Journal of Food Measurement and Characterization (2024) 18:1900–1912
https://doi.org/10.1007/s11694-023-02310-y

ORIGINAL PAPER

# NMR-based analytical methods for quantifying boswellic acids in extracts employed for producing food supplements: comparison of $^{13}$C-qNMR and $^{1}$H-NMR/PLS-R methods

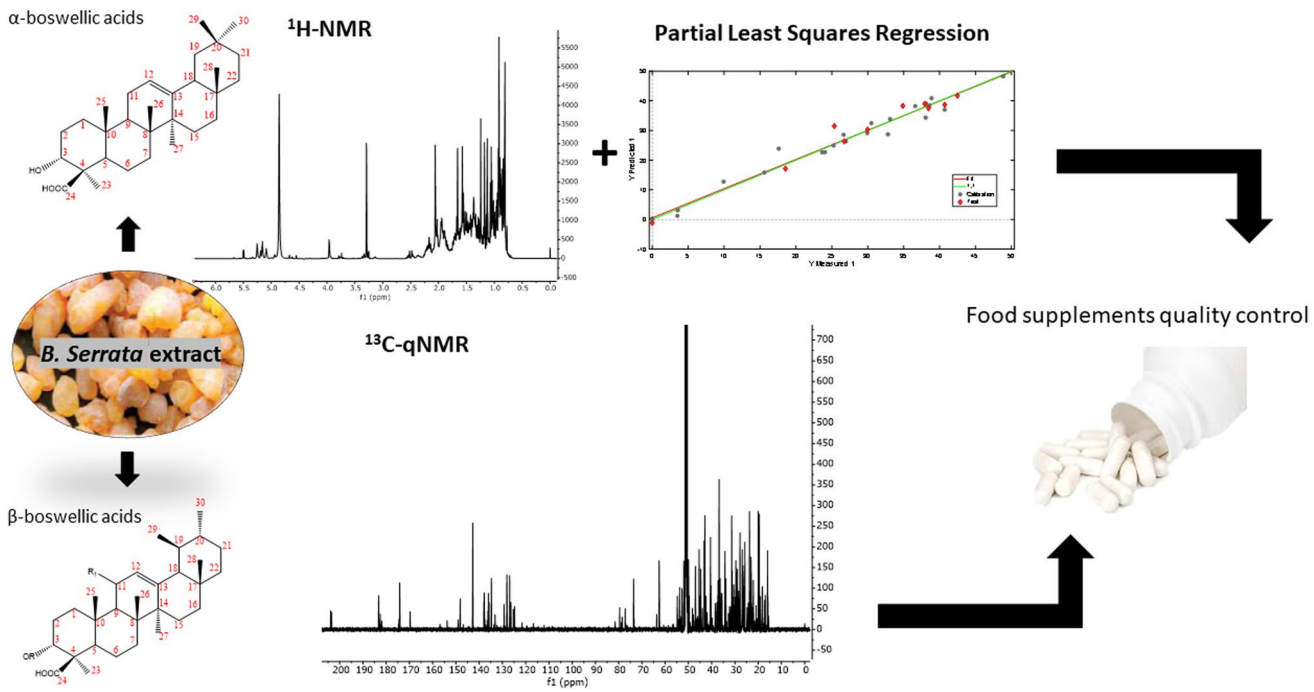
**Eleonora Truzzi[1] · Danny Vincenzo Piazza[2] · Maria Cecilia Rossi[3] · Stefania Benvenuti[2] · Davide Bertelli[2]**

Received: 4 September 2023 / Accepted: 29 November 2023 / Published online: 24 December 2023


## Abstract

The content of boswellic acids (BAs) in *Boswellia serrata* extracts employed for producing food supplements is often overestimated due to the use of conventional non-selective quantification methods. Here, the applicability of NMR spectroscopy for the quality control of *B. serrata* extracts was evaluated by employing different strategies: $^{13}$C-quantitative NMR (qNMR) and $^{1}$H-NMR coupled with chemometrics. The $^{13}$C-qNMR demonstrated high precision and accuracy, but long-lasting acquisition times. $^{13}$C-qNMR quantitative results were used to build a PLS-R model on the $^{1}$H-NMR spectra to generate a faster analytical method. The $R^2$ and the RMSE in prediction were 0.925 and 5.878 respectively, indicating good model performances, which can be improved by increasing the number of extracts. Moreover, the identification of 3 extracts out of 33 without any trace of BAs underlined the importance of proper controls of the starting material to produce BA-based food supplements.

## Graphical abstract




**Keywords** Chemometrics · PULCON · Dietary supplements · Multivariate statistical analysis · Partial least-squares regression

Extended author information available on the last page of the article

## Introduction

*Boswellia serrata* Roxb. is a plant native to India and Pakistan and oleogum resin thereof (also known as frankincense) contains several organic acids among which up to 12 different boswellic acids (BAs). BAs are pentacyclic triterpenoids that occur as two constitutional isomers, α-BA (oleanane type) and β-BA (ursane type). The BAs can also be different in the substitutions at C-3 and C-11 positions. Among the BAs, the α- and β-BAs, acetyl-α-BA (AαBA), acetyl-β-BA (AβBA), 11-keto-β-BA (KBA), and 3-O-acetyl-11-keto-β-BA (AKBA) are the most concentrated and pharmacologically active compounds. *B. serrata* extracts and BAs were investigated in-depth during the last decades and their therapeutic efficacy against chronic inflammatory conditions was confirmed in clinical pilot studies [1]. At the beginning of the twentieth century, *B. serrata* extracts and BAs were approved as a remedy for inflammation in Europe and have been mentioned in the 7th supplement of the European Pharmacopoeia since 2006. Currently, the extracts of *B. serrata* oleogum resin are employed to produce food supplements and medicated feeds for the treatment of chronic inflammatory diseases in humans, pets, and livestock. As a matter of fact, BAs inhibit the expression of lipoxygenases and suppress the activity of cyclo-oxygenase 1. The hydrophobicity of BAs guarantees an extended pharmacological effect and a half-life of about 6 h due to the slow renal clearance [2, 3].

The *Boswellia* extract market was valued at 64.30 million dollars in 2022, and the forecast expects significant growth in only seven years with a CAGR of 4.2% (Compound annual growth rate) [4]. Due to consumers' increasing interest in natural products, food supplements are subjected to counterfeiting. In the case of *B. serrata* extracts, quality control is essential due to the increasing importance of this herbal medicine in traditional and conventional medicines. Manufacturers often determine the total content of BAs in the extracts by a non-selective method based on titration [5]. Consequently, the concentration of BAs in the extracts is overestimated due to the presence of other organic acids in the extracts, such as lupeolic and tirucallic acids or 9,11-dihydro-BAs [6–8]. In 2016, Meins et al. demonstrated the occurrence of the counterfeiting problem by investigating the quality of the top-sold food supplements containing *Boswellia* extracts in European and American markets. From their investigation, 41% of the products did not comply with the label declaration and three samples did not show any trace of BAs [9]. Without proper quality control of the imported extracts, the commercialized food supplements could be inefficient due to the small amounts of BAs. In the literature, several separative techniques were suggested for the quantification of BAs and quality control of resins or commercial products [6, 10–16]. Nuclear magnetic resonance (NMR) spectroscopy could represent a good alternative to chromatographic techniques which are time-consuming and require the construction of calibration curves. In the last decades, NMR spectroscopy was also largely used for the quality control of food matrices and supplements [17, 18]. The main advantage of NMR spectroscopy is the capability to provide qualitative and quantitative information simultaneously [19, 20]. Quantitative NMR (qNMR) allows the concomitant quantification of several compounds by employing an external standard, without requiring any calibration curve [21, 22]. NMR spectroscopy also provides the chemical fingerprinting of complex matrices that can be used for creating untargeted multivariate statistical methods capable to quantify the content of analytes in foods. Recently, the coupling of NMR spectral data with multivariate regression models has been demonstrated to efficiently predict the content of adulterant compounds in complex food matrices, such as fruit juices, wine, edible oils, and chocolate [23–26].

