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Analysis of pentacyclic triterpenic acids from frankincense gum resins and related phytopharmaceuticals by high-performance liquid chromatography. Identification of lupeolic acid, a novel pentacyclic triterpene

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Abstract

An HPLC gradient method with photodiode array detection was developed for the simultaneous analysis of 12 different pentacyclic triterpenic acids in Indian and African frankincense gum resins as well as in related phytopharmaceuticals. The triterpenic acids were obtained by an exhaustive extraction procedure. Identification of the compounds was based on retention times, UV-spectra and add on technique with standards isolated from African frankincense. The method allows differentiation of frankincense of different origin and standardization of frankincense-based phytopharmaceuticals. Further, this is the first report identifying a novel pentacyclic triterpene, lupeolic acid, as a constituent of frankincense gum resins.
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Keywords: Pentacyclic triterpenic acids; Lupeolic acid; Triterpenes

1. Introduction

The gum resins we have analyzed were collected from trees belonging to the genus *Boswellia*. These resins are also called olibanum or, more commonly, frankincense. Of the dozen of species of *Boswellia* that grow in India, on the Arabian peninsula and along the eastern coast of Africa [1] only a few species are pharmaceutically important. Indian frankincense is the gum resin collected from the tree *Boswellia serrata*, while African frankincense contains gum resins from *Boswellia carteri* and *Boswellia frereana* [2,3]. Winterstein and Stein [4] were the first to attempt isolation and characterization of the constituents of frankincense. Further studies came from Pardhy and Bhattacharyya [5] and Fattorusso et al. [6] who analyzed the gum resins from *B. serrata* and *B. carteri*, respectively. For the analysis of boswellic acids in gum resins of frankincense, HPTLC techniques have previously been used [3,7]. However, the chemical structure and purity of most of the isolated substances were at best only partially characterized. Frankincense gum resin contains 5–9% oil, an ether-soluble fraction of 60–70% comprising sesquiterpenes, alcohols, esters, as well as

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boswellic acids and an ether-insoluble fraction of 25–30% containing polysaccharides [3,8]. Apart from the commercial value of frankincense extracts for the perfume industry, the gum resins apparently contain promising pharmacological compounds. An extract from Indian frankincense (*B. serrata*) marketed in Europe as H15 is currently under clinical investigation [9–13]. Indeed, boswellic acids have been reported to possess anti-inflammatory and antitumor activity, which may at least partially be due to inhibition of human leukocyte elastase [14] and/or 5-lipoxygenase inhibitory activity [15], and to topoisomerase inhibition [16] leading to apoptosis-related tumor cell death [17]. In order to evaluate the pharmacological potential of natural compounds derived from frankincense, we have purified boswellic acids belonging to the family of pentacyclic triterpenes as well as a novel pentacyclic triterpene, lupeolic acid, to apparent homogeneity. We have further characterized their structure by mass spectrometry and by nuclear magnetic resonance spectroscopy [18]. The corresponding standards were used to identify the compounds extracted from Indian and African frankincense as well as some readily available related phytopharmaceuticals such as H15 by an HPLC gradient method using photodiode array detection.

2. Experimental

2.1. Chemicals

All chemicals were of analytical reagent-grade unless stated otherwise. Reverse-osmosis type quality water, pureAqua (Schnaitsee, Germany), plus a Milli-Q station from Millipore (Eschborn, Germany) were used throughout. The standard compounds (Fig. 1, **1–12**) were obtained by an isolation technique based on the method of Winterstein and Stein [4]. The standards were characterized by HPLC, TLC, UV spectroscopy, and by mass spectrometry as well as by 1D and 2D nuclear magnetic resonance spectroscopy [18]. As estimated by HPLC and TLC, the purity of the standards was ≥99%. Oleanolic acid, betulinic acid, ursolic acid and glycyrrhetinic acid were purchased from Roth (Karlsruhe, Germany), the corresponding acetyl-derivatives were synthesized according to standard procedures [4]. Methanol, ethanol, ethyl acetate, acetone, isopropanol, methyl ethyl ketone, hexane, and acetic acid 96% were purchased from Merck (Darmstadt, Germany). Indian (*B. serrata*) and African (*B. carteri*, *B. frereana*) frankincense was purchased from a phytopharmaceutical supplier (Klenk, Schwebheim, Germany). The five batches of H15, the phytopharmaceutical A, were supplied by Ayurmedica (Pöcking, Germany), lots: A1—# 139, A2—# 205, A3—# 220, A4—# 263, A5—# 298 and the two batches of African frankincense capsules were from Dr Fernando (Kraichtal-Gochsheim, Germany), lots: B1—# 899040, B2—# 896042.

