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Development, Validation, and Application of a Simple and Rugged HPLC Method for Boswellic Acids for a Comparative Study of Their Abundance in Different Species of *Boswellia* Gum Resins

Alberto Asteggiano [1,2], Loris Curatolo [3], Valentina Schiavo [1], Andrea Occhipinti [3] and Claudio Medana [1,*]

[1] Department of Molecular Biotechnology and Health Sciences, University of Torino, Via Pietro Giuria 5, 10125 Torino, Italy
[2] Biosfered S.r.l., Via Paolo Veronese 202, 10148 Turin, Italy
[3] Abel Nutraceuticals S.r.l., Via Paolo Veronese 202, 10148 Turin, Italy
* Correspondence: claudio.medana@unito.it; Tel.: +39-011-670-5244

Featured Application: We present an HPLC method development and validation for the quantitation of boswellic acids in *Boswellia* resin samples and construction of a multivariate model for the clustering of different sample species.

Abstract: The resin of *Boswellia* plant species has been used since ancient times for its several bioactive curative effects. In modern times, extracts of this resin are still widely commercialized on the food supplements and nutraceuticals market. Nowadays the legislation on botanical extracts and the increasing demand for their safety and traceability are setting new standards for their chemical characterization. In this work we present an easy, feasible and rugged HPLC-UV-based approach for the quantitation of boswellic acids in food-supplement-grade resin extracts of *Boswellia* plant species. This method can be used for quality control purposes as well as for studying their differences in regional origin, years of harvesting and species-related differences. The method employs a C18 (3 × 150 mm, 3 µm) analytical HPLC column, and the separation is carried out through a gradient of acetonitrile/water with 0.1% formic acid. The method was validated following the ICH guidelines and used to quantitate different samples of commercial resin of *Boswellia carteri* and *serrata* together with other samples from unknown species. The six known boswellic acids were identified and quantified in all the analyzed samples. The results were used to build a multivariate model to graphically appreciate their difference through their clustering. The model was then augmented by adding further quantitation data for boswellic acids belonging to different species of *Boswellia* obtained by another scientific publications to increase the number of studied samples.

Keywords: *Boswellia*; frankincense; liquid chromatography; method development; 3-O-Acetyl-11-keto-β-boswellic acid; 11-keto-β-boswellic acid; boswellic acid; multivariate model; PCA

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1. Introduction

Plants of the *Boswellia* genus (Burseraceae) are widely spread in northeast Africa, the Arabian Peninsula and India, and their gum resins have been used since ancient times for spiritual and medical purposes [1]. The genus includes 25 different species, including *Boswellia sacra (B. sacra)*, typical of Arabia and northeast Africa, *Boswellia papyrifera (B. papyrifera)* from Somalia, Eritrea, Sudan and Ethiopia and *Boswellia serrata (B. serrata)*, typical of India [1]. However, the classification of the species within this genus is hard to define. For example, until a few years ago, *B. sacra* and *Boswellia carteri (B. carteri)* were considered

two different species, while recently they have become recognized as a single species [2]. Gum resins from plants of the genus *Boswellia* are widely used for the extraction of several bioactive compounds useful for cosmetics and nutraceutical applications. *Boswellia* gum resins contain up to 30% pentacyclic triterpenic acids (PTA), such as boswellic acids, their acetylated derivates and lupeolic acid [3], as well as volatile compounds that can make up to 15% by weight, including a di-terpenic fraction made from incensole, incensole acetate and cembrenol [4]. As a food supplement, the most used species are *B. carteri* Birdw. (syn. *sacra* Flück.) and *B. serrata* Roxb. which contain a large amount of boswellic acids. The content of boswellic acids is different among the species and therefore among the treatment indications also: *B. serrata* resin is used for the treatment of oxidative and inflammatory damage [5], rhinitis [6], asthma [7], age-related disorders [8], neurorecovery [9], arthritis [10], skin disorders and cancer [11] and against several human pathogenic and plant pathogenic fungi. *B. carteri* is otherwise used in the treatment of gastric and hepatic disorders [12] and skin disorders [13] and for its hepatoprotective activity [14], analgesic effect [15], antiglycation and antioxidant activities [16], tumor suppression [17], anticoagulation effects [18], anti-inflammatory activity [19] and cardioprotective effects [20]. In the market, it is often not possible to trace the origin of the gum resin; therefore, it is important to validate an analytical method to support the identification of the plant species from gum resin samples. The aim of this work is to provide an analytical method able to quantify the boswellic acids within the gum resin by a simple HPLC-DAD analysis and to develop a multi-variate model able to perform the clusterization of the observations reflecting their species. The application of the method presented in this study and the related multivariate analysis approach is focused on samples of resins from *B. carteri* and *B. serrata* due to their wide use as dietary supplements. Furthermore, we decided to augment the multivariate model with the introduction of literature data from another scientific publication.

2. Materials and Methods

2.1. Samples

Samples of *B. carteri* and *B. serrata* resins were purchased from commercial resin vendors in Europe under the market name of "olibanum *Boswellia carteri*" for *B. carteri* and "frankincense resin *Boswellia serrata*" for *B. serrata*. For some of them, data regarding their provenience and species as well as the year of harvesting were not reported by the importing company.