In the present work, two different analytical approaches were developed using an NMR spectrometer for quantifying the BA content in raw *B. serrata* extracts used for producing food supplements and medicated feeds. The first approach aimed at the design of a robust qNMR method on $^{13}$C-NMR spectra for quantifying each BA in the extracts. Then, a faster method based on an untargeted chemometric approach was built on $^{1}$H-NMR spectra. To the best of our knowledge, this is the first evaluation of the applicability of NMR spectroscopy in the quality control of *B. serrata* extracts.

## Methods

### Materials

Standards of β-BA (purity ≥ 97.5%), KBA (purity ≥ 98%), AKBA (purity ≥ 99%), AαBA (purity > 95%), AβBA (purity ≥ 98%), α-BA (purity ≥ 95%), were obtained from Extrasynthese (Genay, France). Pyridoxine (purity ≥ 98%), methanol-*d4* (purity ≥ 99.8%), and 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TSP), were purchased from Sigma Aldrich (Milan, Italy). Thirty-three *B. serrata* raw extracts were supplied from several manufacturers of medicated feeds, who imported thereof from Indian and Pakistan companies.

### Sample preparation

About 40 mg of the extract were accurately weighed, transferred into a conical flask, and dissolved in 1 mL of methanol-*d4* under magnetic stirring for 1 h. The external standard

was prepared by dissolving pyridoxine in methanol-*d4* at a concentration of 10 mM. The solutions were filtered and transferred into a WILMAD® NMR tube, 5 mm, Ultra-Imperial grade, 7 in. L,528-PP (Sigma-Aldrich, Milan, Italy). Finally, the TSP was added.

## NMR spectroscopy and spectra acquisition procedures

All the analyses were performed on a Bruker FT-NMR Avance III HD 600 MHz spectrometer equipped with a CryoProbe BBO H&F 5 mm (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany). All the experiments were carried out at 300 K and non-spinning. One-dimensional $^{1}$H and $^{13}$C spectra and bi-dimensional Heteronuclear Single-Quantum and Multiple Bond correlations spectra (HSQC and HMBC, respectively) were used for the assignment of the resonances of each standard of BA.

$^{1}$H-NMR experiments were acquired using the Bruker sequence "zg30"; the acquisition parameters were as follows: time domain (number of data points), 131,072 K; dummy scans, 0; number of scans, 32; acquisition time, 4.96 s; delay time, 10 s; pulse width, 11.4 µs; spectral width, 22 ppm (13,204 Hz); digitization mode, baseopt. The total acquisition time was 7 min.

$^{13}$C-qNMR quantification experiments were performed using a 1D inverse-gated decoupling sequence to avoid NOE during relaxation [25]. The experiments were acquired using the Bruker sequence "zgpg_pisp_f2.fas" and the acquisition parameters were consequently modified and set as follows: time domain (number of data points), 65,536 K; dummy scans, 0; number of scans, 256; acquisition time, 0.98s; delay time, 50s; spectral width, 220.87 ppm; digitization mode, baseopt. To use this sequence as inverse gated, the proton decoupling power (PLW13) during the recycling delay and experiment time was set to 0 db. The total acquisition time was 3 h and 37 min.

HSQC spectra were acquired by using the Bruker sequence "hsqcedetgpsp.3". The acquisition parameters were as follows: time-domain (number of data points), F2: 2048, F1: 256; dummy scans, 16; the number of scans, 8; acquisition time, F2: 0.1551019s, F1: 0.0040388s; delay time, 2s; pulse width, 10.75 µs; spectral width, F2: 11 ppm (6602 Hz), F1: 209 ppm (31,692 Hz); fid resolution, F2: 6.447376 Hz, F1: 247.599670 Hz; digitization mode, digital. The total acquisition time was 1 h 5 min.

HMBC spectra were acquired using the Bruker sequence "hmbcetgpl3nd". The acquisition parameters were as follows: time-domain (number of data points), F2: 4096, F1: 256; dummy scans, 16; the number of scans, 8; acquisition time, F2: 0.3102037s, F1: 0.0040388s; delay time, 1.5s; pulse width, 10.75 µs; spectral width, F2: 11 ppm (6602 Hz), F1: 209 ppm (31,692 Hz); fid resolution, F2: 3.223688 Hz, F1: 247.599670 Hz; digitization mode, digital; receiver gain, 203. The total acquisition time was 1 h 12 min.

After the sample was inserted into the probe, at least 5 min waited to achieve the thermal equilibrium. Subsequently, the magnetic field was locked, the probe head was tuned and matched, and finally the sample was shimmed. All these procedures were automatically executed to ensure the highest reproducibility. For $^{1}$H-NMR, the correct 90° pulse was calibrated for each sample with the "pulsecal" Bruker AU program, and the receiver gain was set.

### *Quantitative $^{13}$C-NMR analysis*

Due to the complexity of the $^{1}$H-NMR spectra of the extracts, the qNMR method was developed using the $^{13}$C-NMR spectra. Pyridoxine was selected as the external reference compound for the quantification due to its solubility in methanol and signal chemical shifts not overlapping to target molecules which can allow its employment also as an internal standard. Prior to the peak integrations, each spectrum was calibrated according to the TSP signal and then an automatic zero order phase and a baseline correction were applied.

The standard solution in methanol-*d4* (10.155 mM) was prepared and analyzed at each session of acquisition and then used to quantify the BAs in the extracts. The $^{13}$C-NMR signals of pyridoxine used for the quantification were: C2' at 19.19 ppm, C4' at 60.17 ppm, and C5' at 61.47 ppm. $T_1$ was measured for the signals of pyridoxine and BAs to select the correct delay time and the signals with the lower relaxation for the quantification. The delay time was set to seven times the biggest $T_1$. To calculate the carbons $T_1$ the Bruker sequence "t1irig" was used with the following acquisition parameters: temperature 298 K; time-domain (number of data points), F2: 65,536 and F1: 8; dummy scans, 0; the number of scans, 8; acquisition time, F2: 0.9043968 s and F1: 0.0006656 s; delay time, 150 s; pulse width, 10.00 µs; spectral width, F2: 240 ppm (36,231 Hz) and F1: 10 ppm (6009 Hz); fid resolution, F2: 1.105709 Hz and F1: 1502.402832 Hz; digitization mode, digital.

For the quantification, only peaks with a sufficient signal-to-noise ratio (10:1) were used. The quantification of BAs was achieved using the Concentration Conversion Factor (CCF) method, implemented in Mnova® 14.1.2 software (Mestrelab Research, S.L., Santiago de Compostela, Spain). The signals belonging to BAs were automatically integrated and compared to the area of signals generated by the pyridoxine standard solution.

## Method validation

The validation of the qNMR method was evaluated in terms of intra- and inter-day precisions, limit of determination

(LOD) and quantification (LOQ), and recovery on one standard of BA. Only one standard was selected for the validation since all BAs display comparable chemical properties. AβBA was chosen because its content in the extracts is in the middle of the abundance range in most cases. Precision was assessed by analyzing the standard five times for three different days. The results are expressed as average percent relative standard deviations (RSD%). The LOD can be determined from the spectrum by calculating the concentration corresponding to a signal having the S/N is equal to 3. The LOQ value is conventionally set at the concentration corresponding to S/N equal to 10. Finally, the recovery was evaluated at three different concentrations (low, medium, and high) by spiking one extract with one standard before the dissolution of the extract in 1 mL of methanol-*d4*.

### Multivariate statistical analyses

Multivariate statistical analyses were carried out on $^{1}$H-NMR spectra using PLS_Toolbox for MATLAB® (version 8.9.2, Mathworks Inc.). $^{1}$H-NMR spectra of the *B. serrata* extracts were aligned by using Icoshift 1.0 toolbox for MATLAB® and exported as spectral intensities. The dataset was preprocessed by means of baseline correction (automatic weighted least squares, order 2), followed by Pareto-scaling and mean-centering. Principal component analysis was performed to have a general overlook of sample disposition in the space on the whole dataset. A PLS-R model was built for quantifying total BA content in the extracts. The dataset (n = 33) was randomly split into training and test set (70:30) for building and validating the model, respectively. The quantitative results obtained from the $^{13}$C-qNMR were used to build the multivariate regression. For both models, the cross-validation was performed using the leave-one-out method due to the relatively low number of samples in calibration [27]. The number of principal components (PCs) and latent variables (LVs) for the construction of the PCA and PLS-R models respectively were selected depending on the lowest root mean squares errors (RMSE) in calibration and cross-validation.