2.2. Instrumentation and software

The HPLC system consisted of a low-pressure gradient LC-9A Shimadzu pump (Kyoto, Japan), an automatic sample injector Aspec XL (Abimed, Langenfeld, Germany), a column oven IWN CH100 (Junedis, Gröbenzell, Germany) and a photodiode array detector UVD 340S (Dionex, Idstein, Germany) connected to a personal computer equipped with Chromeleon Software version 6.11 (Dionex, Idstein, Germany). Statistical calculations were carried out with the software package Valoo (Applica, Bremen, Germany). The separation was carried out on a ReproSil-Pur 120 ODS-3 column (250×3.0 mm I.D., particle size 5 μm; Dr Maisch, Ammerbuch, Germany). Exhaustive extraction of boswellic acids from frankincense gum resin was carried out with a Soxhlet extraction device (VWR International, Darmstadt, Germany).

2.3. Standards and stock solutions

Standard stock solutions were prepared from each compound (**1–12**) by dissolving 1 mg substance in 1 ml methanol. For further preparation of standard solutions, the stock solutions were mixed and diluted with methanol yielding concentrations from 1 to 25 μg substance/80 μl. The stock solutions are stable for 1 week at −20 °C. An extended stability was achieved by dilution of the standards with dimethyl sulfoxide.

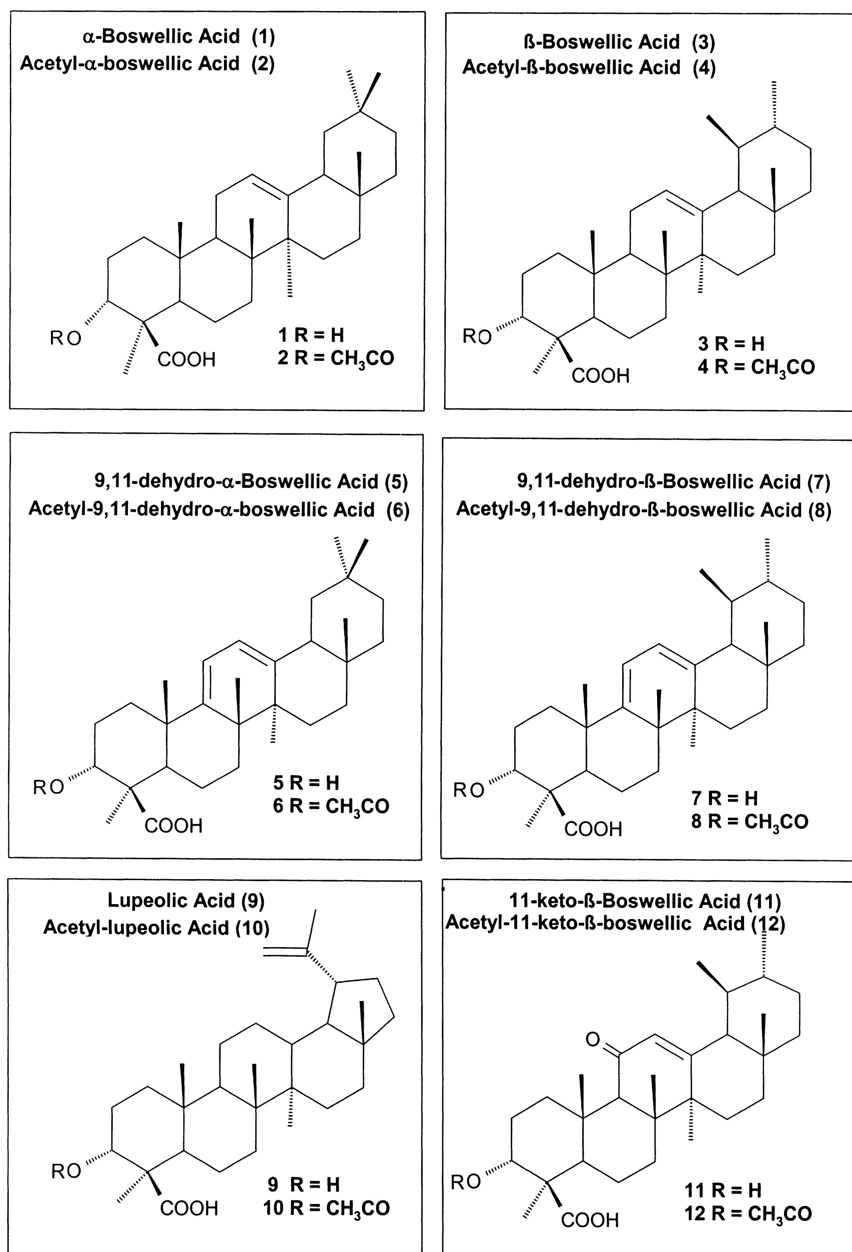

Fig. 1. Structure of different triterpenic acids present in frankincense.