2.2. Samples for Meta-Multivariate Analysis

Boswellic acid concentration data were found and obtained from [21]. Those data were from 2019; our data were obtained in the time span 2020–2022.

2.3. Reagents

Acetonitrile Chromanorm (ACN), formic acid Emparta (FA), ammonium acetate (AA), 1-propanol Reactapur (1-PrOH), orthophosphoric acid Chromanorm (OA) were purchased from VWR (Milan, Italy). Dimethylsulfoxide Emsure (DMSO), ethanol Emparta (EtOH) and methanol Emparta (MeOH) were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained via a Milli-q apparatus by Millipore corporation (Massachusetts, USA). Standards of 3-O-Acetyl-11-keto-β-boswellic acid (AKBA) > 98%, 11-keto-β-boswellic acid (KBA) > 98%, β-boswellic acid (B-BA) > 98%, α-boswellic acid (A-BA) > 98%, 3-O-Acetyl -α-boswellic acid (A-ABA) >98% and 3-O-Acetyl -β-boswellic acid (A-BBA) > 98 were purchased from Extrasynthese (Lyon, France).

2.4. Sample Preparation

For the analysis, 50 g of the sample (gum resin grains of *B. sacra* and *B. serrata* species) were weighted, and each aliquot of sample was extracted with 100 mL of MeOH for 4 h by bath sonication. After 4 h, the resulting solution was filtered with a paper filter. The

filtered solution was concentrated through a rotary vacuum evaporator (Rotavapor R215, Büchi, Flawil, Switzerland). The extraction was repeated for an additional hour. The second aliquot of methanolic solution was concentrated as described before and pooled with the first extraction. The extract was dried in the oven at 100 °C for 4 h to evaporate all the residual solvent. For the analysis, 50 mg of the pulverized extract was weighted in a plastic tube and extracted by dissolution in 10 mL of methanol. The resulting solution was then sonicated in an ultrasonic bath, centrifuged 5000× *g* × 5 min to precipitate the insoluble particles and finally diluted 20-fold in the same solvent used for the analytical extraction in an autosampler 1.5 mL vial.

2.5. HPLC Analysis

For the HPLC analysis, an LC-40 HPLC system from Shimadzu (Milan, Italy) was employed. The instrument was equipped with an HPG binary pump and a Shimadzu SPD40 PDA spectrophotometric detector. To find the best analytical set-up, we compared three different instrumental methods. The protocols have in common the use of the following RP C18 column: Shimadzu GIST-HP C18 (3 × 150 mm, 3 μm) and the detector wavelength settings.

Method 1: The HPLC system was operated in binary gradient mode; solvent A was water:MeOH 5 mM AA and solvent B was 1—PrOH:MeOH 5 mM AA. The chromatographic gradient was set as follows: flow, 0.2 mL/min; initial conditions, t = 0 min B % = 30, t = 2 min B % = 50, t = 35 min B % = 85, t = 37 min B % = 100, t = 43 min B % = 100. The column reconditioning was performed for the next 6 min as follows: t = 43.1 min B % = 30, t = 49 min B % = 30. Total analysis time was 49 min. Column was maintained at RT.

Method 2: HPLC was operated in binary gradient mode, solvent A was 90:9.5: 0.5 water:MeOH: OA, while solvent B was 55 40: 4.5: 0.5 = MeOH:ACN:water:OA. Flow was set at 0.3 mL/min and the solvent ramp was as follows: t = 0 min B % = 70, t = 35 min B % = 100, t = 40 min B % = 100. The reconditioning was as follows: t = 40.1 min B % = 70, t = 45 min B % = 70. Total analysis time was 45 min. Column was maintained at RT.

Method 3: HPLC was operated in binary gradient, A: 0.1% formic acid in water, B: 0.1% formic acid in ACN. The design was as follows: t = 0 min B % = 70, t = 17 min B % = 100, t = 23 min B % = 100. The reconditioning was as follows: t = 23.1 min B % = 70, t = 31 min B % = 70. Total analysis time was 31 min. Flow was set at 0.2 mL/min. Column temperature was set at 60 °C.

In the three different methods, the PDA or the UV-Vis detector were set to acquire at 250 or 205 nm, respectively, for the keto boswellic acids (AKBA, KBA) and the boswellic acids (A-BA, B-BA, A-ABA, A-BBA). Slit width was set at 1.2 nm. Acquisition bandwidth was set at ±4 nm.

2.6. Method Validation Parameters

Method validation was conducted on method 3 following the ICH guidelines for the parameters of linearity, accuracy, precision and sensitivity [22]. To satisfy the validation requirements, 3 calibration curves with 7 points were built on 3 different days and from 2 different operators. For the parameter of linearity, the calibration interval was extrapolated from the range of analytical response with a R^2 linearity coefficient > 0.998. The repeatability of the calibration curves was assessed by comparing the relative standard deviation RSD % of their slopes. The absence of a CRM matrix of *Boswellia* without boswellic acids led us to use the solvent as matrix for the studies of accuracy and precision. The accuracy was expressed in terms of accuracy BIAS % (Acc. BIAS %) using a low (5 mg/L n = 3), a medium (20 mg/L n = 3) and a high concentration level (100 mg/L n = 3). The results were expressed as % of difference between the real and the measured value. Intermediate precision was calculated based on the levels used for the accuracy step as relative standard deviation (RSD %) among the measurements (n = 3) for each level. Sensitivity was measured in terms of LOD and LOQ (limit of detection and limit of quantitation, respectively) based on the standard error σ of the calibration curve. The σ value is divided

by the value of the average slope and multiplied 3-fold for the LOD and 10-fold for the LOQ.