## Results and discussion

The main problem concerning food supplements is the lack of standardization of the natural extracts used for their production. This issue leads to a large variability in the concentration of bioactive compounds, resulting in lack of efficacy or onset of unpredictable side effects. Thus, the quality control of imported *B. serrata* extract is essential to overcome this problem. As explained in the introduction, the content of BAs in the extracts is often determined by using a non-selective titration method. Consequently, the development of a rapid analytical tool is necessary to provide the real content of BA to the manufacturers of food supplements and medicated feeds containing *B. serrata* extracts.

The $^{1}$H and $^{13}$C-NMR spectra of BAs were qualitatively examined to assign all their resonances. The chemical structures of BAs, the spectra, and the peak assignments thereof are reported in Figs. 1 and 2, and Table 1, respectively.

The assignments and the chemical shifts agreed with those reported in the literature [8, 28]. As can be observed in Fig. 2A and Table 1, the signals in $^{1}$H-NMR spectra displayed similar chemical shift due to the high

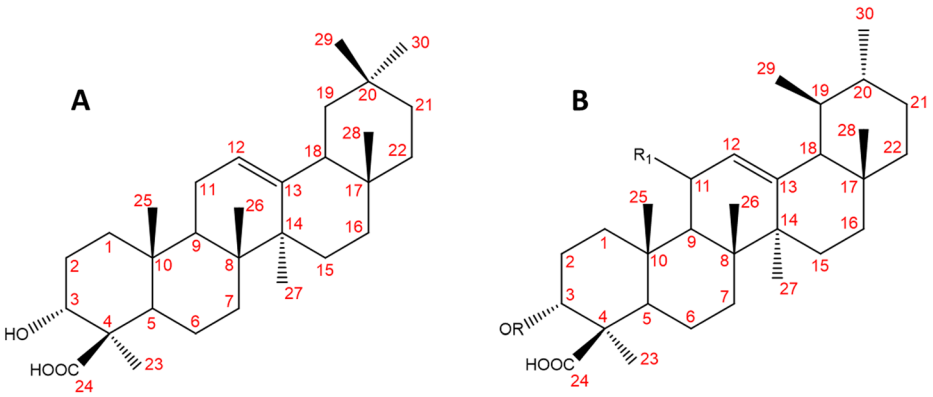



**Fig. 1** Chemical structure of α- (**A**) and β- (**B**) boswellic acids

| | R | $R_1$ |
|---|---|---|
| α-boswellic acid (α-BA) | H | H |
| Acetyl-α-boswellic acid (AαBA) | Ac | H |

| | R | $R_1$ |
|---|---|---|
| β-boswellic acid | H | H |
| Acetyl-β-boswellic acid | Ac | H |
| 11-keto-β-boswellic acid | H | =O |
| Acetyl-11-keto-β-boswellic acid | Ac | =O |

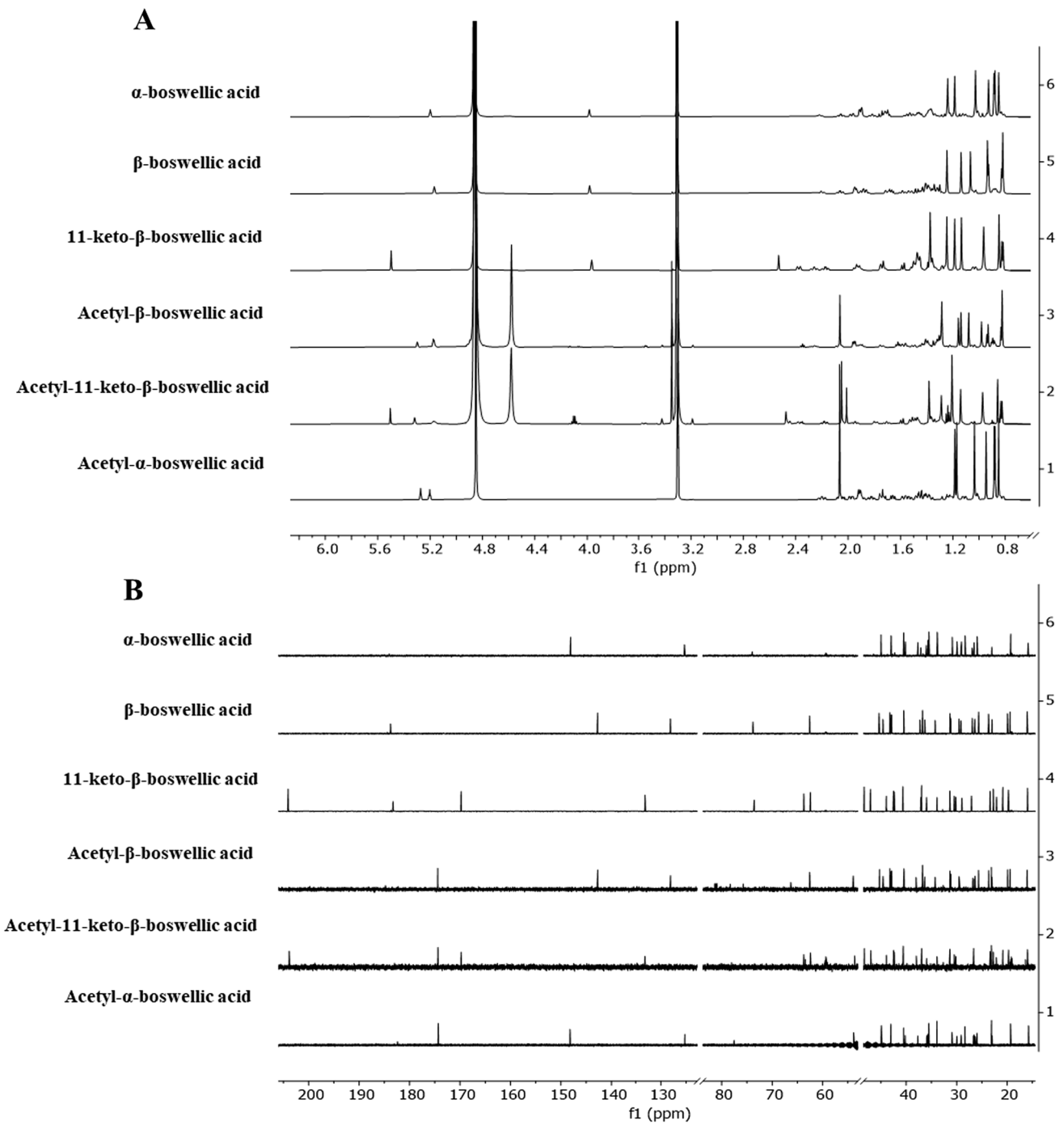



**Fig. 2** $^{1}$H- (**A**) and $^{13}$C- (**B**) NMR spectra of boswellic acids

structural similarity of BAs. The close resonances resulted in extremely complex proton spectra of *B. serrata* extracts where most of the peaks were overlapped (Fig. 3A).

Consequently, the development of a qNMR method on $^{1}$H-NMR spectra for the quantification of BAs could not be possible without the employment of signal deconvolution which can introduce significant errors in the calculation. Conversely, characteristic well-resolved peaks for each BA were observed in the $^{13}$C-NMR spectra (Figs. 2B and 3B). For this reason, the qNMR method was built using carbon signals to quantify each BA.

