures (M1–M3) consisting of different ratios of the pure lactones (santonin and xerantholide) and the standard were prepared and dissolved in 0.6 ml $CDCl_3$. Thus, M1 contained 14.77 mg santonin, 5.05 mg xerantholide and 7.62 mg standard. M2 was prepared with lower concentration of the components – 5.12 mg santonin, 2.64 mg xerantholide and 5.26 mg standard. The composition of M3 was 1.19 mg santonin, 0.51 mg xerantholide and 1.22 mg standard.

2.4.2. NMR spectroscopy

The NMR spectra of the samples were recorded on a Bruker Avance II+ 600 spectrometer with proton operating frequency 600.13 MHz, equipped with a 5 mm TBI probe. The spectra were measured without sample spinning at temperature of 293 K. All ^{1}H NMR spectra for quantitative analysis of the lactone content were measured with the standard 1D pulse sequence using the following experimental parameters, optimized for quantitative measurements as described by Malz and Jancke [12]: 30° hard excitation pulse, spectral width 16 ppm, 6 μs preacquisition delay, 32 K points in the time domain, 256 scans and a relaxation delay of 10 s to ensure complete relaxation for all signals. The spectra were Fourier transformed after zero filling to 64 K, giving a digital resolution in the frequency domain of 0.15 Hz/pt. Careful manual phase and baseline corrections were performed prior to signal integration. For quantitative analysis selected signals of the mixture were integrated three times and the results were averaged. The integration limits were chosen so as to exclude ^{13}C satellites of the neighbouring signals. To assess the influence of the processing procedure on the quality and precision of the results the spectra were also integrated once without baseline correction. The signal/noise (S/N) ratio for the CHO group from standard was 1965.3. The S/N for the total lactone signal (H-2) from all H- and DH-type components was 1094, which is far above the requirements for quantitative analysis.

2.4.3. Quantitative analysis

The quantity of lactone components in the studied mixture, was determined using the following general equation:

$$m_X = m_{ST} \frac{N_{ST}}{N_X} \frac{I_X}{I_{ST}} \frac{M_X}{M_{ST}}$$

where m_X is the unknown mass of the lactone component, m_{ST} is the weighted mass of the standard; M_{ST} and M_X are the molar masses (in Da) of the standard and the lactone component, respectively; I_{ST} and I_X represent the integrated signal area of standard and the lactone component, respectively; N_{ST} and N_X correspond to the number of protons from the respective integrated signal for the standard and the lactone component.

Two approaches have been adopted in the determination of the total lactone content in the crude lactone mixture. In the first approach the integral values of the average signals at 7.61 ppm and 6.00 ppm for H-2 and H-3, respectively, which are common for all lactones present in the analysed fraction, were used. In the second approach the total lactones were calculated as a sum of helenanolides and 11α,13-dihydrohelenanolides using common signals for H-13 of the exomethylene and methyl group, respectively.