2.7. Multivariate Analysis

Data obtained from the sample quantitation were used to build a matrix of observations (samples) and variables (concentration of boswellic acids), and to these data we added several data points from other peer reviewed publications which analyzed the boswellic acid contents in different *Boswellia* gum resin extracts with similar extraction methods. The variables were transformed into principal components with the SIMCA 14.1 software and to fit a PCA model. All the missing values were approximated to 0.01 and the distance scaling used UV for the multivariate analysis for the samples obtained in this study and was Pareto for the meta-analysis approach. The variables were not transformed since their nature and magnitude were similar. The critical R2 and Q2 were set at 0.995.

3. Results

3.1. Method Validation

The method was successfully validated in the ranges of 0.1–100 mg/L for the keto-acids (KBA and AKBA), 1–100 mg/L for the A-BA and B-BA. Finally, the acetyl boswellic acids A-ABA and A-BBA curves were validated in the range 5–100 mg/L. Table 1 reports the results of the validation process, while Figures S1–S3 in the Supplementary Materials report, respectively, an example of a chromatogram for a sample, a calibration standard mix and a blank sample. All the R^2 of the calibration lines exceed the 0.995 value, in particular the values for KBA, AKBA, A-BA and B-BA were higher than 0.999. The parameter of relative standard deviation of the calibration lines, expressed as RSD % of the slopes, shows low values and consequently highlights a good signal stability and repeatability for the three calibration line replicates.

Table 1. Validation data for the boswellic acid quantitative method. The columns report the different molecules studied: KBA, AKBA, A-BA, B-BA, A-ABA and A-BBA. Each row shows the validation parameter. Rt: retention time; R2: linearity coefficient; RSD % of slopes: relative standard deviation of the slopes; Acc. BIAS %: quantitation error expressed as % on three different measurements; RSD %: precision expressed as relative standard deviation of three different measurements; LOD: limit of quantitation; and LOD: limit of detection.

Parameter		KBA	AKBA	A-BA	B-BA	A-ABA	A-BBA
Rt (minutes)		12.3	16.1	19.8	20.3	24.1	24.9
Range (mg/L)		0.1–100	0.1–100	1–100	1–100	5–100	5–100
R2 (n = 3)		0.999	0.999	0.999	0.999	0.998	0.997
		0.999	0.999	0.999	0.999	0.998	0.997
		0.999	0.999	0.999	0.999	0.998	0.997
RSD % of slopes (n = 3)		1.76	−0.25	−0.72	−0.45	0.51	−0.74
		−0.69	1.04	0.84	2.93	−0.43	0.56
		−1.07	−0.79	−0.11	−2.48	−0.08	0.18
Acc. BIAS % (n = 3)	5 mg/L	8.6	0.2	1.52	11	−23.0	−12.2
		2.1	4.3	5.65	8.6	−23.4	−11.2
		3.6	−2.5	3.05	−15.0	−23.5	−11.7
	20 mg/L	0.3	0.9	−10.5	6.0	11.8	4.5
		2.1	4.4	5.59	5.5	26.8	7.7
		−0.6	−2.1	0.3	−5.3	21.9	6.0
	100 mg/L	−3.6	0.01	−0.9	0.2	8.7	0.3
		−2.5	1.0	0.9	3.2	10.4	1.4
		−1.2	−0.8	0.1	−2.74	9.7	1.3
RSD % (n = 3)	5 mg/L	6.3	3.4	2.4	14.5	2.4	2.4
	20 mg/L	2.3	3.3	8.3	6.5	8.3	8.3
	100 mg/L	1,6	0.9	0.9	3	0.9	0.0
LOD (ppm)		0.19	0.45	0.47	0.97	4.6	3.59
LOQ (ppm)		0.62	1.50	1.58	3.25	15.2	11.95

The parameter for accuracy, Acc. BIAS %, shows good values for all the concentrations studied in all the replicates as well as the RSD % describing method precision. LOD and LOQ, as calculated from the residuals of the calibration curves and their slopes, show the lowest concentrations of the boswellic acids that the method can detect and quantify with the minimal error.

3.2. Quantitative Analysis of Boswellic Acids

Eighteen samples of *Boswellia* gum resin were extracted according to the procedure described in Materials and Methods section. After their analytical preparation the solutions were injected into the HPLC apparatus. The concentration of the 6 chemical species investigated is reported in Table 2.