The T1 relaxation times of the signals selected for the quantification were determined before the optimization of the quantitative acquisition parameters on $^{13}$C-NMR spectra. The longest relaxation time (> 6 s for KBA) was observed for the C14 whose signal was employed for the quantification of

**Table 1** Chemical shifts (ppm) of protons and carbons of α-boswellic acid (αBA), β-boswellic acid (βBA), 11-keto-β-boswellic acid (KBA), acetyl-11-keto-β-boswellic acid (AKBA), acetyl-β-boswellic acid (AβBA), and acetyl-α-boswellic acid (AαBA) in methanol-*d4*

| | αBA | βBA | KBA | AKBA | AβBA | AαBA |
|---|---|---|---|---|---|---|
| H1 | ~1.40/1.33 (m) | ~1.431/1.359 (m) | ~1.453/1.357 (m) | ~1.471/1.235 (m) | ~1.497/1.219 (m) | ~1.437 (d)/1.205 (t) |
| H2 | 2.226/1.516 (m) | 2.206/1.527 (m) | 2.261/1.488 (m) | 2.327/1.543 (m) | 2.265/1.560 (m) | 2.198/1.558 (m) |
| H3 | 3.987 (t) | 3.983 (t) | 3.967 (t) | 5.292 (t) | 5.303 (t) | 5.268 (t) |
| H5 | ~1.455 (m) | ~1.475 (m) | ~1.460 (m) | ~1.381 (m) | ~1.395 (m) | ~1.395 (m) |
| H6 | 1.905/1.715 (m) | 1.871/1.713 (m) | 1.922/1.741 (m) | 1.795/1.771 (m) | 1.915/1.744 (m) | 1.910/1.732 (m) |
| H7 | 1.528/1.379 (m) | 1.584/1.407 (m) | 1.732/1.474 (m) | 1.493/1.472 (m) | 1.559/1.397 (m) | 1.556/1.393 (m) |
| H9 | 1.697 (m) | 1.676 (m) | 2.531 (bs) | 2.480 (m) | 1.616 (m) | 1.654 (m) |
| H11 | 1.906 (m) | 1.942 (m) | / | / | 1.952 (m) | 1.975 |
| H12 | 5.199 (t) | 5.168 (t) | 5.498 (s) | 5.505 (s) | 5.1726(t) | 5.198 (t) |
| H15 | 1.815/1.009 (m) | 1.884/1.037 (m) | 1.929/1.072 (m) | 1.945/1.046 (m) | 1.918/1.045 (m) | 1.908/1.043 (m) |
| H16 | 2.056/0.818 (m) | 2.062/0.881 (m) | 2.174/1.036 (m) | 2.182/0.849 (m) | 2.067/0.892 (m) | 2.061/0.812 (m) |
| H18 | 1.973 (dd) | 1.342 (bs) | 1.581 (bs) | 1.589 (bs) | 1.338 (bs) | 1.931 (dd) |
| H19 | 1.752/1.027 (m) | 1.388 (m) | 1.474 (m) | 1.456 (m) | 0.887 (m) | 1.733/1.032 (m) |
| H20 | / | 0.930 (m) | 0.965 (m) | 0.970 (bs) | 0.927 (m) | / |
| H21 | 1.368/1.113 (m) | 1.410/1.317 (m) | 1.453/1.358 (m) | 1.461/1.364 (m) | 1.389/1.300 (m) | 1.328/1.276 (m) |
| H22 | 1.467/1.222 (m) | 1.432/1.317 (m) | 1.518/1.392 (m) | 1.518/1.369 (m) | 1.433/1.315 (m) | 1.451/1.371 (m) |
| H23 | 1.238 (s) | 1.245 (s) | 1.246 (s) | 1.206 | 1.154 (s) | 1.182 (s) |
| H25 | 0.930 (s) | 0.935 (s) | 1.135 (s) | 1.184 (s) | 0.978 (s) | 0.943 (s) |
| H26 | 1.027 (s) | 1.065 (s) | 1.186 (s) | 1.204 (s) | 1.079 (s) | 1.032 (s) |
| H27 | 1.186 (s) | 1.137 (s) | 1.374 (s) | 1.381 (s) | 1.139 (s) | 1.167 (s) |
| H28 | 0.850 (s) | 0.818 (s) | 0.848 (s) | 0.855 (s) | 0.820 (s) | 0.848 (s) |
| H29 | 0.879 (s) | 0.824 (d) | 0.821 (d) | 0.826 (d) | 0.829 (d) | 0.873 (s) |
| H30 | 0.887 (s) | 0.930 (d) | 0.965 (s) | 0.970 (s) | 0.933 (d) | 0.881 (s) |
| 3-O-Ac | / | / | / | 2.055 (s) | 2.059 (s) | 2.061 (s) |
| C1 | 37.107 | 37.268 | 36.955 | 37.989 | 38.01 | 37.702 |
| C2 | 29.136 | 29.145 | 28.970 | 26.641 | 26.469 | 26.542 |
| C3 | 73.965 | 73.825 | 73.562 | 77.966 | 77.361 | 77.550 |
| C4 | 51.433 | 51.456 | 51.465 | 51.733 | 50.107 | 49.814 |
| C5 | 52.090 | 52.069 | 51.644 | 53.636 | 53.938 | 53.855 |
| C6 | 23.038 | 22.981 | 22.097 | 22.133 | 22.999 | 22.960 |
| C7 | 35.711 | 36.325 | 35.983 | 35.960 | 36.324 | 35.761 |
| C8 | 42.985 | 43.214 | ***48.327**** | ***46.996**** | 43.217 | 43.014 |
| C9 | 49.871 | ***49.935**** | 63.717 | 63.724 | 51.452 | 50.022 |
| C10 | ***40.501**** | ***40.467**** | ***40.629**** | 40.582 | ***40.423**** | 40.524 |
| C11 | 26.540 | 26.401 | 204.047 | 203.852 | ***26.373**** | 26.313 |
| C12 | ***125.318**** | 128.105 | 133.141 | 133.147 | 128.085 | 125.260 |
| C13 | ***148.100**** | 142.743 | 169.795 | 169.792 | 142.730 | ***148.194**** |
| C14 | ***44.967**** | ***45.385**** | ***47.090**** | ***48.300**** | ***45.307**** | ***44.932**** |
| C15 | 29.044 | 29.530 | 30.176 | 30.165 | 29.524 | 29.094 |
| C16 | 29.924 | 31.148 | 30.463 | 30.459 | 31.142 | 29.962 |
| C17 | ***35.489**** | 36.766 | 37.103 | 36.948 | 36.769 | 35.532 |
| C18 | 51.435 | ***62.565**** | ***62.433**** | 62.423 | 62.564 | 51.492 |
| C19 | 49.952 | 42.975 | 42.371 | 42.375 | 42.874 | 50.075 |
| C20 | 33.831 | 42.870 | 42.509 | 42.510 | 42.985 | 33.898 |
| C21 | 37.679 | 34.255 | 33.878 | 33.881 | 34.265 | 37.729 |
| C22 | 40.157 | ***44.603**** | 43.932 | 43.922 | 44.593 | 40.200 |
| C23 | 26.925 | 26.887 | 27.062 | / | 26.790 | 26.676 |
| C24 | 184.017 | 183.770 | 183.285 | 182.967 | 183.022 | 182.372 |
| C25 | 15.842 | 16.027 | 15.960 | 15.960 | 16.034 | ***15.744**** |

**Table 1** (continued)

| | αBA | βBA | KBA | AKBA | AβBA | AαBA |
|---|---|---|---|---|---|---|
| C26 | 19.290 | 19.431 | 20.850 | 20.847 | 19.428 | 19.330 |
| C27 | 28.320 | ***25.650**** | 22.728 | 22.725 | 25.673 | 28.371 |
| C28 | ***30.864**** | 31,320 | 31.346 | 31.336 | 31.312 | 30.910 |
| C29 | ***25.933**** | 23.672 | 23.372 | 23.112 | 23.661 | 25.986 |
| C30 | 36.011 | 19.917 | 19.728 | 19.711 | 19.896 | 35.937 |
| 3-O-Ac | / | / | / | 174.380 | 174.420 | 174.312 |
| 3-O-Ac | / | / | | 22.744 | 23.112 | 23.095 |

In brackets, the multiplicity of proton signals is reported: *bs* broad signal; *d* doublet; *m* multiplet; *s* singlet; *t* triplet

*Peaks used to quantify BAs in *B. serrata* extracts

each BA. Therefore, the D1 was set to seven times the maximum T1 at 50s accordingly to Bruker guidelines to achieve the complete relaxation of carbon nuclei and thus quantitative results. The long D1 led to a time-consuming analysis of $^{13}$C-NMR spectra of the extracts. The method used for the quantification is based on PULse Length-based CONcentration (PULCON) determination. The PULCON method correlated the absolute intensity of the peaks originating from two different spectra, belonging to the sample and the external standard. The concentration conversion factor (CCF) can be calculated due to the principle of reciprocity, compensating for the signal intensity according to the different acquisition parameters (such as number of scans, receiver gain, pulse length, and tip angle) [29].