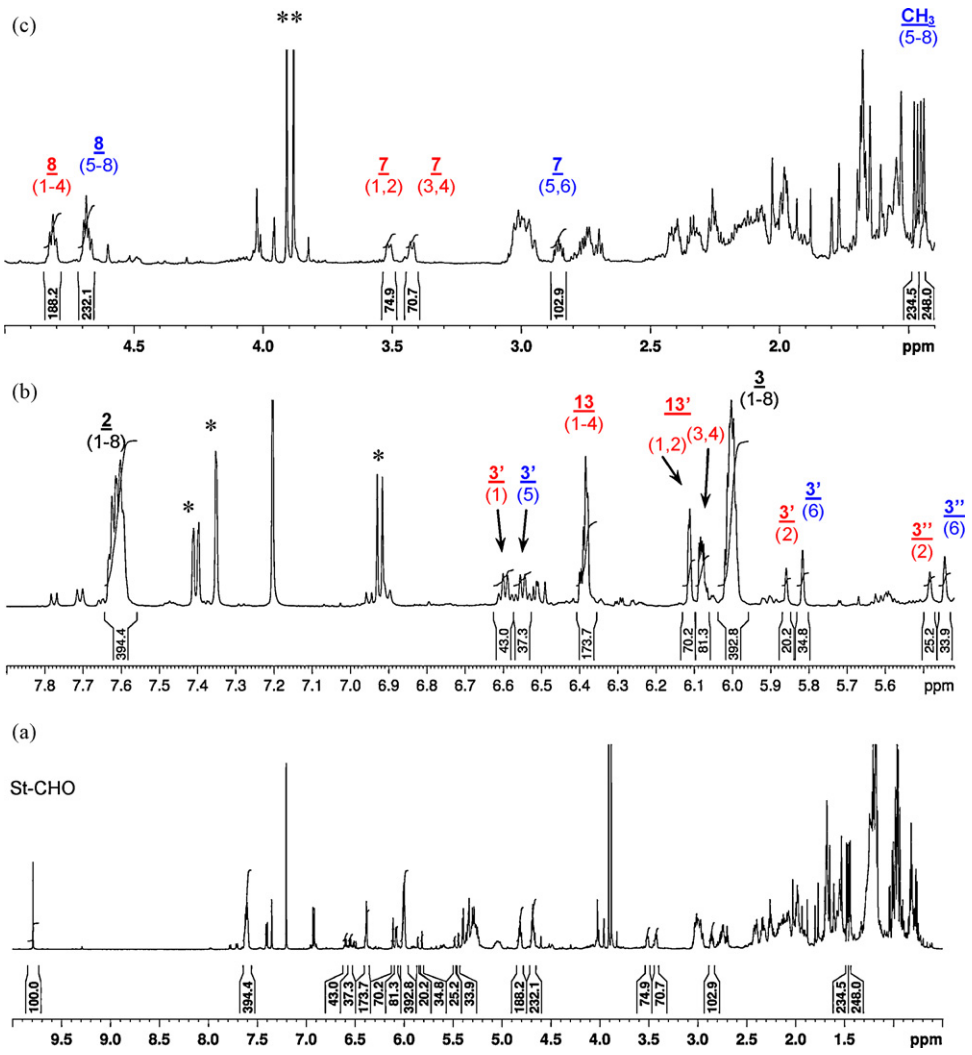

Fig. 2. ^{1}H NMR spectrum (600.13 MHz) of the crude lactone fraction: (a) full spectral range; the signal of the CHO group from the standard is indicated (St-CHO); (b) and (c) expanded parts of the spectrum with assignment of selected key signals used for the quantitative analysis, given with bold, underlined numbers (see Fig. 1 for signal numbering). The signals from helenalin (H) components are in red, while the signals from the 11α,13-dihydrohelenalin (DH) components are given in blue. The numbers in brackets indicate the numbering of the components as given in Fig. 1. The asterisks indicate the two OCH_3 groups and the aromatic signals from the standard. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4.4. Supporting information

^{1}H NMR spectrums of fractions: F1 (Fig. S1), F2 (Fig. S2), and F3 (Fig. S3). Expanded parts from ^{1}H NMR spectrum model mixtures: M1 (Fig. S4-A), M2 (Fig. S4-B) and M3 (Fig. S4-C). Integrated signals and calculated quantities for model mixtures: M1 (Table S1-A), M2 (Table S1-B) and M3 (Table S1-C).

3. Results and discussion

A fundamental characteristic of NMR spectroscopy is that integrated signal intensity (signal area) in the spectrum is proportional to the number of nuclei giving the corresponding resonance. This relationship makes NMR spectroscopy an important tool for quantitative analysis, with practical application for mixture analysis and purity assessment.

In the present study ^{1}H NMR spectroscopy was used to determine the total lactone content in a crude lactone fraction obtained from *A. montana*. For this purpose an accurately weighted amount of 3,4-dimethoxybenzaldehyde (M = 166.17) was added to the studied sample as an internal standard. This compound, which is a stable crystalline substance, was chosen as an internal standard for our measurements because its NMR spectrum is simple and its signals do not overlap with the resonances from the studied sample. The strong singlet at 9.79 ppm for the CHO group is in a well isolated part of the spectrum and presents an ideal reference signal.

3.1. Lactone composition and signal assignment

It is known that the main sesquiterpene lactones present in *A. montana* are helenalin (H) and 11α,13-dihydrohelenalin (DH) esters. The crude lactone mixture obtained from flower heads was separated into four fractions (F1–F4) to determine the qualitative lactone composition. Assignment of the signals for each individual compound was performed using a combination of 1D and 2D NMR techniques, including ^{13}C, DEPT, COSY, HSQC and HMQC spectra. ^{1}H and ^{13}C chemical shift values determined in our study for helenalin and 11α,13-dihydrohelenalin skeleton types as well as for ester residues are in agreement with literature data [10,13,14]. The first fraction (F1) consists of almost pure compound identified as 6-O-(2-methylbutyryl)-helenalin (HMB) (**4**) (Fig. S1 in Supporting information). Analysis of the second fraction (F2) showed that it contained a mixture of both helenalin and 11α,13-dihydrohelenalin type structures which were determined as tigloyl- (HT) (**1**), methacryloyl- (HM) (**2**) and isobutyrylhele-

Table 1
^{1}H and ^{13}C NMR data of helenanolides **1**–**4**.