Table 2. Table listing all the samples analyzed in the study with the validated method. Each row reports the sample ID on the first column; declared species reports, for each sample, its species, with "unk" if unknown; provenience reports the national or territorial origin of the sample, with "unk" if unknown. The remaining column reports the concentrations of each boswellic acid studied expressed as weight percentage or g/100 g, and all the observations are an average value of 2 or more analytical repetitions with an RSD value lower than 10%.

Sample ID	Declared Specie	Provenience	% AKBA	% KBA	% A-BA	% B-BA	%A-ABA	%A-BBA
1	*B. carteri*	Somalia	1.43	0.09	0.36	3.6	0.43	1.58
2	*B. carteri*	Somalia	1.45	0.1	0.29	3.19	0.84	3.04
3	*B. carteri*	Somalia	1.04	0.96	0.39	4.35	0.69	2.84
4	*B. carteri*	Africa	0	0.06	3.24	6.72	1.7	9.73
5	*B. carteri*	Africa	0.47	0.12	2.55	5.95	2.86	11.8
6	*B. carteri*	Somalia.	4.29	0.43	1.7	2.34	4.28	6.6
7	*B. carteri*	Somalia	8.42	1.19	1.93	3.71	2.8	7.15
8	*B. serrata*	India	2.76	2.82	3.14	10.31	1.3	3.3
9	*B. serrata*	India	2.45	2.41	2.9	7.63	1.2	3.1
10	*B. serrata*	India	3.62	1.1	1.74	9.07	1.1	2.3
11	unk.	Somalia	3.89	0.41	1.97	4.94	3.55	8.92
12	unk.	Kenya	0.18	0.06	2.26	5.81	3.53	14.55
13	unk.	Africa	5.63	1.08	1.08	0.78	0.6	0.07
14	unk.	Africa	0.77	0.06	2.36	4.64	3.53	6.85
15	unk.	Africa	0.42	0.02	3.24	5.29	3.19	5.56
16	unk.	Africa	4.58	0.47	2.11	0.917	5.8	1.6
17	unk.	unk.	5.29	0.51	1.31	3.44	3.69	2.89
18	unk.	unk.	5.61	0.47	1.76	1.98	2.62	4.94

In total, 10 gum resin samples were reported by the supplier as *B. carteri* (7) and *serrata* (3), 6 samples coming from the African region did not show any information about the botanical species and, finally, 2 samples were purchased without any information about their provenience or species. The 18 analyzed samples show an appreciable diversity in their composition of boswellic acids. It is possible to notice how the KBA concentration is generally higher in *B. serrata* (2.11 ± 0.9% by weight $n = 3$) than *B. carteri* (0.42 ± 0.5% by weight $n = 7$); on the other hand, AKBA has shown more disperse results in samples of *B. carteri* (2.44 ± 2.9 by weight $n = 7$). The samples coming from the African region, but not reporting any further detail about the species, show concentration values of AKBA and KBA which are like the African *B. carteri* samples: the average content of AKBA is 2.57 ± 2% by weight ($n = 6$) and KBA is 0.35 ± 0.4% by weight ($n = 6$). Finally, the two totally unknown samples report a smaller variation and respective values of AKBA and KBA of 5.45 ± 0.22% by weight and 0.49 ± 0.2% by weight. A-BA values increase in the *B. serrata* samples (2.6 ± 0.7% by weight $n = 3$) as well as B-BA (9 ± 1.3% by weight $n = 3$), while their acetylated forms are more concentrated in certified *B. carteri* and the ones for which the species was not clarified, namely for A-ABA 1.9 ± 1.4% by weight ($n = 7$) and 3.4 ± 1.3% by weight ($n = 6$) and for A-BBA 6.1 ± 3.8% by weight ($n = 7$) and 6.3 ± 5.2% by weight ($n = 6$). Finally, the totally unknown samples show a boswellic acid concentration pattern which can resembles the African *B. carteri* certified samples.

3.3. Multivariate Analysis

The score plot in Figure 1 presents the spatial distribution of the analyzed samples together with the loading of their variables. A clear separation between *B. carteri* and *B. serrata* samples is visible and takes place mostly on the vertical component. As already mentioned in the last paragraph, KBA concentration seems to play a role in discriminating the two species since the *B. serrata* contains a higher concentration of this keto-triterpenic acid. The samples of *B. carteri* show, on this score plot, a heterogeneous composition that was also mentioned in the last paragraph. Their left to right spreading is appreciable on the lower side of the plot, and it is like the one of the unknown species sample. Interestingly, samples 17 and 18 (which were not supplied with any information about species or provenience) have a very close grouping. Their spatial belonging to the *B. carteri* group, as well as their proximity with certified *B. carteri* samples, suggests that those two resins belong to the species of *B. carteri*. The concentration of AKBA drives the clustering of 5 samples of *B. carteri* (1, 2, 3, 6, 7) and 4 unknown samples (16, 17, 18, 13). A second group of African samples, among which 4 and 5 are certified *B. carteri,* also contains the samples 11, 12, 14 and 15, which are highly probably *B. carteri.* Those samples are shifted towards the left side of the lower part of the plot, where the A-ABA, A-BBA and A-BA variables carry the highest effect.