The reliability of the qNMR method was assessed by validation. The percent relative standard deviations (RSD%) were 5.54 and 8.36% for the intra- and inter-day precision, respectively, suggesting that the results provided by the spectrometer are reproducible through time. The accuracy determined by the recovery test could be considered satisfactory being in the range between 80 and 120 (81.51, 87.07, and 106.52% for the low, medium, and high concentrations respectively) [30]. Finally, the LOD and LOQ for AβBA were 0.1453 and 0.42 mg/mL respectively.

After the method validation, a total of thirty-three extracts of *B. serrata* were analyzed and each BA was quantified (Table 2).

The total percentage of BAs in the *B. serrata* extracts was compared to the percentage of BAs declared by the manufacturers on the label (in the range from 60 to 70%). As can be observed in Table 2, all the extracts contained a total concentration of BAs lower than that declared on the label, confirming the overestimation of their content by the producers [31]. The total content of BAs in the extracts agreed with those observed by Zwerger et al. [14], which ranged between 2.6 and 43.2%, and were higher than that observed by Katragunta et al. [15]. Additionally, three extracts did not show any trace of BAs, supporting the importance of the necessity of the quality control of the imported *B. serrata* extracts used to produce food supplements and medicated feeds. The $^{1}$H-NMR spectra of these samples are reported in Figure S1.

After the quantification of total BA content in *B. serrata* extracts by the qNMR method, the employment of multivariate statistical analysis on $^{1}$H-NMR spectra was attempted. The creation of a multivariate statistical model for quantifying the BA concentration in the extracts could represent a more rapid and valid alternative to conventional analytical methods and the qNMR approach.

The unsupervised PCA was performed to evaluate the similarities and differences between the extracts considered in this study. The first two PCs explained 76.11% and 6.15% of the total variance, respectively. In the score plot, the extracts were separated on the PC1 depending on the concentration of total BAs (Fig. 4A). Indeed, the resonances ascribable to BAs protons were the most important variables on positive values of PC1 (Fig. 4B). Consequently, samples were separated from right to left depending on the total concentration of BAs. Moreover, on the most negative values of PC1, two samples without any trace of pentacyclic triterpenoids were observed.

The PC2 played a central role in separating one extract from the others, namely ext31, which displayed a completely different $^{1}$H-NMR spectrum (Figure S1).

Then, a regression model was built using the PLS algorithm. For the construction of the PLS-R model, the x-matrix was generated by using the spectral intensities for each spectral point, as for the PCA. Conversely, the y-matrix was composed of the quantitative results determined by the $^{13}$C-qNMR method expressed as total percentage of BAs. The algorithm extracted four LVs which captured 98.03% of the variance of the x-block. The Hotelling $T^2$ and Q residual control chart (95% of confidence interval) was examined to assess the presence of outliers (Fig. 5A). Hottelling $T^2$ values express the distance of each sample from the center of regular samples. Conversely, Q residuals summarize the remaining variation of each sample that is undescribed by the model. In the chart, no

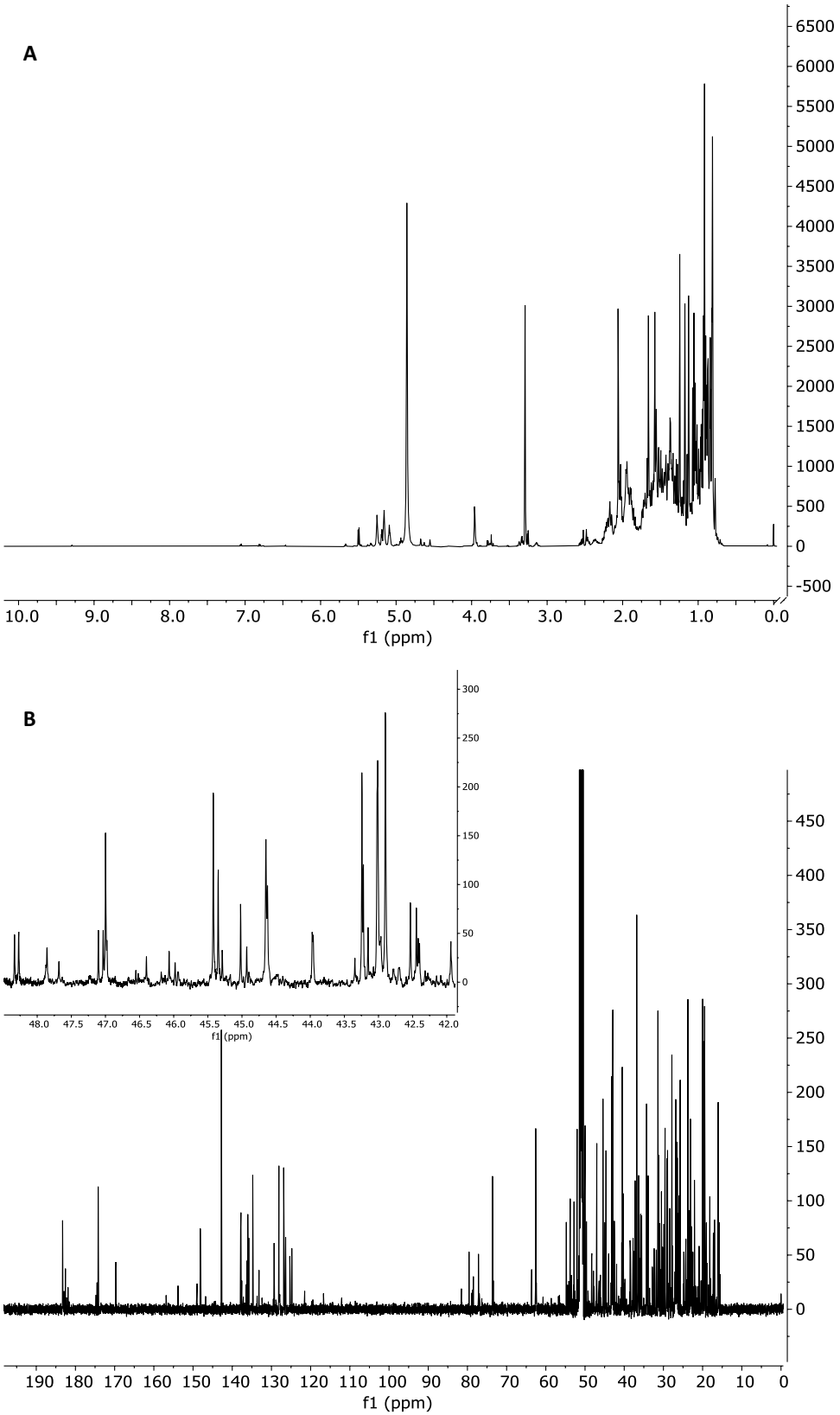



**Fig. 3** $^{1}$H (**A**) and $^{13}$C-NMR (**B**) spectra of a *B. serrata* extract. Enlargement on a spectral region with well-resolved peaks used for the quantification