2.4. Sample preparation

2.4.1. Extraction procedure

The extraction procedure was optimized with respect to solvent and extraction time. As test material African frankincense was used. The solvents employed were methanol, ethanol, ethyl acetate, acetone, isopropanol, methyl ethyl ketone, and hexane. For every test 10 g of freshly ground frankincense gum resin was extracted with 150 ml solvent by a Soxhlet method. First we analyzed the extraction with methanol for different periods of time.

Subsequently, we compared different solvents at the optimal point of time of 3 h as established for the methanol extraction with respect to extraction efficiency and extract composition.

2.4.2. Sample pretreatment for comparison of frankincense of different origin

Four grams of ground Indian or African frankincense gum resin were placed in a Soxhlet extraction tube and were subsequently extracted with 60 ml methanol at room temperature for the optimal time of 3 h. After evaporation of the solvent, the residues were weighed and 10 or 20 mg dry extract were dissolved in 1 ml dimethyl sulfoxide. For the analysis of boswellic acids, 65 μl of each frankincense extract was injected onto the HPLC column without any further purification; analysis was carried out in duplicates.

2.4.3. Sample pretreatment for the analysis of phytopharmaceuticals

For the analysis of the phytopharmaceutical preparations from each lot, either four tablets or capsules were separately tested according to the extraction procedures described in Section 2.4.1. The higher boiling point of ethanol compared with that of methanol warranted a better reproducibility of the extraction procedure and allowed better solubility of the lipophilic compounds and pharmaceutical adjuvants in the phytopharmaceuticals. For these reasons, ethanol was used for all further tests. Sample solutions were prepared by stirring one tablet or capsule at 500 rpm in 6 ml ethanol in a sealed glass container at room temperature for 40 min. The supernatant was centrifuged at 4300 *g* for 5 min. From this solution, 80 μl was transferred to a sampler vial. The solvent was evaporated under a stream of nitrogen and the residue was dissolved in 1 ml dimethyl sulfoxide. For further details of the procedure, see Section 2.4.2.

2.5. HPLC conditions

Due to the stability of the ReproSil-PUR ODS-3 column as well as the efficient elution of matrix compounds by the gradient program used, guard columns were not required. For the elution of the compounds, the following mobile phase and gradient program was used. Mobile phase A: methanol–water–acetic acid (80:20:0.2, v/v/v). Mobile phase B: methanol–acetic acid (100:0.2, v/v). Initial conditions were 62% A and 38% B at a flow-rate of 0.56 ml/min. Linear gradient to 51% A over 20 min, then 39% A until 35 min, 32% A until 40 min, 31% A until 45 min, 100% B until 50 min. At the end of this program, all remaining matrix compounds were eluted from the column with 100% B at a flow-rate of 0.9 ml/min. At 60 min, the flow-rate was reduced to 0.56 ml/min with 62% A. In order to shorten analysis time and to stabilize the chromatographic system, the column was maintained at 28 °C. Tests runs were carried out between 21 and 30 °C; in our configuration, 28 °C proved to be the most suitable temperature in terms of resolution, analysis time and selectivity. A new sample injection was feasible 66 min after the previous run.

The chosen gradient program separates keto boswellic acids from 0 to 20 min, deacetylated boswellic acids from 20 to 35 min, and acetylated boswellic acids from 35 to 45 min.

2.6. Identification and quantification

The eluent was continuously scanned at 210, 250 and 280 nm and the three-dimensional data fields were recorded. The peaks of pentacyclic triterpenic acids in the frankincense samples were positively identified by three different parameters. First, the retention time of the peak was required to be in the range of ±0.1 min of the corresponding standard. Second, the resulting photodiode array spectrum of the sample was on-line compared with a stored reference spectrum of the corresponding standard at a concentration of 1 μg/80 μl, and third the peak area of the tested peak had to be enlarged at the same retention time after adding the corresponding pure standard substance to the real sample. Calibration curves of the standards ranging from 0.1 ng/injection up to 6 μg/injection revealed linearity with R values exceeding 0.999 (peak area vs. concentration). Generally, quantification was carried out on the basis of external standards with several concentrations that were analyzed in duplicate before and after each sample.

