Exploiting the versatility of vacuum-assisted headspace solid-phase microextraction in combination with the selectivity of ionic liquid-based GC stationary phases to discriminate Boswellia spp. resins through their volatile and semivolatile fractions

Exploiting the versatility of vacuum assisted headspace solid-phase microextraction in combination with the selectivity of ionic liquids-based GC stationary phases to discriminate *Boswellia* ssp. resins through their volatile and semi-volatile fractions

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Running title: Vac-HS-SPME and IL-GCMS for frankincense characterization

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Keywords

Boswellia spp resins, fast GC, ionic liquid-based stationary phase, vacuum assisted headspace solid phase microextraction

List of abbreviations and acronyms

HS-SPME: headspace solid phase microextraction, Reg-HS-SPME: HS-SPME under regular conditions, Vac-HS-SPME: HS-SPME under reduced pressure, IL: ionic liquid

Abstract

The frankincense resins, secreted from *Boswellia* species, are an uncommon example of a natural raw material where every class of terpenoids is present in similar proportions. Diterpenoids (serratol, incensole, and incensole acetate) are used to discriminate samples from different species and origins. Headspace solid phase microextraction (HS-SPME) has been used for frankincense analysis although requires long sampling time for medium-to-low-volatility markers; HS-SPME under vacuum (Vac-HS-SPME) can overcome this limit. GC is used for analysis but the separation of incensole and serratol needs polar stationary phases. This study develops a method to discriminate frankincenses based on Vac-HS-SPME combined with Fast GC-MS with ionic liquid-based stationary phases. Optimized Vac-HS-SPME parameters for solid samples included a temperature during air-evacuation that must be below 0°C, a sampling time of 15 minutes for pre-equilibrium and 15 minutes of extraction, and a sample amount above 100mg to obtain significant recoveries of all classes of markers. Fast GC provides the baseline separation of all markers in 20 minutes. The total analysis time thus drops to about 50 minutes instead of 120 (60 minutes of sampling plus 60 minutes of analysis) reported in the literature. The method was successfully applied to commercial frankincense samples.

1. Introduction

The burning of incense is probably the oldest perfuming method in existence, and frankincense gum oleoresins (also known as olibanum) are a central ingredient in many incense mixtures. Frankincense resins are secreted from trees of the genus *Boswellia*, and typically appear as pea-to-thumb-size grains with a translucent, whitish-yellow to dark brown color. About 30 species of *Boswellia* are known and many of them are used to produce frankincense resins. The main commercial sources are: *Boswellia sacra* Flueck. (Arabian Frankincense), which is the top quality product and native to Oman and Somalia; *Boswellia serrata* Roxb. ex Colebr. (Indian Frankincense) from India and widely used in Ayurvedic, Hindu and Buddhist medicines; *Boswellia papyrifera* (Caill. ex Delile) Hochst., which grows in coastal regions of Sudan, Eritrea, Ethiopia and northern Somalia; and *Boswellia frereana* Birdw., which is typically found in Somalia, but is of lower commercial interest [1]. Besides the most commercially relevant plants, other rarer species are available, such as the endemic *Boswellia* species from the island of Socotra, *B. ameero* Balf. f., *B. dioscoridis* Thulin, *B. elongata* Balf. f. and *B. socotrana* Balf. f. [2].

Frankincense resin is an uncommon example of a natural raw material in which each terpenic class (mono-, sesqui-, di-, and triterpenoids) is present in rather similar proportions. Boswellic acids, which are pentacyclic triterpenic acids, are non-volatile markers and have been shown to possess anti-inflammatory properties, while the essential oil from frankincense resins is a common raw material in perfumery. The market value of frankincense is strongly influenced by differences in composition, and approximately varies from 5 US$ kg^{-1} for *B. serrata* to 150 US$ kg^{-1} for *B. sacra* [3].

The volatile and semi-volatile fractions of *Boswellia* species are rather complex and differ notably in composition and odor. In general, the resins mainly contain mono-, sesqui- and diterpenoids, with *B. papyrifera*, in particular, being characterized by n-octyl acetate, n-octanol and the significant presence of diterpenoids. These diterpenoids are cembrane derivatives; in particular serratol (also known as cembrenol), incensole and incensole acetate (see Figure 1). They are present in most *Boswellia* resins although in different amounts and proportions [2-6]. They can therefore be used to discriminate between samples of different species and origins, and act as a complement to the fast screening method, which is based on a quality evaluation of odor and a visual assessment of color, that is commonly used.

The characterization of the frankincense volatile fraction is generally carried out via the direct use of GC-MS on the essential oils that are obtained from the steam- or hydro-distillation of the resins. This, however, is not a convenient approach to fast screening as it is time consuming and requires considerable sample amounts.