**Table 2** Quantitative results of α-boswellic acid (αBA), β-boswellic acid (βBA), 11-keto-β-boswellic acid (KBA), acetyl-11-keto-β-boswellic acid (AKBA), acetyl-β-boswellic acid (AβBA), acetyl-α-boswellic acid (AαBA), and total BAs in *B. serrata* extracts

| | αBA | βBA | KBA | AKBA | AβBA | AαBA | Total BAs |
|---|---|---|---|---|---|---|---|
| ext1 | 4.90 | 9.54 | 3.22 | 2.46 | 6.83 | 1.73 | 28.68 |
| ext2 | 6.12 | 12.58 | 3.56 | 2.24 | 6.01 | 3.77 | 34.27 |
| ext3 | 6.42 | 13.19 | 4.13 | 2.74 | 6.33 | <LOQ | 32.80 |
| ext4 | 7.24 | 15.53 | 4.29 | 3.54 | 10.10 | 4.41 | 45.12 |
| ext5 | 4.69 | 11.14 | 3.40 | 1.92 | 5.56 | 2.21 | 28.92 |
| ext6 | 5.03 | 8.67 | 3.92 | 2.42 | 5.19 | 2.14 | 27.37 |
| ext7 | 4.18 | 16.13 | 2.51 | 3.18 | 6.72 | 3.17 | 32.40 |
| ext8 | 3.96 | 11.74 | 2.55 | 2.32 | 10.61 | 2.47 | 29.10 |
| ext9 | 4.70 | 13.70 | 3.13 | 1.59 | 6.81 | 2.67 | 32.59 |
| ext10 | 4.56 | 9.61 | 4.20 | 1.90 | 3.76 | <LOQ | 24.03 |
| ext11 | 3.37 | 6.42 | 3.07 | 1.79 | 3.89 | <LOQ | 18.53 |
| ext12 | 5.84 | 13.61 | 5.50 | 4.31 | 8.68 | 3.00 | 40.92 |
| ext13 | 5.77 | 13.79 | 4.39 | 1.98 | 7.17 | 3.10 | 36.20 |
| ext14 | 0.94 | 0.93 | <LOQ | 0.60 | 1.02 | <LOQ | 3.50 |
| ext15 | 6.47 | 9.20 | 3.94 | 2.57 | 7.79 | 3.38 | 33.35 |
| ext16 | 6.36 | 14.57 | 5.19 | 3.16 | 9.17 | 3.18 | 41.62 |
| ext17 | 8.13 | 14.63 | 4.10 | 4.39 | 9.46 | 3.14 | 43.84 |
| ext18 | 1.53 | 0.41 | 0.59 | 0.71 | 0.32 | n.d | 3.57 |
| ext19 | 7.94 | 14.24 | 6.23 | 3.31 | 6.87 | 2.81 | 41.40 |
| ext20 | 6.53 | 17.90 | 4.67 | 2.80 | 6.22 | 2.48 | 35.28 |
| ext21 | 6.97 | 14.30 | 4.06 | 3.62 | 9.93 | 4.06 | 42.93 |
| ext22 | 1.89 | 3.53 | 1.58 | 0.89 | 2.06 | <LOQ | 9.94 |
| ext23 | 4.30 | 8.66 | 2.86 | 1.98 | 5.84 | 4.04 | 27.68 |
| ext24 | 2.66 | 5.22 | 2.01 | 1.76 | 3.95 | <LOQ | 15.60 |
| ext25 | 8.03 | 16.43 | 5.12 | 2.98 | 9.92 | 3.46 | 45.94 |
| ext26 | 4.51 | 8.61 | 2.25 | 1.82 | 5.15 | 1.74 | 24.08 |
| ext27 | 5.50 | 13.31 | 3.91 | 3.66 | 8.50 | 2.30 | 37.17 |
| ext28 | 6.19 | 19.47 | 4.91 | 4.50 | 7.63 | 2.74 | 44.91 |
| ext29 | 3.21 | 4.31 | 2.45 | 2.55 | 5.08 | <LOQ | 17.61 |
| ext30 | 7.45 | 20.03 | 5.57 | 6.22 | 9.57 | 3.73 | 52.58 |
| ext31 | n.d | n.d | n.d | n.d | n.d | n.d | n.d |
| ext32 | n.d | n.d | n.d | n.d | n.d | n.d | n.d |
| ext33 | n.d | n.d | n.d | n.d | n.d | n.d | n.d |

Results are expressed as a percentage (w/w)

samples were collocated on the upper-right side, indicating the absence of outliers.

In Fig. 5B, the regression fit of the model is displayed. The samples were plotted depending on the percentage of total BAs measured (supplied in the y-block) and predicted by the model. The coefficient of regressions ($R^2$) in calibration, cross-validation, and prediction were 0.967, 0.746, and 0.925, respectively. The RMSE in calibration, cross-validation, and prediction were 2.436, 10.040, and 5.878, respectively. Considering that the RMSE is the deviation from the fit and has the same unit of measurement of x- and y-axes, the estimation error in prediction was lower than 6% [24].

The variable's importance in projection (VIP) score plot revealed which variables were essential for the regression (signals above the significance threshold) (Fig. 5C). Specifically, $-CH_3$ signals got the highest scores as expected. Indeed, these peaks were characteristic for all BAs and their intensity was higher than that of $-CH_2-$and $-CH$ signals. The resonances in the spectral region above 2.5 ppm did not display any relevant role in the regression model.

The two developed approaches proved to be promising analytical methods for the quality control of *B. serrata* extracts. The $^{13}$C-qNMR method provided accurate quantitative results for each type of BA present in the extracts; however, due to the long relaxation time of the carbon nuclei this kind of approach might be not suitable for the rapid control of the raw material employed for the production of food supplements. $^{1}$H-NMR/PLS-R method could be a valid and rapid tool for the purpose. As a matter of fact, it can furnish the total content of BAs which is the most important