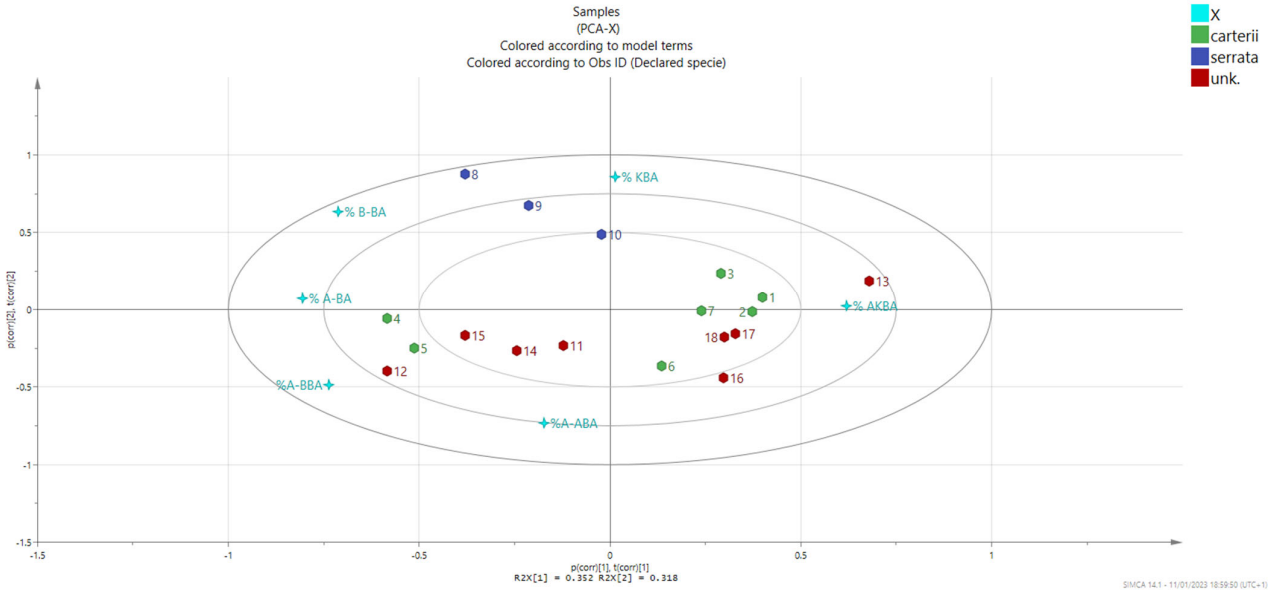

Figure 1. Biplot of the PCA model for the set of analyzed samples belonging to *B. carteri*, *B. serrata* and unknown species; each circular dot represents a sample, while each violet diamond represents the loading of each variable.

3.4. Meta-Multivariate Analysis

To increase the number of studied cases as well as the sample classes, we decided to augment the models obtained with our data with additional data from a peer reviewed scientific publication which adopted a similar extraction and validated quantitation method [21]. These data are concordant with ours in terms of the abundance profile of the six molecules, and they represent a useful tool to improve the sample clusterings and their species-specific comparisons. Data came from 8 different *Boswellia* species and added 40 new samples to our study and are presented in Table 3.

Table 3. Table listing all the samples used for the meta-multivariate approach. Each row reports the sample ID on the first column; declared species reports sample species, with "unk" if unknown; provenience reports the national or territorial origin of the sample, with "unk" if unknown. The remaining column reports the concentrations of each boswellic acid studied expressed as weight percentage or g/100 g. All the quantitation data are obtained from [21].