	^{1}H MNR δ, ppm (J, Hz)				^{13}C NMR			
	1 (HT)	**2** (HM)	**3** (HIB)	**4** (HMB)	**1**	**2**	**3**	**4**
1		[a]2.94–3.0 m		2.96 m	53.55	53.52	53.33	53.38
2		[a]7.58–7.62 m		7.59 dd (1.53; 5.97)	162.14	162.022	162.12	162.10
3		[a]5.98–6.03 m		6.00 dd (2.97; 6.03)	129.68	129.65	129.63	129.60
4		–		–	208.97	208.82	208.88	208.80
5		–		–	55.62	55.57	55.49	55.44
6	5.34 brs	5.35 brs	5.29 brs	5.31 brs	77.88	78.10	77.52	77.50
7	3.51 (brd 6.60)	3.51 (brd 6.60)	3.42 (brd 7.80)	3.42 (brd 7.80)	47.68	47.43	47.50	47.67
8		[a]4.81 td (1.94; 7.44)		4.81 td (1.87; 7.39)	78.16	78.19	78.07	78.10
9		[a]2.35–2.43 m		2.33 ddd (3.39; 7.08; 15.36)	40.35	40.35	40.28	40.35
9′		[a]1.63–1.71 m		1.66 ddd (2.21; 8.75; 15.36)				
10		[a]2.07–2.13 m		2.11 m	26.13	26.11	26.08	26.08
11		–		–	137.42	135.77	137.34	137.35
12		–		–	169.54	169.54	166.58	169.57
13	6.40 brd (2.88)	6.40 brd (2.76)	6.39 brd (2.82)	6.38 brd (2.61)	125.02	125.02	125.09	125.11
13′	6.11 m	6.11 m	6.08 m	6.09 brd (2.61)				
14	[b]1.21 d (6.44)	[b]1.20 d (6.56)	[b]1.19 d (6.44)	1.20 d (6.72)	20.02	19.99	19.99	19.99
15	0.95 s	0.96 s	0.94 s	0.93 s	18.36	18.13	18.30	18.34
Ester groups								
1′	–	–	–	–	166.58	165.94	175.68	175.29
2′	–		2.40 septet (7.02)	2.23 m (6.96)	128.17	126.31	33.92	40.90
3′	6.60 qq (1.23; 7.12)	5.86 brs	1.04 d (6.96)	1.50 m	138.26	126.36	18.54	26.56
3″		5.48 brs		1.32 m				
4′	1.69 brd (7.12)	1.80 brs	1.00 d (7.02)	0.77 t (7.44)	11.99	18.53	18.53	11.27
5′	1.68 brs			1.01 d (6.96)	14.47			16.17

[a] Overlapped signals for the three compounds.
[b] Signals could be interchanged.

nalin (HIB) (**3**) and 2-methylbutyryl dihydrohelenalin (DHMB) (**8**) (Fig. S2 in Supporting information). The third fraction (F3) was found to consist of tigloyl- (DHT) (**5**), methacryloyl- (DHM) (**6**) and isobutyryl (DHIB) (**7**) esters of 11α,13-dihydrohelenalin as well as, non-lactone structures (Fig. S3 in Supporting information). The last fraction (F4) isolated from the crude extract did not contain any sesquiterpene lactones (^{1}H NMR spectrum and IR control). The structures of the lactones found in the studied extract are presented in Fig. 1.

The expanded parts of the spectrum of the crude lactone fraction with assignment of selected key signals used for the analysis are given in Fig. 2. For clarity the signals from helenalin type components (**1**–**4**) are in red, while the signals from 11α,13-dihydrohelenalin derivatives (**5**–**8**) are given in blue. The asterisks indicate the two OCH_3 groups and the aromatic signals from the standard. The abbreviation St-CHO indicates the CHO group from the standard.

^{1}H and ^{13}C NMR data for helenanolides and 11α,13-dihydrohelenanolides present in the crude lactone mixture are presented in Tables 1 and 2, respectively.