Solid phase microextraction (SPME), and in particular when combined with headspace (HS)-SPME, can well fit to this aim. HS-SPME is a solvent-free high-concentration-capacity sampling technique [7] where target analytes are transferred from the matrix to a polymeric fibre coating via two consecutive steps (matrix/headspace and headspace/fibre) and recovery is maximized when the equilibrium of the full process is reached. The time needed to reach equilibrium depends on several factors, such as the properties of the analytes, matrix and fibre coating. Although conventional HS-SPME was already applied to characterize frankincense resins [6, 8], it shows obvious limits to achieve equilibrium with semi-volatiles and implies long extraction times and high extraction temperatures. These limits can be overcome by vacuum assisted HS-SPME (Vac-HS-SPME), a technique where a low sampling pressure is applied during HS-SPME sampling. Vacuum facilitates the volatilisation of semi-volatiles by reducing the resistance found the thin gas-film layer adjacent to the sample-headspace interface. Analytes are transferred to the headspace faster and the time needed to reach equilibrium is reduced. Sampling under vacuum greatly improves the extraction kinetics (speed) of lower volatility compounds resulting in high extraction efficiency and sensitivity over time [9, 10].

The complexity of the volatile fraction of frankincense resins means that it is mandatory that a GC stationary phase (SP) with the appropriate selectivity to separate all olibanum components, in particular the characterizing markers, is chosen for use. Most studies involve GC analyses carried out with apolar columns because of their efficiency, stability at high temperature and high amount of available data (i.e. retention indices) for analyte identification. Unfortunately, apolar SPs do not separate two of the discriminating diterpenic markers (incensole and serratol), which often leads to erroneous identification (i.e. the coelution peak has also been hypothesized to be isoincensole) [5]. Polar columns are therefore mandatory for the correct characterization of the resins. In this respect, the introduction of ionic liquids (ILs) for use as SPs has opened up new possibilities in the separation of critical pairs of compounds in natural matrices [11-15]. These SPs show peculiar selectivity and a comparable, or higher, polarity than conventional polydimethylsiloxane- and polyethyleneglycol-(PEG) based columns, and, at the same time, provide similar, or even higher, maximum allowable operating temperatures and lower bleeding than most PEG-based SPs.

This article describes the development of a simple and fast method to sample volatiles and semi-volatiles markers characteristic of *Boswellia* ssp. resins. The proposed method uses Vac-HS-SPME combined with Fast GC-MS with narrow bore columns coated with IL SPs able to discriminate between frankincenses and applicable to quality control that aims [16-19].

2. MATERIALS AND METHODS

2.1 Chemicals and Samples

Incensole, incensole acetate and serratol (cembrenol) were all provided by Prof. G. Appendino (Università del Piemonte Orientale, Novara, Italy).

Standard solutions of incensole, incensole acetate and serratol were prepared in cyclohexane at a concentration of 100 mg L^{-1} and stored at 4°C.

Two authentic *Boswellia* spp. resin samples (one *Boswellia socotrana* Balf. f. and one *Boswellia papyrifera* (Caill. ex Delile) Hochst) were provided by Prof. G. Appendino (Università del Piemonte Orientale, Novara, Italy), while a further three commercial frankincense samples, labeled as *B. sacra*, were bought from a local herbalist's shop and were called frankincense 1, 2 and 3. Each frankincense sample was first frozen using liquid nitrogen, then pulverized with a mortar and pestle and finally stored at -18°C.

The essential oils of the *B. socotrana* and *B. papyrifera* samples, obtained via hydrodistillation according to the European Pharmacopoeia procedure [20], were also analyzed to optimize the chromatographic method.

2.2 Vacuum-assisted HS-SPME and regular HS-SPME

The experimental set-up adopted to perform vacuum assisted (Vac-)HS-SPME experiments consisted of a commercial headspace 20 mL vial hermetically sealed with a joint stainless-steel cap containing a hole that could tightly accommodate a Thermogreen® LB-1 septum with half-hole (6 mm diameter x 9 mm length; Merck-Sigma Aldrich). The stainless-steel gastight cap was provided by Professor Eleftheria Psillakis [21]. The air-evacuation step and the HS-SPME sampling were carried out using Thermogreen® LB-1 septa. The solid sample was placed inside the vial, the vial was then stored at -18°C for one hour and finally air-evacuated. The air-evacuation step was carried out using a 22 gauge hypodermic needle sealed to a 5 mL syringe that was tightly secured to the tubing of a N 820.3 FT.18 (7 mbar ultimate vacuum) pumping unit manufactured by KNF Lab (Milan, Italy). The needle was then inserted through the septum and the vial was air-evacuated. Two air-evacuation times (45 and 120 seconds respectively) were tested. After air-evacuation the vial was directly transferred to the autosampler. GC desorption lasted 10 minutes to avoid carry-over. To remove the cap, atmospheric pressure was restored inside the vial by piercing the septum with a disposable syringe needle.

Regular (Reg-)HS-SPME experiments were performed with the previously described experimental set-up, while omitting the air-evacuation step.

Divinyl benzene/carboxen/polydimethylsiloxane fibers (DVB/ CAR/PDMS l: 2 cm long, d_f: 50/30 mm) were used for both Vac-HS-SPME and Reg-HS-SPME. The fibers were purchased from Supelco Co. (Bellafonte, PA, USA) and conditioned before use as recommended by the manufacturer.

Three different sample amounts, 5 mg, 40 mg and 100 mg, and two sampling temperatures, 50°C and 80°C, were tested. Sampling-time profiles were obtained by sampling 100mg of the investigated matrices at 80°C for 5, 15, 30 and 60 minutes. All extractions were run in triplicate. The HS-SPME parameters were optimized using the frankincense resin from *B. socotrana*. All other frankincense resins were sampled by adopting the optimized conditions (100 mg of matrix extracted for 15 minutes at 80°C).