2.7. Validation procedures

2.7.1. Determination of linearity, repeatability, and limit of detection

To test the linearity of all compounds, we used standard solutions in DMSO in the range from 0.1 ng/injection to 6 μg/injection. Each sample was measured in duplicate. The regression and the limit of detection (Table 2) were calculated with the Valoo software (Applica, Bremen, Germany) based on the standardization criteria DIN 32645 as defined by the German standardization committee [19,20]; this procedure warrants that the limit of detection is with 99% probability different from the background.

For the determination of repeatability, a standard mixture of all compounds in the range of 5 ng to 1 μg dissolved in DMSO was measured with a coefficient of variation ±2% (n=6).

2.7.2. Determination of analytical specificity

The specificity of the separation method was assessed by analyzing eight structurally similar substances, namely oleanolic acid, acetyloleanolic acid, ursolic acid, acetylursolic acid, betulinic acid, acetylbetulinic acid, glycyrrhetinic acid, acetylglycyrrhetinic acid, combined with the known standards (**1**–**12**) in a single test solution. Separation was achieved with the HPLC method described in Section 2.5. None of the tested substances coeluted with any of the triterpenic acid standards (**1**–**12**).

3. Results and discussion

3.1. Extraction method of pentacyclic triterpenic acids

Initially, we extracted the frankincense resins with solvents quite different in selectivity and polarity. Using methanol as solvent for the analysis of the extraction kinetics, we observed that the extraction efficiency in terms of weight was basically maximal after 3 h (Fig. 2). However, it was astonishing that the various solvents used, yielded at least in terms of weight approximately the same amount of dry extract (Table 1). The extraction yield ranging from approximately 62–75% compares favourably with that described for the ether-soluble fraction, i.e. 60–70% [3,8]; from its polarity, diethyl ether would range between methyl ethyl ketone and hexane. Moreover, further analysis of the resulting extracts by the HPLC gradient method (see Section 2.5) showed only slight differences in the extract composition compared with methanol (data not shown). From the point of polarity and selectivity of the solvents used, one would expect a quite different extraction profile of the frankincense compounds. The reason for this unexpected extraction behavior is probably due to some constituents of the frankincense oil that seem to serve as emulsifier for the pentacyclic triterpenes allowing their solution in both polar and apolar solvents. With this background, it was not unexpected that further trials to purify the primary Soxhlet extract by a solvent–solvent extraction method failed. Only precipitation of the triterpenic acids by barium hydroxide [4], or by a novel method

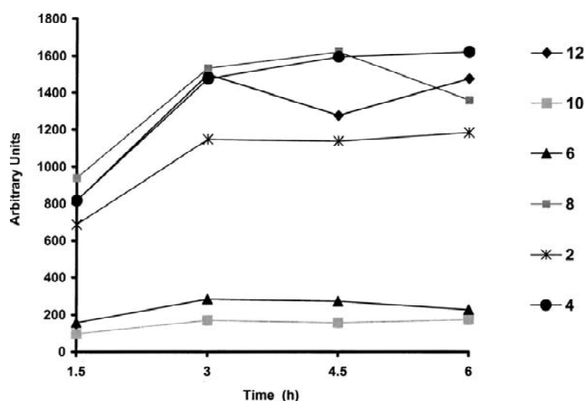

Fig. 2. Time-dependent methanolic extraction of acetylated triterpenic acids from African frankincense. Results for the corresponding deacetylated triterpenic acids were similar.

Table 1
Extraction yield from African frankincense after extraction with different solvents

Solvent	Amount of extract (g)
Methanol	7.44
Ethanol	7.44
Ethyl acetate	7.49
Acetone	7.46
Isopropanol	7.50
Methyl ethyl ketone	7.50
Hexane	6.15

Ten grams of ground African frankincense gum resin were extracted with 150 ml of each solvent.

developed in our laboratory (in preparation) yielded a pure pentacyclic triterpenic acid mixture, which allowed further purification and separation at the semi-preparative scale.