3.2. Quantity of the individual sesquiterpene lactones

The quantities of individual lactones were determined from the integral areas of some well isolated key signals. Most of the signals for helenalin and 11α,13-dihydrohelenalin structures are overlapped in the ^{1}H NMR spectrum of the crude lactone fraction. That is why the signals corresponding to the ester fragments were used mainly for the quantification of individual compounds. Analysis of the different fractions (F1–F3) obtained from the crude lactone mixture indicates the presence of tigloyl-, methacryloyl-, isobutyryl- and 2-methylbutyryl-esters of helenalin (H) and 11α,13-dihydrohelenealin (DH). The quantities of HT (**1**) and DHT (**5**) were determined from the signals at 6.60 ppm and 6.55 ppm, respectively. Using the above formula, the masses of HT (**1**) and DHT (**5**) were calculated to be 1.1 mg (5.6%) and 1.0 mg (5.1%), respectively. Other well defined signals are those for HM (**2**) (5.86 ppm and 5.48 ppm) and DHM (**6**) (5.82 ppm and 5.45 ppm), which were used for their quantification. The results show that the measured sample contains 0.5 mg (2.5%) of HM (**2**) and 0.8 mg (4.1%) of DHM (**6**). No individual signals for HIB (**3**), HMB (**4**), DHIB (**7**) and DHMB (**8**) were found for separate integration. Components HIB (**3**) and HMB (**4**) give a common signal for H-7 at 3.42 ppm, which was used to asses their quantity, assuming an average molar mass of 339.40. The averaged quantity of HIB (**3**) and HMB (**4**) in the studied mixture was calculated to be 1.7 mg (8.6%). The total quantity of H-type sesquiterpene lactones calculated as a sum of the individual quantities of each identified H-esters was 3.3 mg (16.8%), while their amount found on the basis of the integral of H-13 multiplet at 6.39 ppm for all helenanolides was 4.1 mg (20.8%), assuming an averaged molecular mass of 338.4 for all H-components. The observed difference could be explained by the presence of some unidentified H-type components.

The signal for H-7 of DHIB (**7**) and DHMB (**8**) cannot be used for the quantitative determination, because they were overlapped with other resonances in the interval 2.72–2.77 ppm. Their amount could be determined by subtracting the integrals for DHT (**5**), DHM (**6**) from the integral for all DH-type esters of C-13 methyl groups at 1.45 ppm and 1.48 ppm. The obtained integral was considered to correspond to the sum of DHIB (**7**) and DHMB (**8**), as well as some eventually unidentified DH-type components. Their quantity was found to be 3.4 mg (17.2%) using an average molar mass of 341.42.

The total quantity of DH-type lactones (including possible non-identified components) determined from the signal of C-13 methyl groups was 5.2 mg (26.4%), assuming an average molar mass of 340.41.

3.3. Total lactone content

Determination of the total sesquiterpene lactone content was based on the average area of the signals at 7.61 ppm and 6.00 ppm assigned to H-2 and H-3, respectively, which are common for the lactones presented in the studied fraction. For the quantitative calculation an average molar mass of 339.4 for all identified components was used. The small differences in the molar masses of the

Table 2
^{1}H and ^{13}C NMR data of 11α,13-dihydrohelenanolides **5**–**8**.

	^{1}H NMR δ, ppm (J, Hz)				^{13}C NMR			
	5 (DHT)	**6** (DHM)	**7** (DHIB)	**8** (DHMB)	**5**	**6**	**7**	**8**
1	[a]2.96–3.05 m				54.14	54.10	53.95	53.95
2	[a]7.59–7.63 m				162.04	162.01	162.04	162.09
3	[a]5.98–6.02 m				129.49	129.47	129.44	129.38
4	–				209.43	209.39	209.54	209.37
5	–				54.96	54.92	54.83	54.73
6	5.40 s	5.41 s	5.34 s	5.35 s	72.05	72.41	71.72	71.60
7	2.86 m	2.86 m	2.76 m	2.75 dd (6.51; 10.23)	48.72	48.67	48.89	48.93
8	4.68 brt (5.87)			4.69 brt (6.09)	79.51	79.43	79.43	79.42
9	[a]2.39–2.43 m				40.94	40.88	40.97	40.95
9′	1.55–1.63 m			1.56 m				
10	[a]2.13–2.23 m				25.81	25.69	25.76	25.76
11	[a]2.96–3.05 m				40.47	40.47	40.47	40.46
12	–				179.11	178.99	179.02	179.03
13	1.48 d (7.50)	1.48 d (7.50)	1.45 d (7.50)	1.45 d (7.44)	10.92	10.91	10.86	10.85
14	1.15–1.19			1.17 d (6.72)	19.69	19.69	19.71	19.68
15	0.98 s	0.99 s	0.96 s	0.96 s	17.52	17.53	17.57	17.53
Ester groups								
1′	–				166.59	165.94	175.63	175.23
2′	–	–	2.34 septet (6.99)	2.18 m	128.29	135.91	33.87	40.86
3′	6.55 qq (7.02; 1.08)	5.82 brs	1.01 d (6.90)	1.48 m	137.89	126.01	18.83	29.67
3″		5.45 brs		1.28 m				
4′	1.68 brd (7.02)	1.77 brs	0.97 d (7.02)	0.76 t (7.44)	11.99	18.15	18.89	11.30
5′	1.65 brs	–	–	0.98 d (7.02)	14.45	–	–	16.18