2.3 Instrumental set-up

Analyses were carried out using a MPS-2 multipurpose sampler (Gerstel, Mülheim a/d Ruhr, Germany) installed on a Shimadzu GC-FID-MS system consisting of a Shimadzu GC 2010, equipped with FID, in parallel with a Shimadzu QP2010-PLUS GC–MS system; data were processed and elaborated using Shimadzu GCMS Solution 2.51 and GC Solution 2.53SU software (Shimadzu, Milan, Italy).

2.3.1 Columns

GC analyses were carried out using two 30 m × 0.25 mm d_c, 0.25 µm d_f conventional columns coated with 95% methyl-polysiloxane 5%-phenyl (SE-52) and autobondable nitroterephthalic-acid-modified polyethylene glycol (FFAP-EXT) from Mega (Legano, Mi, Italy). A conventional IL-based SLB-IL60i (30 m × 0.25 mm d_c, 0.25 µm d_f) column, and a narrow bore SLB-IL60 (15 m × 0.10 mm d_c, 0.08 µm d_f) column from Supelco (Bellefonte, PA, USA) were also used.

2.3.2 GC-FID-MS conditions

Analyses were carried out under the following conditions: temperatures - injector, 250°C, transfer line, 270°C, ion source, 200°C; carrier gas - He; flow control mode - constant linear velocity; flow rate - 1 ml min^{-1}; injection mode - split; split ratio - 1:20. The MS was operated in electron ionization mode (EI) at 70 eV, scan rate: 666 u/s, mass range: 35–350 m/z. FID temperature, 250°C; sampling rate, 40 ms. Temperature programs: i) 50°C//5°C min^{-1}//250°C (5 min) for conventional SE-52, FFAP-EXT, SLB-IL60i columns; ii) 40°C//10°C min^{-1}//180°C//15°C min^{-1}//230°C (2 min) for the narrow bore SLB-IL60 column. The GC system was alternatively operated with MS or FID as detectors. Identification was performed via comparisons of linear retention indices and mass spectra

either with those of authentic standards, or with data stored in commercial and in-house libraries and the results were confirmed using those of previous publications [2, 4, 6].

2.4 Data elaboration

All elaboration was carried out using Excel (Microsoft) with the exception of the heat map, which was created using Morpheus software (https://software.broadinstitute.org/morpheus).

3. RESULTS AND DISCUSSION

The development of a fast semiautomatic method entails the investigation of each analytical step. In this study, two *Boswellia* essential oils (*B. socotrana* and *B. papyrifera*) were analyzed first on different stationary phases (SPs) in order to find a column that could separate most frankincense components, and, in particular, the diterpenoid markers. The second step dealt with the careful optimization of sampling conditions (sample amount, sampling time and temperature) using *B. socotrana* as the model sample. Vac-HS-SPME and regular HS-SPME (Reg-HS-SPME) were tested in this step. Finally, the chromatographic method was sped-up to fast-GC by translating the analytical conditions from conventional to narrow bore columns. The developed method was then validated on a series of commercial frankincense samples.

3.1 Choice of the GC stationary phase

The choice of a SP that separates as many components as possible is, of course, a crucial step for the effective characterization of a sample. The diterpenoid markers incensole, incensole acetate and serratol, and the essential oils of *B. socotrana* and *B. papyrifera* were analyzed with a range of commercially available columns to define the most appropriate SP for their analysis.

As has already been mentioned, apolar columns are not appropriate for the analyses of frankincense resins because of the coelution of incensole and serratol (Figure 2a). Two polar columns were therefore tested, the first was a polyethylene-glycol-based SP (FFAP-EXT) and the second was an IL-based SP (SLB-IL60i). Figures 2b and 2c show that both columns separate diterpenoids with a good resolution but the SLB-IL60i was chosen because of its higher resolution (22 with SLB-IL60i *vs*. 14 with the FFAP-EXT column) and low bleeding. This column not only separates the diterpenic markers but also the other characterizing components. Figures 3a and 3b report the GC-MS profiles, using the SLB-IL60i column, of the *B. socotrana* and *B. papyrifera* frankincense resins, as sampled by Vac-HS-SPME (see paragraph 3.2). The SLB-IL60i column was therefore used in this study. Table S1 reports the list of the compounds identified, their retention times in the conventional and narrow-

198 bore IL-based columns, together with their molecular formula, molecular weight and main
199 physicochemical properties (LogP, boiling point and vapor pressure).

200 **3.2 Sample preparation - Optimization of the Vac-HS-SPME method**

201 The suitable characterization of frankincense resins by HS-SPME entails the optimization of the
202 sampling conditions to ensure that all classes of volatiles and semi-volatiles are recovered
203 (mono/sesqui/diterpenoidic compounds) with extraction times that are compatible with those of the
204 chromatographic run. In this section, the performance of the Reg-HS-SPME sampling of a *B.*
205 *socotrana* resin is compared to that of Vac-HS-SPME. Preliminary experiments showed that the 2
206 cm long DVB/CAR/PDMS fiber provided a complete picture of frankincense composition, and it was
207 therefore chosen for use in the following tests. The next experiments aimed to optimize the conditions
208 for the pre-equilibration of the frankincense with the headspace for the subsequent Reg- and Vac-HS-
209 SPME samplings. This step is critical because it influences the repeatability of the results, in particular
210 with Vac-HS-SPME. An equilibration time of 15 minutes was chosen as it was the minimum time for
211 which repeatability gave an RSD% of below 15% (except for compounds in traces) over five
212 experiments under any applied conditions (data not reported).