3.2. HPLC method

So far, HPLC analysis of boswellic acids in frankincense or biological matrices was attempted only by a few authors [21–23]. Previously published HPLC methods have at best been used to analyze up to six different boswellic acids in native frankincense [21], but the respective HPLC tracings showed many more unknown peaks. In contrast, our method allows separation and quantification of 12 different pentacyclic triterpenic acids. The additional compounds identified were 9,11-dehydro-α- and -β-boswellic acids (**5–8**) and lupeolic acid (**9, 10**) both in its deacetylated and acetylated forms. The structure and existence of the boswellic acids **5–8** have been described earlier [23], whereas **9, 10** are novel compounds, which can be isolated using the procedure of Winterstein and Stein [4]. Only recently we have reported the structural and physical data for various boswellic acid derivatives characterized by 1D and 2D nuclear magnetic resonance [18]. In accordance with the observation of other authors [23], 3-*O*-acetyl-11-hydroxy-β-boswellic acid was detected as well. Unpublished observations from our laboratory indeed support the claim by Schweizer et al. [23] of a work-up-dependent formation of (**8**). Thus, we observed transformation of 3-*O*-acetyl-11-hydroxy-β-boswellic acid into the thermodynamically more stable 3-*O*-acetyl-9,11-dehydro-β-boswellic acid (**8**) by dehydratization in the presence of acetic acid indicating a precursor function of 3-*O*-acetyl-11-hydroxy-β-boswellic acid. In addition, mass spectrometry in the EI mode of 3-*O*-acetyl-11-hydroxy-β-boswellic acid showed among others, mass fragments 496 and 514; the spectrum of 3-*O*-acetyl-11-hydroxy-β-boswellic acid differs from that of (**8**) only by the mass fragment 514 suggesting that fragment 496 is generated by dehydratization during electron bombardment. Because the extracts also contained the corresponding 3-*O*-acetyl-9,11-dehydro-α-boswellic acid (**6**), the 3-*O*-acetyl-11-hydroxy-α-boswellic acid might exist in the gum resins too; however, so far we have been unable to identify the latter compound. Analysis of pharmaceutical preparations containing extracts from frankincense such as the commercial H15, revealed greater amounts of the 9,11-dehydro compounds (**5–8**) than that present in the gum resin (Table 4) suggesting that the extraction procedures operated at acidic pH. As the 9,11-dehydro compounds might be pharmacologically active, such activity must be tested and it might therefore be necessary to quantify these in addition to the other known boswellic acids.

In accordance with the spectral properties of the tested substances (Fig. 3), we analyzed absorbance at three different wavelengths, 210 nm for α- and β-boswellic acid as well as lupeolic acid, 250 nm for 11-keto-β-boswellic acid and 280 nm for 9,11-dehydro-α- and -β-boswellic acids.

To obtain sufficient separation of the different pentacyclic triterpenic acids from interfering compounds, it was necessary to test different stationary and mobile phases. After testing a number of columns including Spherisorb ODS2 (3 μm), Spherisorb ODS2 (5 μm), Nucleosil 100 C_{18} (5 μm), Hypersil 120 ODS (5 μm), ReproSil-Pur C_{18}-AQ (5 μm), ReproSil-Pur ODS-3 (5 μm), Superspher 100 RP (4 μm), and Superspher 100 RP-EC (4 μm) (all 250×4 mm I.D., with the gradient program described in Section 2.5), we finally chose the ReproSil-Pur ODS-3 on the basis of the performance criteria, stability, selectivity, and reproducibility. As to the development of the mobile phase, it was important to take into account that some of the compounds do not possess conjugated double bonds. Therefore, they showed only weak UV absorption and the separation had to be monitored at 210 nm. We tested methanol as well as acetonitrile as solvent systems. Basically acetonitrile offers the advantage of a better UV transparency at the wavelength of 210 nm. However, in long-term tests, methanol proved to be superior, because in contrast to acetonitrile it prevented irreversible adsorption of matrix material to the column and therefore significantly increased the column stability.

It is well known that lowering the apparent pH of the mobile phase significantly improves the peak shape of eluting acidic compounds. As compared to phosphoric acid [21], acetic acid has the advantage of being easily removed in case one intends to collect the compounds after separation. In order to