Sample ID	Declared Specie	Provenience	% AKBA	% KBA	% A-BA	% B-BA	%A-ABA	%A-BBA
Sch_1	*B. sacra*	Oman	4.18	0.27	1.03	2.72	2.91	4.86
Sch_2	*B. sacra*	Oman	2.92	0.28	0.73	2.02	1.73	3.06
Sch_3	*B. sacra*	Oman	3.44	0.12	0.41	1.31	2.07	3.59
Sch_4	*B. sacra*	Oman	3.29	0.11	0.64	2.11	2.53	4.95
Sch_5	*B. sacra*	Oman	3.59	0.22	1.33	2.94	3.85	6.23
Sch_6	*B. sacra*	Oman	2.18	0.23	0.77	2.48	1.69	3.12
Sch_7	*B. sacra*	Oman	3.75	0.16	0.76	2.24	2.72	3.85
Sch_8	*B. sacra*	Oman	3.33	0.21	0.67	1.82	1.90	3.24
Sch_9	*B. sacra*	Oman	2.61	0.10	0.58	1.91	2.31	4.45
Sch_10	*B. sacra*	Oman	2.18	0.11	0.47	1.86	1.88	3.99
Sch_11	*B. sacra*	Oman	3.01	0.12	0.46	1.38	2.37	3.67
Sch_12	*B. dalzielli*	Burkina Faso	5.34	1.17	1.33	1.74	2.73	3.14
Sch_13	*B. dalzielli*	Nigeria	7.22	0.99	1.62	2.03	3.92	3.80
Sch_14	*B. dalzielli*	Senegal	6.83	1.39	1.61	1.68	2.97	2.81
Sch_15	*B. papyrifera*	Ethiopia	4.39	0.44	1.09	2.36	2.14	3.00
Sch_16	*B. papyrifera*	Eritrea	2.78	0.34	0.55	1.64	1.43	1.99
Sch_17	*B. papyrifera*	Senegal	1.68	0.32	1.03	3.18	2.02	4.65
Sch_18	*B. serrata*	India	1.13	0.53	1.64	4.97	1.17	3.65
Sch_19	*B. serrata*	India	1.87	2.23	2.53	4.97	1.17	3.06
Sch_20	*B. serrata*	India	1.36	0.31	1.56	4.79	1.62	4.19
Sch_21	*B. serrata*	India	0.86	0.58	1.32	5.40	0.74	2.31
Sch_22	*B. serrata*	India	1.33	0.98	1.53	4.27	1.01	2.94
Sch_23	*B. serrata*	India	0.93	0.63	0.89	2.83	0.62	1.98
Sch_24	*B. serrata*	India	0.81	0.40	1.22	3.83	0.84	2.36
Sch_25	*B. carteri*	Somalia	0.01	0.01	1.18	3.81	1.95	4.64
Sch_26	*B. carteri*	Somalia	0.01	0.00	1.55	5.73	3.38	8.46
Sch_27	*B. carteri*	Somalia	0.00	0.01	2.89	6.67	0.00	0.01
Sch_28	*B. carteri*	Somalia	0.00	0.09	3.65	8.39	0.00	0.01
Sch_29	*B. carteri*	Somalia	4.93	0.65	2.07	1.81	1.74	2.55
Sch_30	*B. neglecta*	Somalia	0.00	0.14	2.68	8.10	0.08	0.37
Sch_31	*B. neglecta*	Somalia	0.01	0.16	2.67	7.58	0.26	1.31
Sch_32	*B. neglecta*	Somalia	0.00	0.17	2.98	8.59	0.06	0.43
Sch_33	*B. neglecta*	Somalia	0.01	0.06	2.48	7.36	0.18	0.71
Sch_34	*B. neglecta*	Somalia	0.00	0.02	0.72	2.06	0.05	0.26
Sch_35	*B. neglecta*	Kenya	0.00	0.01	0.19	0.32	0.01	0.08
Sch_36	*B. neglecta*	Kenya	0.00	0.01	0.01	0.01	0.01	0.01
Sch_37	*B. rivae*	Ogaden	0.01	0.00	0.01	0.03	0.01	0.02
Sch_38	*B. rivae*	Somalia	0.01	0.01	0.01	0.01	0.01	0.01
Sch_39	*B. rivae*	Somalia	0.01	0.01	0.01	0.01	0.01	0.01
Sch_40	*B. rivae*	Somalia	0.01	0.01	0.01	0.01	0.01	0.01

After the addition of those further data, the model assumes a more complex aspect (see Figure 2). The increased number of classes can be deceiving at a first glance. A separation on the second principal component between the *serrata* samples (higher) and the *carteri* samples occurs. This phenomenon was already seen in the first PCA model (Figure 1) and the addition of further samples seem to confirm this trend. In the paper by Schmiech et al., the authors decided to separate the samples of *B. carteri* and *sacra* into two classes by their provenience from the regions of Somalia and Oman. Even if *B. carteri* is considered as a synonym of *sacra* [23], we decided to keep this separation in the construction of our model in order to understand if those samples belonging to different regions show different clusterization. *B. sacra* samples are shifted nearby and within the *B. carteri*, *Boswellia dalzielli* (*B. dalzielli)* and *B. papyrifera* ones. *Carteri* samples are widely spread on the plot and carry the biggest variability. On the other hand, *Boswellia neglecta* (*B. neglecta)*

samples show a narrow grouping on the highest side of the plot. *Boswellia frereana* (*B. frereana*) samples, which are characterized, as well as *B. neglecta*, for a very low abundance of AKBA, are very narrowly spread in the left side of the plot.