213 The sample amount was first optimized by sampling 5, 40 and 100 mg of frankincense for 15 min
214 with both Reg- and Vac-HS-SPME at a sampling temperature of 80°C [6, 8]. With Reg-HS-SPME,
215 the abundance of the high volatility analytes (mono and sesquiterpenoids) increased with sample
216 amount, while that of the diterpenoids was always very low and did not seem to be significantly
217 affected. The results with 5 mg of sample show that: i) for monoterpenoids, the performance of regular
218 sampling is about 50% higher than that of Vac; ii) for sesquiterpenoids, the difference is lower, but
219 Reg sampling is still more effective than Vac; while iii) for diterpenoids, the peak areas with Vac
220 sampling are double than those of Reg. Table 1 reports the relative analyte abundances obtained by
221 sampling with Vac-HS-SPME *vs*. Reg-HS-SPME. The poorer performance of Vac-HS-SPME
222 compared to Reg-HS-SPME with the most volatile components is due to the air evacuation step in
223 which they are significantly aspired with air. This loss is not observed for the less volatile compounds
224 for which the reduced pressure of Vac-HS-SPME promoted vaporization to the headspace, although
225 with longer extraction times (i.e. closer to the equilibrium), the two techniques provide similar results
226 (data not reported).

227 Since suitable enrichment, in particular for the diterpenoids, cannot be achieved with 5 mg of resin,
228 the sample amount was increased to 40mg. The results show that some monoterpenoids are still lost
229 during the air evacuation step with Vac-HS-SPME, but that the medium volatility compounds show
230 a good improvement (Table 1).

Finally, 100mg of sample was evaluated and the results show: i) Reg-HS-SPME and Vac-HS-SPME have comparable responses for the high volatility components; ii) slightly improved recovery of medium volatility components (about 1.5 higher); and iii) drastic improvements in diterpenoids, with peak areas being almost four times higher than Reg-HS-SPME. Further experiments were carried out to exclude any discriminative loss from the headspace over time; air evacuation times of 45 and 120 sec were applied, resulting in perfectly overlapping patterns even for the most volatile compounds. The results show that the slight peak-area differences between Reg-HS-SPME and Vac-HS-SPME are probably related to competition with the adsorbent [10]. The sample amount was then fixed at 100mg.

The sampling time was then optimized by checking the behavior of the frankincense markers when processed with the two investigated techniques with 5, 15, 30 and 60 minutes of sampling. The results are summarized in Figure 4. α-Pinene and limonene (monoterpene hydrocarbons) were taken as a reference for the most volatile frankincense components; these compounds reached equilibrium with both Reg-HS-SPME and Vac-HS-SPME, but the longer extraction times necessary with Reg sampling produce a decrease in the extraction efficiency, which is probably related to adsorption competition. Similar behavior can also be observed for intermediate volatile compounds (*trans*-β-caryophillene, α-humulene and β-selinene), although the extraction performance of Vac-HS-SPME was slightly better than that of Reg-HS-SPME. Finally, the extraction-time profiles of the diterpenoid markers (serratol, incensole and cembrene A) show that sampling under reduced pressure permits recovery to be sped-up even with short extraction times (15 min), although equilibrium could not be reached, even over 60 minutes, by either technique. There were extreme differences in the extraction times needed to reach equilibrium in the different classes of terpenoids with Reg-HS-SPME, and these are in agreement with literature data [22]. The use of Vac-HS-SPME for 15 min was then chosen for the following experiments as it provides the suitable recovery of all compound groups in a short sampling time.

The possibility of decreasing the extraction temperature to 50°C was also explored, but a significant decrease in the abundance of diterpenoids was observed (see Figure S1), meaning that higher extraction temperatures were mandatory.

3.3 Speed-up of the analysis step

The third part of the study was focused on speeding-up the GC analysis to make it compatible with the sampling time. The above chromatographic method was translated to a 15m × 0.10 mm d_c, 0.08 µm d_f column using the method translation approach [16, 17, 19]. The analysis time was thus reduced to 19 minutes, while the separation of all the markers was maintained. Figure 5 reports the translated

GC-FID patterns, with the narrow-bore SLB-IL60 column, of the diterpenic markers and the *B. socotrana* and *B. papyrifera* resins.