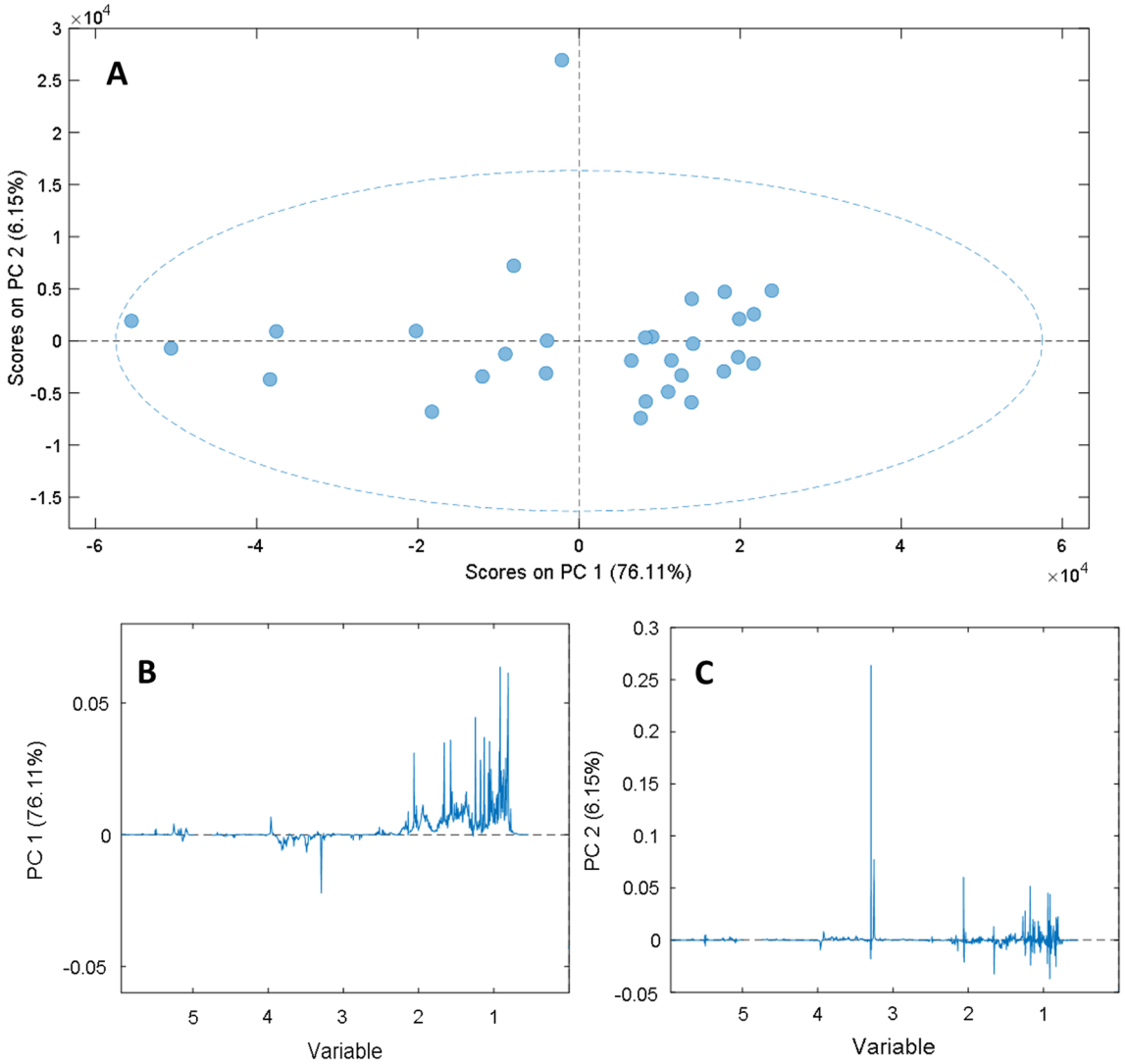



**Fig. 4** Score (**A**) and loading plots of principal component 1 (**B**) and principal component 2 (**C**)

information for food supplement producers to predict the correct dilution of the extract before the preparation of the dosage form. On the other hand, the presence of overlapped signals belonging to different BAs and the occurrence of the same resonances in some cases impaired the quantification of the individual BAs.

To the best of our knowledge, NMR spectroscopy was never applied for the quantification of BAs in *B. serrata* extracts. Conversely, several analytical methods based on high-performance liquid chromatography (HPLC) or high-performance thin-layer chromatography coupled with UV-based or mass-based detectors were proposed [6, 11–16]. These conventional and well-recognized methods are certainly efficacious in the quantification of all BAs in extracts and biological fluids [32–34]; however, the reliability of the quantitative results is strictly connected to the construction of freshly prepared calibration curves for each analyte. On the opposite, qNMR spectroscopy based on the PULCON approach allows the quantification of analytes without the employment of any calibration curve [29]. Moreover, the spectral re-acquisition of the expensive analytical standards is not necessary. Overall, chromatographic methodologies are time-consuming, but the acquisition time is notably lower than that of the $^{13}$C-qNMR method. Indeed, due to the long relaxation time of certain carbons of BAs, the qNMR approach is certainly inconvenient. Instead, $^{1}$H-NMR/PLS-R approach guarantees the fast acquisition of sample proton fingerprinting (7 min) which can be submitted to the statistical model. In the literature, other fingerprinting methods were investigated for the quantification of BAs. Near-infrared spectroscopy coupled with PLS-R was proposed for the quantification of KBA and AKBA in *B. sacra* extracts. The statistical model showed excellent $R^2$ and RMSE values with a prediction error lower than our model; however, the

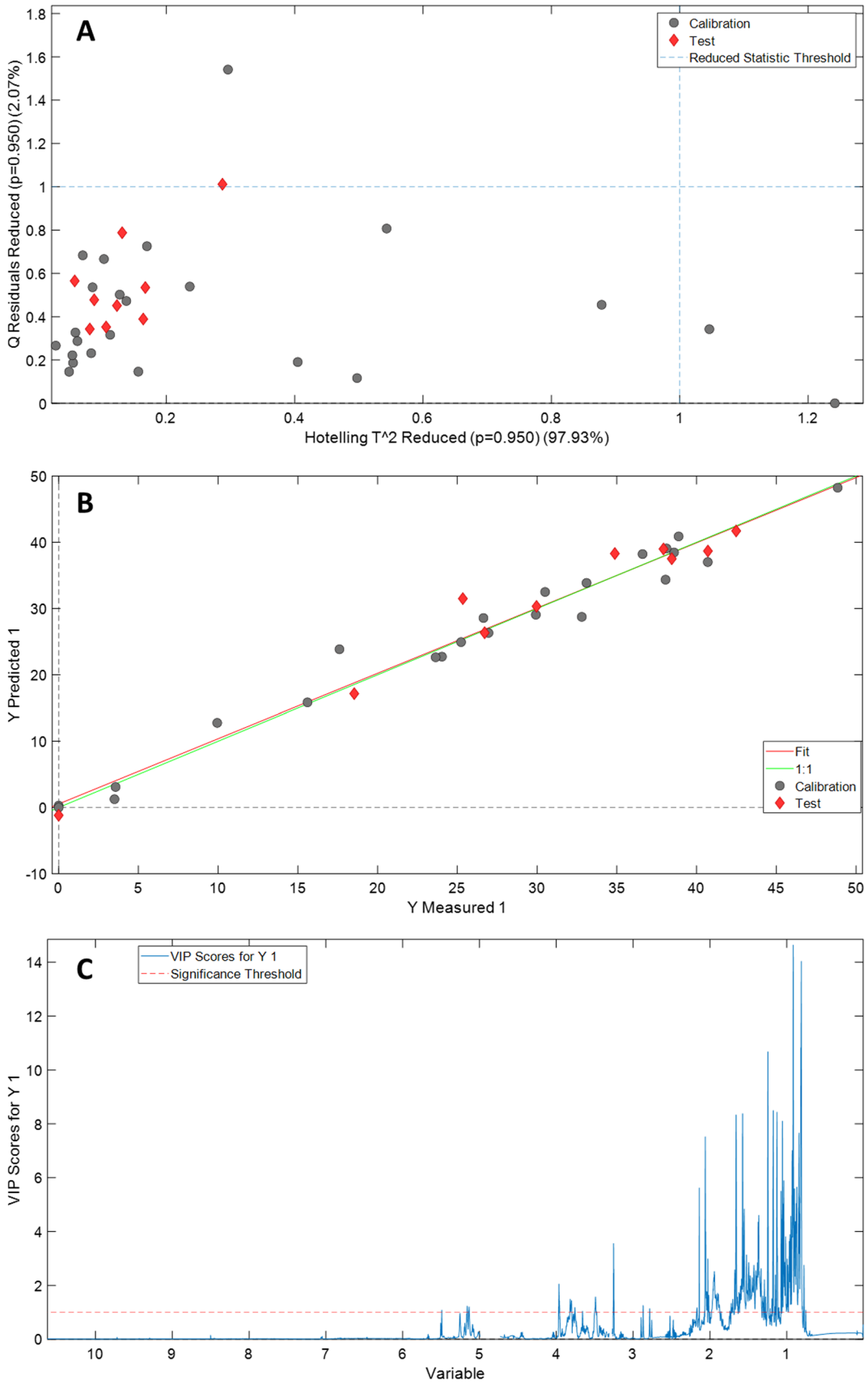



**Fig. 5** Hotelling $T^2$ and Q residual control chart (**A**), regression (**B**), and variable's importance in projection (**C**) of the partial least-squares regression model

quantification of total BA content was not considered and the construction of calibration curves was necessary [35, 36].

## Conclusions

The $^{13}$C-qNMR method demonstrated high precision and accuracy but long acquisition times which impair the applicability of the method for the quality control of *B. serrata* extracts. The $^{1}$H-NMR/PLS-R model could estimate the total content of BAs for the rapid quality control of the extracts. The predictive performances can be certainly ameliorated by increasing the number of extracts to be included in the model construction. The results underlined the urgency to impose stricter quality controls on the content of bioactive compounds within food supplements or medicated feeds, to assure the safety of consumers and the reproducibility of the beneficial effects. The developed methods might also be applied to complex mixtures or food supplements containing compounds other than BAs without the necessity of selective extractions or extract purification.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11694-023-02310-y.