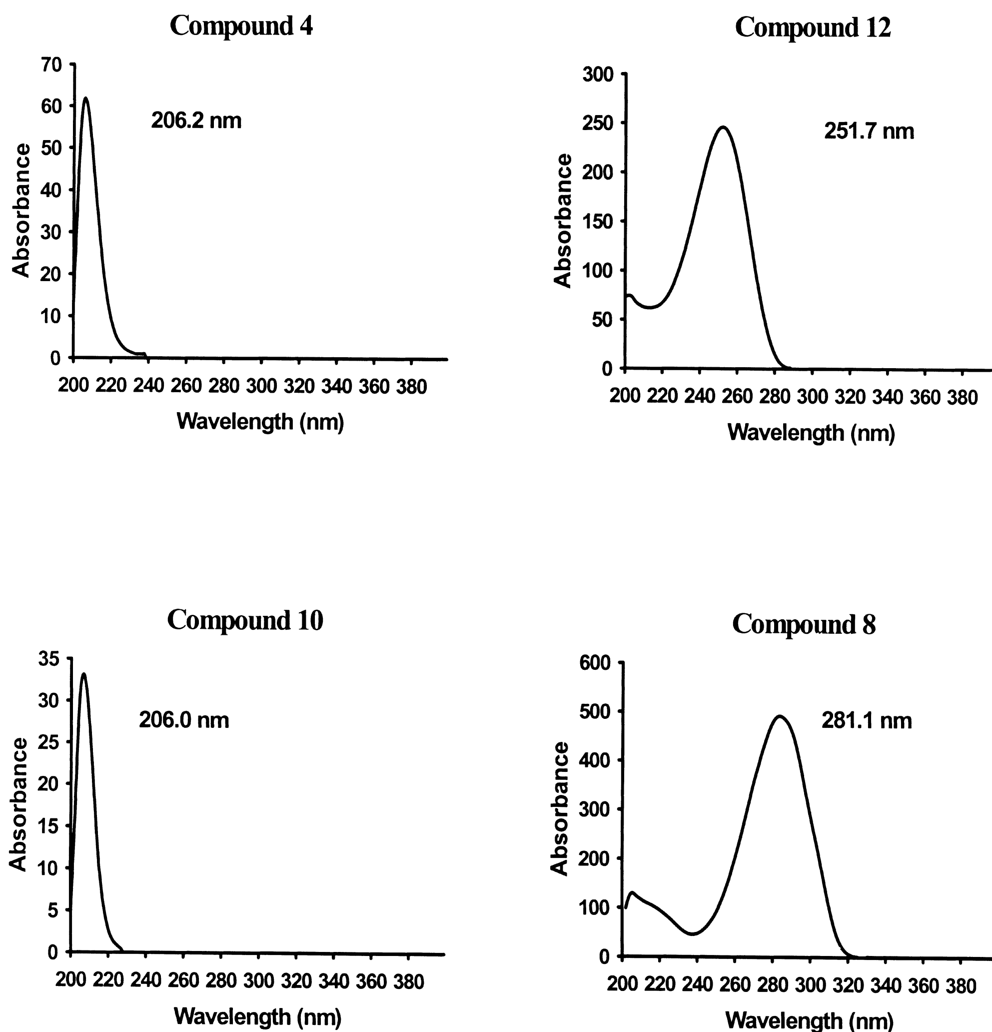

Fig. 3. UV-spectra of the major acetylated triterpenic acids from frankincense. The non-acetylated forms display identical spectra.

obtain reproducible retention times with the long elution times, it was imperative to run the column at a constant temperature of 28 °C. These conditions warranted a proper separation of the compounds investigated (Fig. 4). In addition, this method allowed a very reproducible detection of the compounds, as standard curves had generally a correlation coefficient >0.999 (Table 2). Moreover, the method is highly sensitive; for many of the compounds tested, such as 11-keto-β-boswellic acid (**11, 12**) and 9,11-dehydro-derivatives (**5–8**), the limits of detection were even below 1 ng per injection (Table 2).

3.3. Analysis of triterpenic acids in Indian and African frankincense

The gum resins of Indian or African frankincense are most commonly used for phytopharmaceutical preparations. Therefore, we tested the triterpene composition in Indian and African frankincense in relation to our characterized standards (Table 3). A