3.4 Application of the optimized method to real-world frankincense samples

The two authentic and the three commercial frankincense samples were analyzed with the optimized Vac-HS-SPME-fast-GC-FID-MS method, and the resulting patterns were compared to those obtained with Reg-HS-SPME. The optimal conditions adopted with Vac-HS-SPME were 100mg of frankincense resin, sampled at 80°C for 15 min combined with 15 min of pre-equilibrium (total sampling time 30 min). Tables S2 and S3 reports the mean peak area of each analyte in each sample together with the repeatability (%RSD). The results show that Vac-HS-SPME is more repeatable than Reg-HS-SPME reaching %RSD in general below 10% with the exception of traces. Due to the high number of analytes, the results were summarized in a heat map (Figure 6) where the areas of each analyte (row) is scaled in function of their abundance in each sample from minimum (blue) to maximum (red). The samples were also hierarchically clustered by applying the one minus Pearson correlation. The results of the heat map show that: i) the commercial frankincense 3 sample labelled as *B. sacra*, was actually *B. papyrifera* as proved not only by the high abundance of markers such as octyl acetate, octanol, incensole and incensole acetate, but also by its clustering with the authentic *B. papyrifera*, ii) Vac-HS-SPME when compared to Reg-HS-SPME provides a drastic increase of recovery of both hydrocarbons and oxygenated diterpenoids and most oxygenated sesquiterpenoids as it is clear, for instance, from the difference in intensity of a) incensole, incensole acetate, cembrene, cembrene A and the other diterpene hydrocarbons in *B. papyrifera* samples or b) caryophillene oxide, α and β-eudesmol and serratol in *B. socotrana* and in the commercial sample 2.

4. CONCLUDING REMARKS

A fast and versatile method has been optimized to discriminate *Boswellia* spp. resins using their volatile and semi-volatile fractions.

The method is based on HS-SPME under reduced pressure (Vac-HS-SPME), which increases the release of semi-volatile compounds (i.e. diterpenoidic compounds) in the headspace and enables their sampling in 30 minutes. Vac-HS-SPME was successfully combined with fast GC, with columns coated with an IL-based SP, providing the baseline separation of all markers in 20 minutes.

The latest geometry of Vac-HS-SPME sampling vials makes it possible to include it in robotic autosamplers, i.e. to comprise it in inclusive platforms, the so-called “Total Analysis System” (TAS),

namely systems where the three main steps of an analytical procedure (sample preparation-analysis-data elaboration processing) are on-line merged in a single step [23]. With an integrated platform, after a preliminary air evacuation, it is possible to overlap sampling and analysis steps enabling to analyze up to five samples of *Boswellia* resins in two hours that is the analysis time commonly required for a single sample with conventional methods (60 min sampling and 60 min analysis). The reported method is simple, fast, automated and compatible with the processing of a large number of samples, as is required in a routine quality control laboratory.

This study shows, for the first time, that Vac-HS-SPME can successfully be applied to the analysis of semi-volatiles in solid samples in the plant and natural products fields by applying a low temperature (below 0°C) during the air-evacuation step, and a suitable sample amount (above 100 mg) for a suitable recovery of the most volatile analytes. Further studies are under way to optimize the method for quantitation.

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Figure 1 – Chemical structures of the main markers of the frankincense resins.