**Acknowledgements** The authors want to express their thanks to the C.I.G.S. staff (Centro Interdipartimentale Grandi Strumenti, Modena, Italy) for the precious assistance during the experimental work and to Fondazione Cassa di Risparmio di Modena for the purchase of Bruker FT-NMR Avance III HD 600 MHz spectrometer.

**Author contributions** Conceptualization: ET, DB; Methodology: MCR, ET; Formal analysis and investigation: DVP, ET; Writing—original draft preparation: ET; Writing—review and editing: SB, DB; Funding acquisition: DB; Resources: SB, DB; Supervision: DB.

**Data availability** The data presented in this study are available privacy on request from the last author.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

## References

1. T. Efferth, F. Oesch, Semin. Cancer Biol.. Cancer Biol. **80**, 39 (2022)
2. S. Sharma, V. Thawani, L. Hingorani, M. Shrivastava, V.R. Bhate, R. Khiyani, Phytomedicine **11**, 255 (2004)
3. K. Huang, Y. Chen, K. Liang, X. Xu, J. Jiang, M. Liu, F. Zhou, Evidence-Based Complement. Altern. Med. **2022**, 1 (2022)
4. Maximize Market Research, (2022).
5. V. Rajpal, *Standardization of Botanicals*, 2nd edn. (Business Horizons, New Delhi, 2011)
6. N. Sharma, V. Bhardwaj, S. Singh, S.A. Ali, D.K. Gupta, S. Paul, N.K. Satti, S. Chandra, M.K. Verma, Chem. Cent. J. **10**, 49 (2016)
7. V. Bampidis, G. Azimonti, L. de Bastos, H. Christensen, M. FašmonDurjava, M. Kouba, M. López-Alonso, S. López Puente, F. Marcon, B. Mayo, A. Pechová, M. Petkova, F. Ramos, Y. Sanz, R. Edoardovilla, R. Woutersen, P. Brantom, A. Chesson, J. Westendorf, P. Manini, F. Pizzo, B. Dusemund, EFSA J. (2022). https://doi.org/10.2903/j.efsa.2021.6615
8. K. Belsner, B. Büchele, U. Werz, T. Syrovets, T. Simmet, Magn. Reson. Chem.. Reson. Chem. **41**, 115 (2003)
9. J. Meins, C. Artaria, A. Riva, P. Morazzoni, M. Schubert-Zsilavecz, M. Abdel-Tawab, Planta Med. Med. **82**, 573 (2016)
10. M. Paul, G. Brüning, J. Bergmann, J. Jauch, Phytochem. Anal.. Anal. **23**, 184 (2012)
11. S.A. Shah, I.S. Rathod, B.N. Suhagia, S.S. Pandya, V.K. Parmar, J. Chromatogr. Sci.Chromatogr. Sci. **46**, 735 (2008)
12. S. Mukadam, C. Ghule, A. Girme, V.M. Shinde, L. Hingorani, K.R. Mahadik, J. Chromatogr. Sci.. Chromatogr. Sci. (2023). https://doi.org/10.1093/chromsci/bmad012
13. K. Gerbeth, J. Meins, S. Kirste, F. Momm, M. Schubert-Zsilavecz, M. Abdel-Tawab, J. Pharm. Biomed. Anal. **56**, 998 (2011)
14. M. Zwerger, M. Ganzera, J. Pharm. Biomed. Anal. **201**, 114106 (2021)
15. K. Katragunta, B. Siva, N. Kondepudi, P.R.R. Vadaparthi, N. Rama Rao, A.K. Tiwari, K. Suresh Babu, J. Pharm. Anal. **9**, 414 (2019)
16. A. Asteggiano, L. Curatolo, V. Schiavo, A. Occhipinti, C. Medana, Appl. Sci. **13**, 1254 (2023)
17. R. Sacchi, L. Paolillo, *Advances in food diagnostics* (Blackwell Publishing, Ames, 2007), pp.101–117
18. A.P. Minoja, C. Napoli, Food Res. Int. **63**, 126 (2014)
19. E. Truzzi, L. Marchetti, D.V. Piazza, D. Bertelli, Foods **12**, 1467 (2023)
20. E. Truzzi, L. Marchetti, S. Benvenuti, V. Righi, M.C. Rossi, V. Gallo, D. Bertelli, Molecules **26**, 5439 (2021)
21. C. Simmler, J.G. Napolitano, J.B. McAlpine, S.-N. Chen, G.F. Pauli, Curr. Opin. Biotechnol.. Opin. Biotechnol. **25**, 51 (2014)
22. Z.-F. Wang, Y.-L. You, F.-F. Li, W.-R. Kong, S.-Q. Wang, Molecules **26**, 6308 (2021)
23. L. Marchetti, F. Pellati, S. Benvenuti, D. Bertelli, Molecules **24**, 2592 (2019)
24. E. Truzzi, L. Marchetti, A. Fratagnoli, M.C. Rossi, D. Bertelli, Food Chem. **404**, 134522 (2023)
25. E. Truzzi, L. Marchetti, S. Benvenuti, A. Ferroni, M.C. Rossi, D. Bertelli, J. Agric. Food Chem. **69**, 8276 (2021)
26. G. Papotti, D. Bertelli, R. Graziosi, M. Silvestri, L. Bertacchini, C. Durante, M. Plessi, J. Agric. Food Chem. **61**, 1741 (2013)
27. Inc. Eigenvector Research, Eigenvector Research Wiki (n.d.).
28. M. Ota, P.J. Houghton, Nat. Prod. Commun.Commun. **3**, 21 (2008)
29 R. Watanabe, C. Sugai, T. Yamazaki, R. Matsushima, H. Uchida, M. Matsumiya, A. Takatsu, T. Suzuki, Toxins (Basel) (2016). https://doi.org/10.3390/toxins8100294
30. ICH Expert Working Group, ICH HARMONISED TRIPARTITE GUIDELINE 1 (2005).
31. G. Mannino, A. Occhipinti, M. Maffei, Molecules **21**, 1329 (2016)
32. J. Meins, D. Behnam, M. Abdel-Tawab, NFS Journal **11**, 12 (2018)
33. K. Reising, J. Meins, B. Bastian, G. Eckert, W.E. Mueller, M. Schubert-Zsilavecz, M. Abdel-Tawab, Anal. Chem. **77**, 6640 (2005)
34 K.R. Vijayarani, M. Govindarajulu, S. Ramesh, M. Alturki, M. Majrashi, A. Fujihashi, M. Almaghrabi, N. Kirubakaran, J. Ren, R.J. Babu, F. Smith, T. Moore, M. Dhanasekaran, Front. Pharmacol.Pharmacol (2020). https://doi.org/10.3389/fphar.2020.551911
35. A. Al-Harrasi, N.U. Rehman, F. Mabood, M. Albroumi, L. Ali, J. Hussain, H. Hussain, R. Csuk, A.L. Khan, T. Alam, S. Alameri, Spectrochim Acta A Mol Biomol Spectrosc **184**, 277 (2017)

36. N.U. Rehman, L. Ali, A. Al-Harrasi, F. Mabood, M. Al-Broumi, A.L. Khan, H. Hussain, J. Hussain, R. Csuk, Phytochem. Anal.. Anal. **29**, 137 (2018)



## Authors and Affiliations

**Eleonora Truzzi[1] · Danny Vincenzo Piazza[2] · Maria Cecilia Rossi[3] · Stefania Benvenuti[2] · Davide Bertelli[2]**

✉ Eleonora Truzzi
eleonora.truzzi@unimore.it

Danny Vincenzo Piazza
240674@studenti.unimore.it

Maria Cecilia Rossi
mariacecilia.rossi@unimore.it

Stefania Benvenuti
stefania.benvenuti@unimore.it

Davide Bertelli
davide.bertelli@unimore.it

[1] Department of Chemical and Geological Sciences, University of Modena and Reggio Emilia, via G. Campi 103, 41121 Modena, Italy

[2] Department of Life Sciences, University of Modena and Reggio Emilia, via G. Campi 103, 41125 Modena, Italy

[3] Centro Interdipartimentale Grandi Strumenti, University of Modena and Reggio Emilia, via G. Campi 213/A, 41125 Modena, Italy