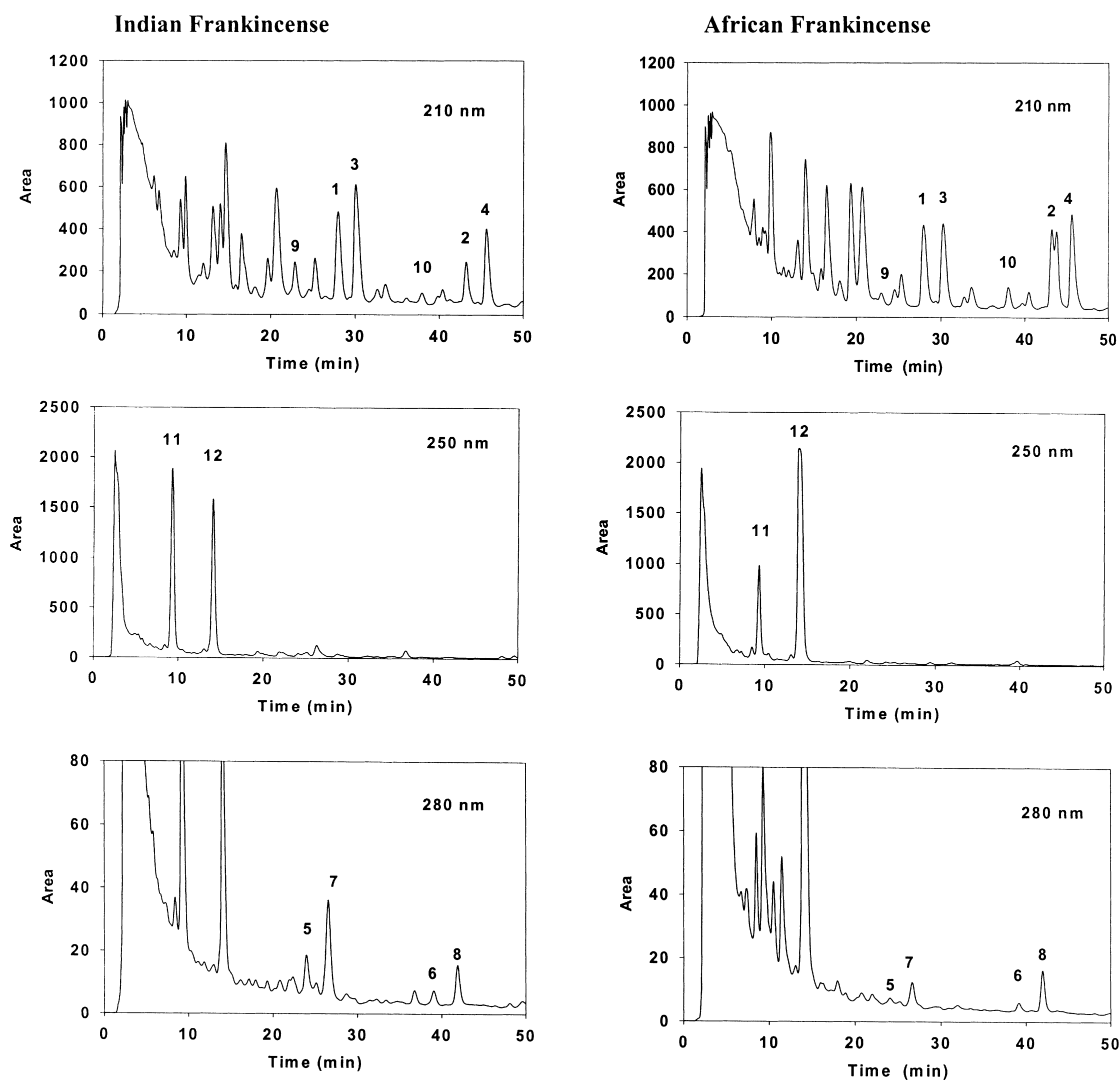

Fig. 4. Differential profiles of pentacyclic triterpenic acids in African and Indian frankincense gum resins.

significant difference was seen with regard to the amount of acetyl-11-keto-β-boswellic acid (**12**) predominating in African frankincense, while twice as much 11-keto-β-boswellic acid (**11**) was found in Indian frankincense; the ratio between both compounds (**12**:**11**) was approximately 0.7 and 4.7 in Indian and African frankincense, respectively (Table 3). A further typical sign for African origin is elution of a peak with a retention time very close to that of acetyl-α-boswellic acid (**2**). The total amount of the detected pentacyclic triterpenic acids was about 25% lower in Indian than in African frankincense (Table 3). Whereas the ratio of **12**:**11** may allow differentiation between frankincense gum resins of African or Indian origin, the differences concerning the total amounts of boswellic acids must be regarded with reservation. Thus, it is well-known that secondary plant metabolites may vary a great deal depending on the climate, the soil, and other environmental parameters. Unfortunately, so far the various *Boswellia* species have not been successfully cultivated, thereby precluding analysis of frankincense gum resins

Table 2
Calibration data for compounds **1**–**12**

Compound	Slope (±SEM)	Intercept (±SEM)	Correlation coefficient	LOD (ng/injection)
1	0.003 (0.0001)	0.07 (0.0546)	0.99983	47
2	0.004 (0.0001)	0.082 (0.0698)	0.99986	47
3	0.003 (0.0001)	0.121 (0.0457)	0.99985	44
4	0.003 (0.0001)	0.579 (0.1078)	0.99960	84
5	0.02 (0.0001)	0.002 (0.0052)	0.99979	0.8
6	0.038 (0.0004)	0.03 (0.0210)	0.99955	1
7	0.03 (0.0001)	−0.003 (0.0054)	0.99991	0.6
8	0.023 (0.0001)	−0.008 (0.0035)	0.99997	0.4
9	0.003 (0.0001)	−0.19 (0.0651)	0.99983	58
10	0.002 (0.0001)	−0.2 (0.0443)	0.99989	42
11	0.03 (0.0001)	0.12 (0.0097)	0.99999	0.5
12	0.03 (0.0001)	0.02 (0.0070)	0.99996	0.9

$n=12$, number of data points for each compound; SEM, standard error of the mean.

from *Boswellia* species grown under controlled conditions [24].