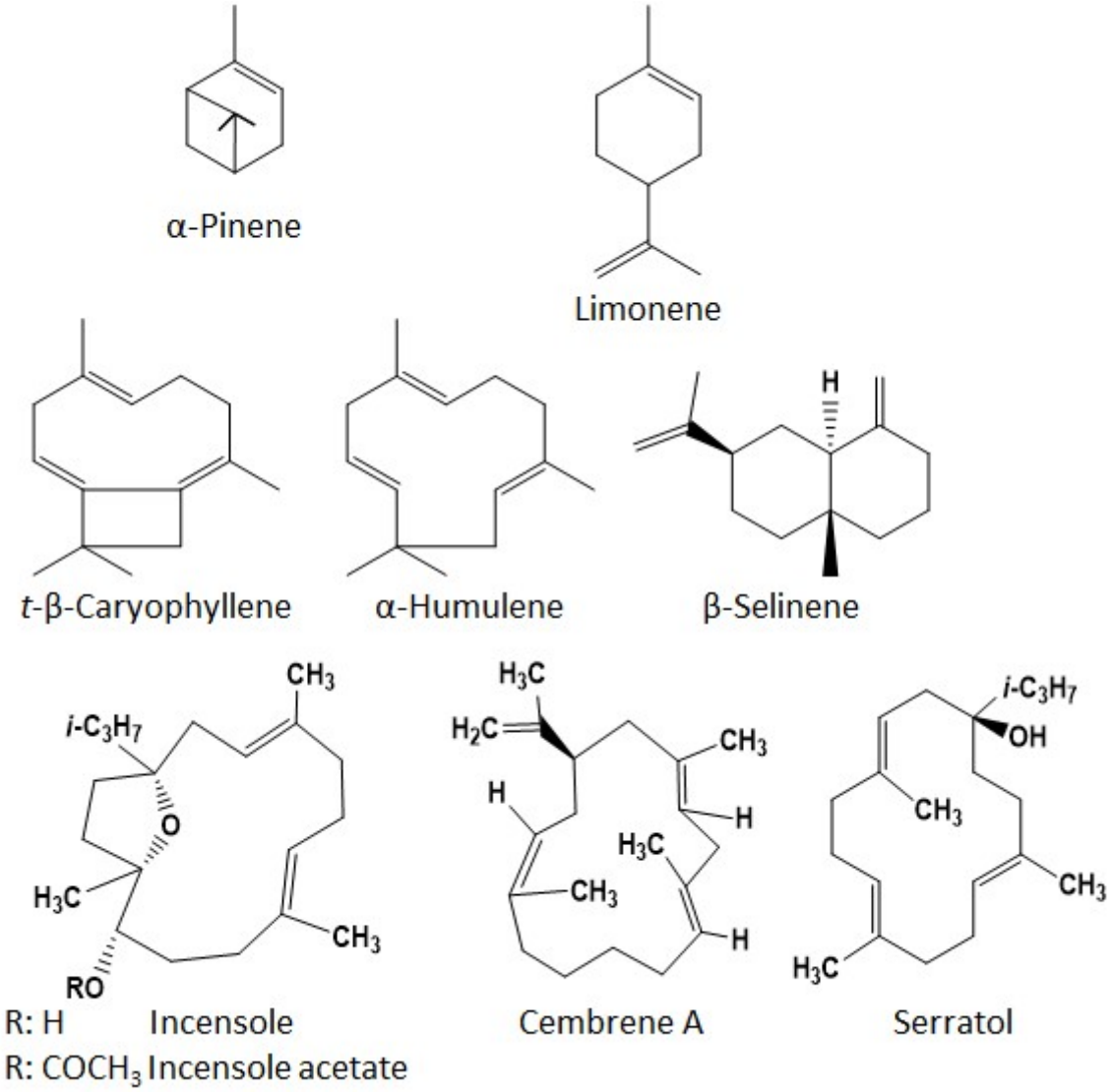


Figure 2 – GC-MS profiles of incensole (pink), serratol (blue) and incensole acetate (black) on the conventional SE-52 (a), FFAP-EXT (b) and SLB-IL60i (c) columns. Analysis conditions: see experimental section.