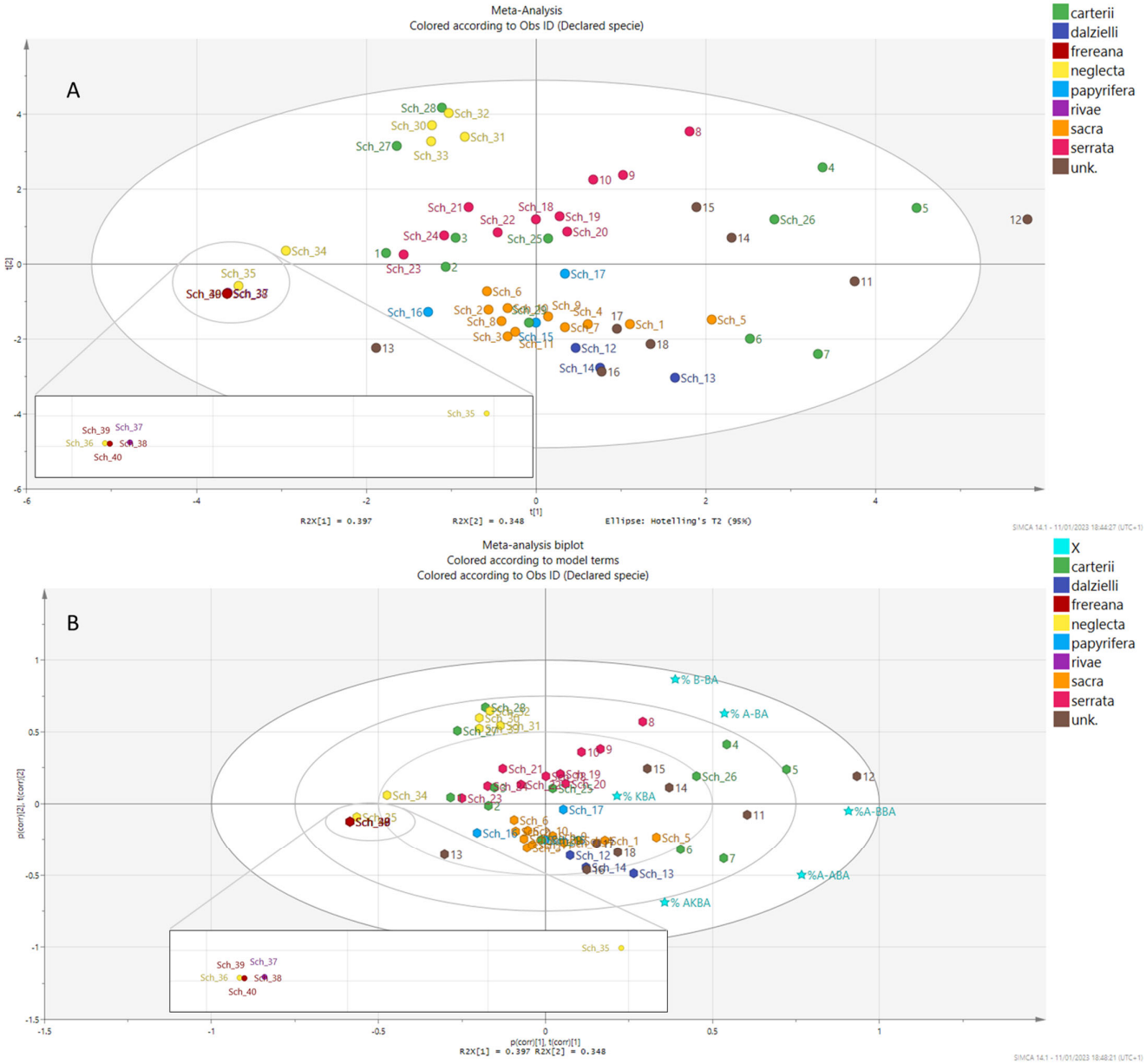

Figure 2. (**A**) PCA score plot for the analysis of the samples in the meta-multivariate study. Each circular dot represents a sample, and its color represents the species, as reported in the legend. (**B**) Biplot of the PCA model for the set of samples in the meta-multivariate study. Each circular orange dot represents a sample, while each green dot represents the loading of each variable. To better observe the overlapped regions, a magnification of the overlapped area is proposed for both plots.

The contribution of each variable is presented in the biplot in Figure 2. AKBA loading influences the sample clusters to shift in the lower-right part of the plot, and this region is mainly populated from *B. dalzielli*, *B. carteri*, *B. sacra* and some of the unknown samples analyzed in our study. The KBA variable influences mostly the clustering of the *B. serrata* samples, together with the A-BA and B-BA. The left side of the plot is populated, on the other hand, by samples which have a low concentration of all the six species investigated.

4. Discussion

The growing interest in botanical extracts based on *Boswellia* plant resin is resulting in the development of several food supplements with this ingredient. However, the spontaneous nature of the *Boswellia* tree, together with the visual similarity of the crude resins, can act as a confounding factor in the species identification both in the field during harvesting and in the market chain. To avoid species misidentification, the analytical chemistry approach can play a fundamental role. Different methods have been compared to define and quantify the boswellic acids within the oleogum resin samples [1]. The crude resin from different Burseraceae was extracted with methanol and analyzed with thin-layer chromatography (TLC). The authors could separate and identify different compounds such as AKBA and other terpenes by using, respectively, TLC and mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). An interesting approach was used by MALDI-TOF fingerprinting analysis to perform the identification of commercial frankincense [24]. High-performance liquid chromatography is more often used for this purpose because it can separate the constitutional isomers of the different PTAs [21]. In this work, we decided to present a validated reverse-phase HPLC method for the quantitative determination of six boswellic acids and use their quantitation data to build a model for the species-related samples clustering.