[a] Overlapped signals for the four compounds.

individual compounds justify this assumption. The possible error in the quantitative analysis resulting from the average molar mass is less important for HT (**1**) and DHIB (**7**), for which the difference is 1.5%. The largest error can be expected for the HM (**2**) where the actual molar mass of the compound differs by 2.7% from the assumed average molar mass. Thus, the determined lactone content was 9.5 mg (48.2%). The amount of sesquiterpene lactones represented as a sum of helenanolides and 11α,13-dihydrohelenanolides calculated from H-13 of the exomethylene and methyl groups was found to be 9.3 mg (47.2%), which is in good agreement with the latter value.

3.4. Analysis of validation samples

Three model mixtures, containing santonin and xerantholide in different ratios were prepared for the validation and a comparison of the results. The total quantity of the components of the first mixture (M1) was 19.8 mg, which is equal to the total quantity of the studied lactone fraction. The total quantity of the components for the second mixture (M2) was 7.76 mg, which is lower as compared to the total lactone content determined for the studied sample. In the third mixture M3, the quantities of the two lactone components, 1.19 mg and 0.51 mg, respectively, were chosen so as to correspond to components with the highest and lowest amount in the crude lactone fraction. The proton spectra of the three samples are presented in Fig. S4-A–C in the Supporting information. The key signals used for the analysis are indicated with S and X for santonin and xerantholide, respectively. The signals were integrated three times – once without baseline correction (columns I_1 and m_1) and twice with baseline correction but using narrow (columns I_2 and m_2) and more wide integral area (columns I_3 and m_3). The calculations were performed for each individual integration value and the average value obtained for each component was taken for comparison with its actual input quantity. The results are presented in Table S1 in the Supporting information.

For the first mixture, M1, the differences between the actual and measured quantity of santonin from the three consecutive integrations were 3.0%, 1.1% and 1.2%, while for xerantholide the differences of 3.6%, 0.4% and 0.2% were found. For the two components the largest discrepancy was found when no baseline correction was applied prior signal integration.

For the second mixture, M2, which contained lower quantity of the two components, the differences between the calculated and actual amount from the three different integrations were 1.0%, 1.0% and 0.8% for santonin, which is the component with larger weighted mass (5.12 mg). For xerantholide, the component with lower input quantity (2.64 mg), the differences were 3.8%, 4.2% and 3.0%. This larger discrepancy could be explained with the larger contribution of noise level to the total integral of the signals used for calculations, although the S/N ratio for xerantholide was high enough (2405).

For the third mixture, M3, the differences between the input quantity of santonin and the calculated on the basis of three different integrations were negligible (less than 1%) and independent of the processing procedure. For the component with lower input amount – xerantholide, the difference was 3.9% in all calculations.

These results imply that for the particular experiments and systems in our investigation the main source of error for the component with lower relative quantity is the spectral noise, while the baseline correction and the width of the integral area have less influence when the S/N is good enough. The above calculations could be considered as a measure of the experimental and processing error, which for all samples was less than 5% even for the component with lowest concentration.

4. Conclusions

A ^{1}H NMR method for the quantitative estimation of sesquiterpene lactones in *A. montana* extract was applied for the first time. Our study demonstrates that two approaches can be used to determine the total amount of lactones. The first one is based on the total area of the signals for H-2 and H-3, which are common for the H- and DH-type sesquiterpene lactones, giving a value of 9.5 mg. The second one represents the lactone content as a sum of the H- and DH-type of compounds determined on the basis of the H-13 exomethylene and methyl group, respectively (9.3 mg). The results from the two experiments are in good agreement, but the obtained values are higher than the amount calculated as a sum of the individual lactones measured (8.3 mg). The observed difference is due

to the presence of unidentified components with lactone structure, for which it is not possible to select specific signals for NMR measurement and indirectly gives information about the quantity of the non-identified components. None of the methods used so far give more accurately the quantity of the total lactones as well as the two types (H and DH) in the sample. Furthermore, four (**1**, **2**, **5**, and **6**) of the identified lactones were quantified as individual compounds. However, the impossibility to measure separately components **3**, **4**, **7**, and **8** in this particular case has to be pointed as a disadvantage of the method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.08.018.

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