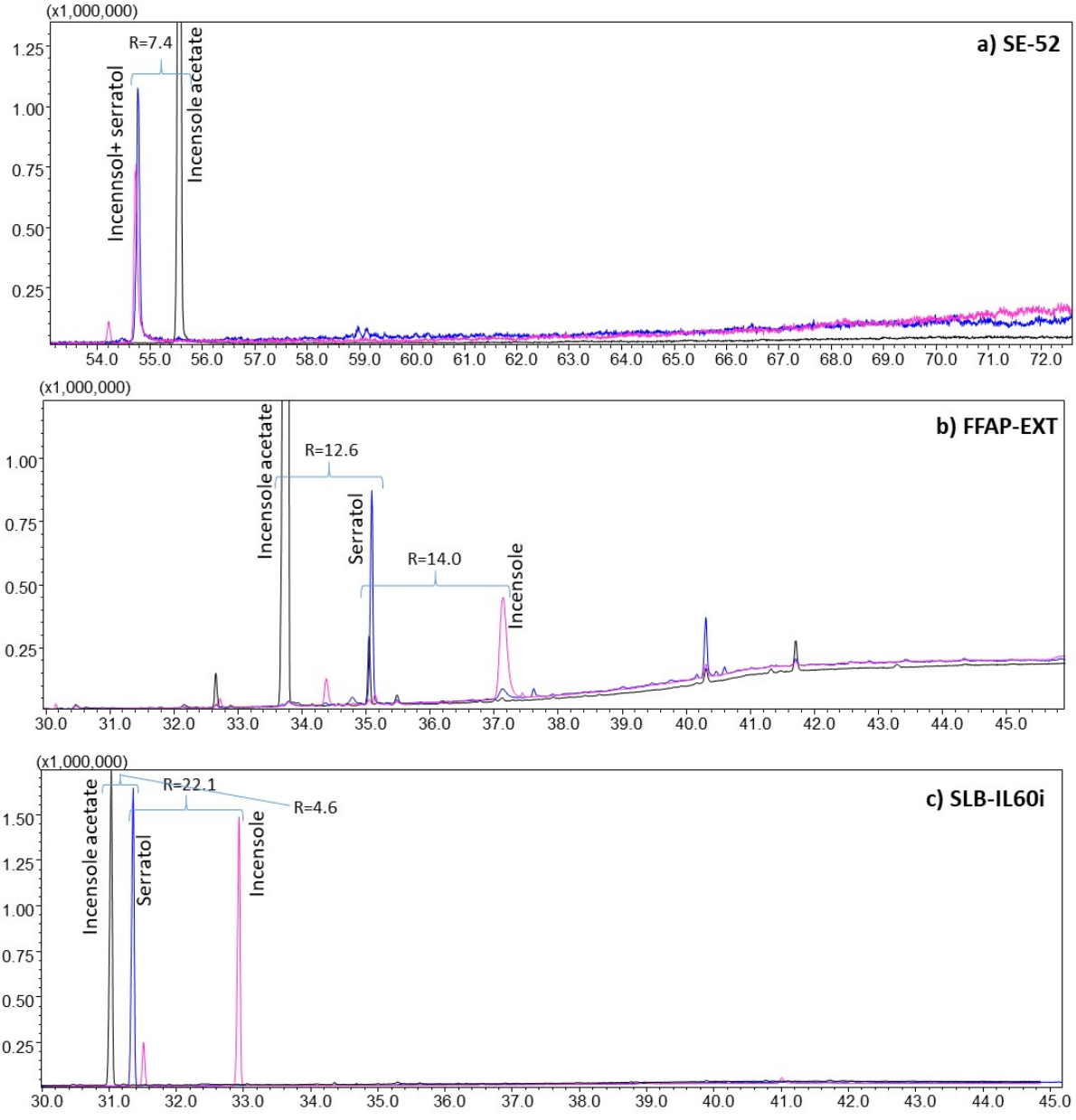


Figure 3 – Vac-HS-SPME GC-MS profiles of *B. socotrana* (black) and *B. papyrifera* (pink) obtained with the SLB-IL60i column. Analysis conditions: see experimental section. Legend: 1. α-Thujene, 2. α-Pinene, 3. Camphene, 4. β-Pinene, 5. Sabinene, 6. β-Myrcene, 7. α-Phellandrene, 8. Limonene, 9. β-Phellandrene, 10. 1,8 - Cineole, 11. *trans*-β-Ocimene, 12. *para*-Cymene, 13. *o*-Methylanisole, 14. α–Cubebene, 15. *cis*-Sabinene hydrate, 16. *p*-Cymenene, 17. α-Terpinolene, 18. α-Copaene, 19. β-Bourbonene, 20. Octanol, 21. β-Elemene, 22. *trans*–Pinocarveol, 23. *cis*-Verbenol, 24. Octyl acetate, 25. 1,8-Menthadien-4-ol, 26. *trans*-Verbenol, 27. *trans*-β-Caryophyllene, 28. Aromadendrene, 29. γ-Selinene, 30. Germacrene D, 31. α-Fenchol, 32. α-Humulene, 33. α-Selinene, 34. β-Selinene, 35. Myrtenol, 36. *trans*-Carveol, 37. *cis*-Carveol, 38. Carvone, 39. Verbenone, 40. Cembrene, 41. Limonene-1,2-diol, 42. Caryophyllene oxide, 43. Hydrocarbon diterpene 1, 44. α-Eudesmol, 45. Hydrocarbon diterpene 2, 46. β-Eudesmol, 47. Hydrocarbon diterpene 3, 48. Incensole acetate, 49. Serratol, 50. Cembrene A, 51. Incensole.