4.1. Optimization and Validation of Quantitative HPLC Method

Quantitation of boswellic acids by HPLC-based techniques has been widely reported in peer-reviewed literature [21,25–27]. In this work, we presented an optimized and fast method to separate all the main analytes in the shortest time span. To find the most-suitable method for the separation of boswellic acids by HPLC, we investigated three different chromatographic ramps with different combinations of solvents, as described in the Materials and Methods section. The first method was inspired by the one used by Schmiech et al. [21], while the second and the third ones are, respectively, the previously used and the newly proposed instrumental quantification methods used in our analytical laboratory. The column choice was easy and, in a certain way, forced since, for this class of molecules, the non-polar interaction is the strongest (also seen in several other scientific publications [21,28–30]). The first method was efficient for separating all the boswellic acids present in the samples [21]; however, it had the disadvantage of long run times. The second method was faster than the first one because of its increased flow, but it was not efficient for achieving an acceptable peak separation. The method showed itself as not suitable for separating all the components of the sample mixture, and, in addition, the solvents could not be used with mass spectrometers (MS). The third method is MS-compatible and it can separate all the analytes in a short run thanks to the regulation of column temperature (60° C). Among the previous published works, Schmiech, M. et al., 2019 [21] is the most similar to ours in terms of scope and methodology. With our approach, we wanted to improve the analytical conditions and propose an overall more robust and cheaper instrumental setup, from MS to the HPLC-UV Vis detector. We succeeded obtaining a fast and robust method with a duration of about 30 min, significantly faster than the methods described in [21,25–27]. The strategy of raising the column temperature was rewarding in terms of run duration and simple solvent choice. In addition, the peaks separations were revealed as very good for all the analytes without coelutions. The validation step for the developed method was led according to the ICH guidelines. All the validation parameters received satisfactory results in the chosen linearity range. Furthermore, the validation results are concordant with the ones described by Schmiech, M. et al. [21].

4.2. Boswellic Acid Quantitation and Multivariate Analysis

The quantitative analysis of boswellic acids has been handled in several scientific publications [21,31–34] and its importance resides in the high commercial value of those molecules, which are used in the food supplement market mostly as anti-inflammatory

agents [5,30,35–37]. By using the HPLC method developed and validated herein, we were able to quantify the six molecules from our samples. The results show a general higher concentration of KBA in the samples of *B. serrata*, which is concordant with other results obtained in the scientific literature [21,31]. The species of *B. carteri* was characterized by a higher variability in the concentration of the boswellic acids. For instance, the concentration of AKBA showed a huge standard deviation value, ranging from more than 8% by weight to traces. The variability in those samples affects the other boswellic acids as well. The high variability of secondary metabolite content can be led by plant metabolic plasticity in response to environmental biotic and abiotic stress (e.g., drought, UV light, temperature, herbivores). In this case the environment plays a central role in the abundance and concentration of those molecules, and it is responsible for their variability [38–42]. Therefore, the spontaneous nature of the *B. carteri* plant as well as the environmental differences in the Horn of Africa and Arabian Peninsula regions may explain the phytochemical variability of *B. carteri* (syn sacra) and the dispersion of their results. The study of a single variable at a time to unveil differences among samples, classes and groups, which are influenced by several variables, can be reductive and ineffective since it does not consider all the descriptors that simultaneously affect the samples. The use of a multivariate approach can overcome this issue and perform the sample clustering considering simultaneously multidimensional variable classification [43–45]. The samples presented in Table 2, influenced by the variables of the six boswellic acids (KBA, AKBA, A-BA, B-BA, A-ABA and A-BBA) have been used to build a multivariate unsupervised model. The model built with our quantification data was able to group samples of *B. serrata* and *B. carteri* and to spatially compare the unknown species samples with the certified *B. carteri* or *B. serrata* ones. With this strategy we were able to validate the hypothesis that the unknown samples belonged to the species of *B. carteri* or *B. sacra*. This model was limited by the lack of other species of *Boswellia* resin to be compared with the unknown samples of our study. To overcome this limitation, we decided to add to the model a number of quantitative data obtained with a similar preparative and analytical approach using the results published by Schmiech et al. [21]. The inclusion of those data in our model confirmed the trend observed in our first model, the separation between the clusters of *B. carteri*/*sacra* and *B. serrata*. Although the paper from Schmiech et al. [21] differentiates the species of *B. sacra* and *carteri*, it seems that the cluster composed from B. sacra samples is only narrower and less spread than the *B. carteri* one, while its distribution on the second principal component remains the same. On the other hand, samples classified as carteri show the widest spread of all the other species. To our knowledge, our work is the first which uses this meta-multivariate approach for the cluster analysis of *Boswellia* samples. With this strategy, we were able both to confirm the trend of our data by comparing them with the ones already published [21] and to increase the numbers of their group by adding our new observations.

5. Conclusions

The work presents a fast and simple method of detection and quantitation of boswellic acids based on a HPLC instrumentation; the method was used to quantitate the 6 most abundant triterpenic acids in 18 different samples of *Boswellia resin* extracts. Obtained data were used to build a multivariate model which was then combined with some data from a peer-reviewed paper. The final model contained 58 observations from 7 different *Boswellia* species. The grouping results from this meta-multivariate analysis show that the chemical profiles of diverse *Boswellia* species can be used to build a spatial clusterization based on the principal component analysis. This approach was useful to distinguish groups of samples of resin of different species growing in proximal areas considering only six variables. A natural progression of this work is represented by the addition of other variables to the model, such as volatile compounds or other terpenic molecules.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13031254/s1. Figure S1: Chromatogram for the samples; Figure S2: Chromatogram for the standard mix; Figure S3: Chromatogram for the blank.

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