3.4. Analysis of triterpenic acids in phytopharmaceutical preparations

With the methods developed, we analyzed the contents of pentacyclic triterpenic acids in two different phytopharmaceutical frankincense preparations (Table 4). Various batches of preparation A that have been most commonly used as phytopharmaceutical medication reveal, especially in batches A2–A5, a quite uniform distribution of pentacyclic triterpenic acids suggesting that the manufacturer employed a standardized extraction procedure and a relatively homogeneous raw material. From the **12**:**11** ratio of approximately 0.73, the material can be clearly identified as Indian frankincense; the enrichment of pentacyclic triterpenic acids in extracts A is about 1.9-fold compared to native Indian frankincense (Table 3). Preparation B displayed a **12**:**11** ratio of 6.3 that is similar to that seen in native African frankincense. However, it contained even less pentacyclic triterpenic acids than the African frankincense batch analyzed.

Furthermore, it is obvious that the contents of 9,11-dehydro-α- and -β-boswellic acids (**5**–**8**) vary considerably depending on the phytopharmaceutical preparation providing further evidence for work-up-dependent generation of these compounds. In the light of potential pharmacological activities, generation of these compounds during the extraction process requires proper control.

Table 3
Comparison of the contents of triterpenic acids in Indian and African frankincense gum resins after exhaustive Soxhlet extraction using methanol as solvent

Compound	Indian (mg/g frankincense)	African (mg/g frankincense)
1	24.5	26.9
2	4.7	21.1
3	46.0	37.2
4	25.4	39.8
5	0.24	0.04
6	0.12	0.06
7	0.54	0.15
8	0.18	0.28
9	3.2	2.3
10	3.5	7.8
11	20.2	10.1
12	14.3	47.0
Total	142.9	192.7

Analysis was carried out in duplicates at two different concentrations.

3.5. Application of the method

The present method can be used for the analysis of pentacyclic triterpenes and similar compounds in different matrices, for example frankincense gum resins, phytopharmaceuticals based on frankincense extracts, plasma, urine, and different tissue samples

Table 4
Comparison of the pentacyclic triterpenic acid contents in phytopharmaceuticals from different suppliers

Supplier	Pentacyclic triterpenic acids (mg/400 mg medication)											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A1	10.1	5.0	30.9	12.3	0.6	0.14	1.6	0.8	1.6	1.1	12.5	8.2
A2	12.3	10.8	36.3	27.6	0.4	0.08	1.2	0.7	1.4	2.5	9.6	8.7
A3	13.6	10.5	36.8	25.6	0.4	0.06	1.2	0.6	2.1	2.5	11.8	9.3
A4	12.0	9.6	33.5	22.2	0.1	0.02	0.7	0.5	1.6	2.2	10.3	9.5
A5	13.0	5.9	37.6	14.3	0.5	0.08	1.2	0.5	5.2	1.3	15.6	8.4
B1	8.8	3.5	6.9	13.0	n.d.	n.d.	0.09	0.1	0.6	2.5	2.2	13.5
B2	10.8	3.3	7.4	13.5	n.d.	n.d.	0.09	0.1	0.4	2.5	2.0	13.1

n.d., not detectable. The preparations provided by the suppliers contained either 400 mg of dry extract (A) or ground African frankincense resin in a single dosage (B).

(in preparation). We have tested this method over a prolonged period of time with respect to stability, selectivity, reproducibility, accuracy and applicability on diverse analytical problems with great success.

4. Conclusion

The procedure described in this paper represents a specific and sensitive method for the determination of pentacyclic triterpenes in different frankincense samples. To our knowledge, so far this is the first method, which allows simultaneous identification and quantification of 12 different triterpenic acids in frankincense gum resins. Further, this method offers the opportunity to standardize frankincense extracts in terms of a defined composition of the different pentacyclic triterpenic acids in phytopharmaceuticals intended for clinical testing.

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