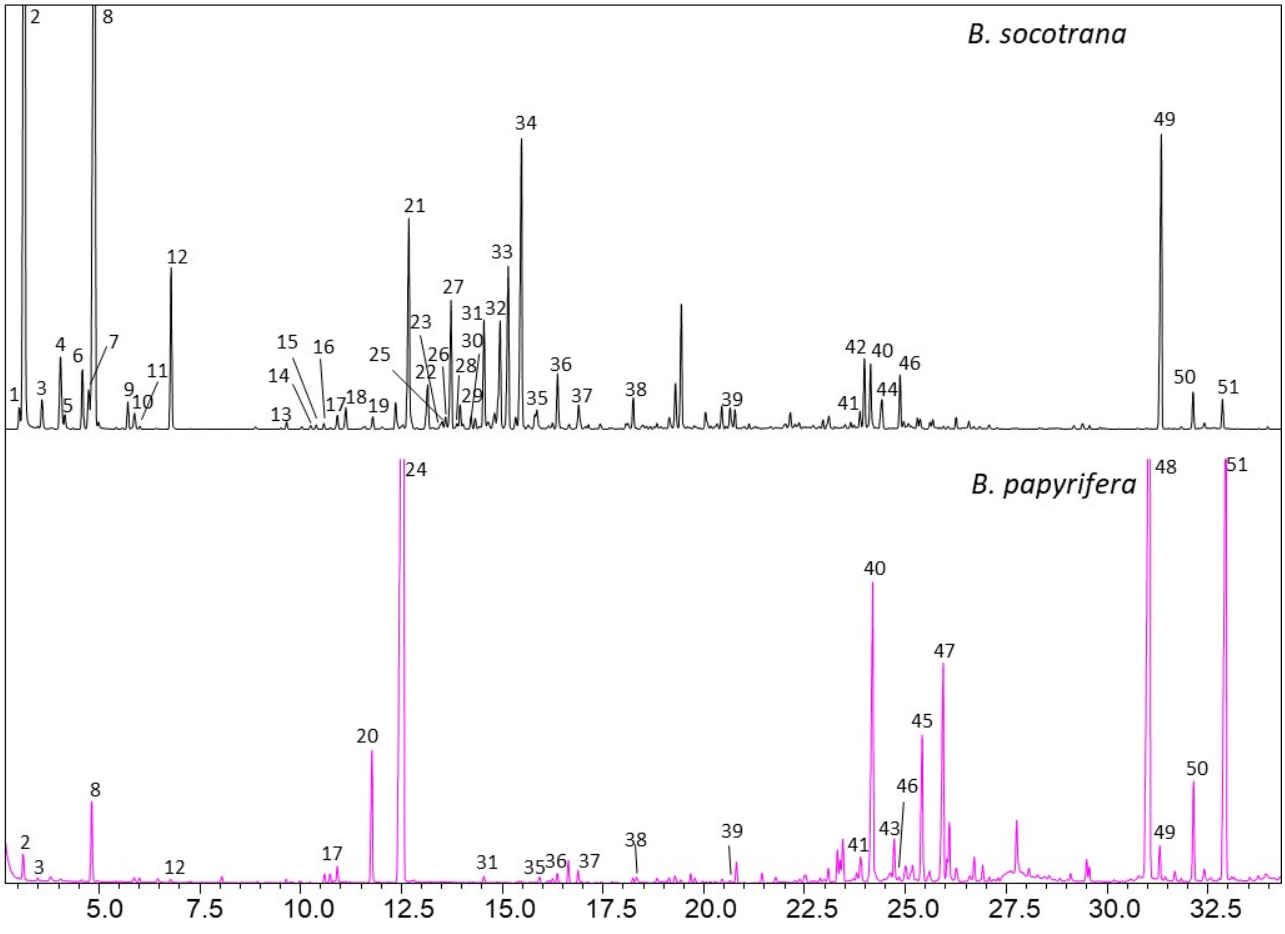


Figure 4 – Extraction time profiles of α-pinene, limonene (monoterpenoids), *trans*-β-caryophyllene, α-humulene and β-selinene (sesquiterpenoids), serratol, incensole and cembrene A (diterpenoids) obtained with Vac-HS-SPME (blue) and Reg-HS-SPME (orange). Sampling amount: 100 mg, sampling temperature: 80°C.

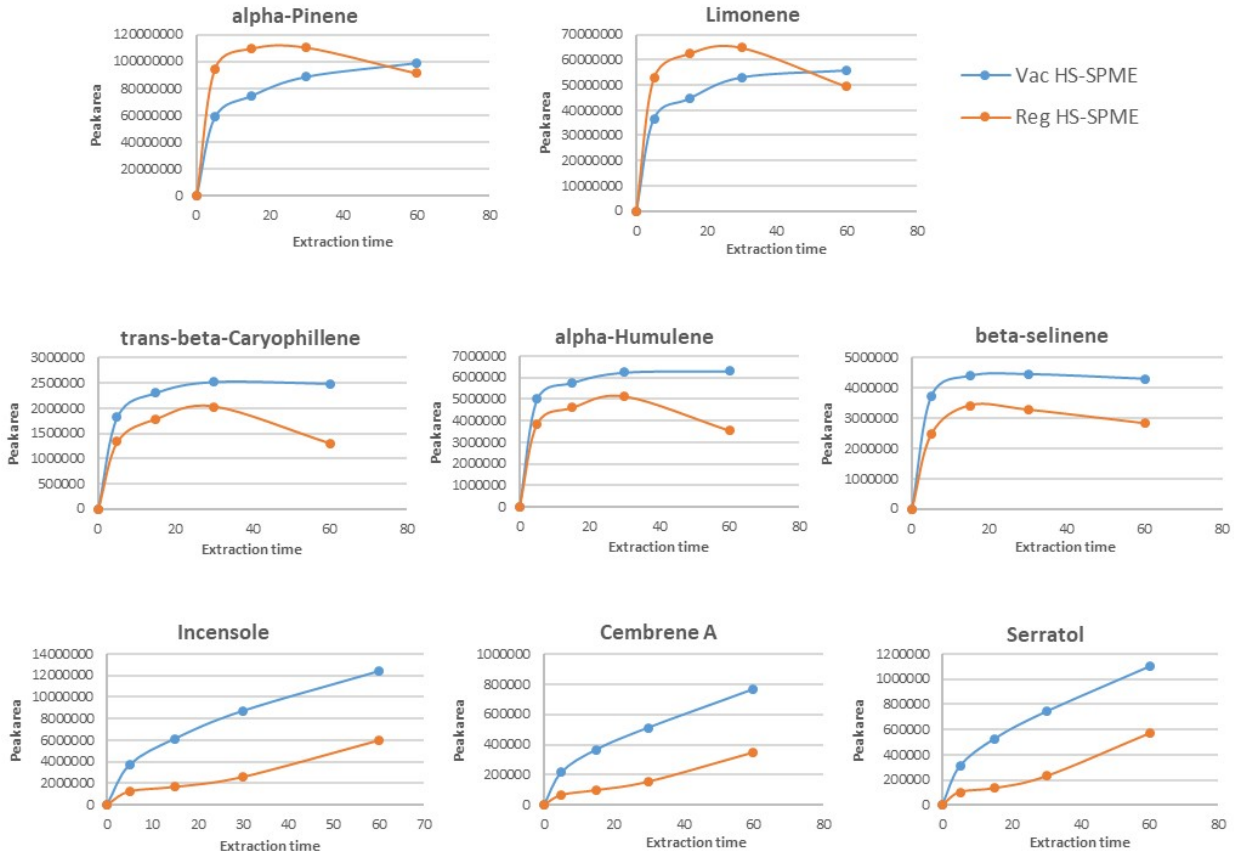


Figure 5 – Vac-HS-SPME Fast-GC-MS profiles of *B. socotrana* (black), *B. papyrifera* (pink), incensole (brown), serratol (green) and incensole acetate (blue) obtained with the SLB-IL60i column. Analysis conditions: see experimental section. Legend: see caption to figure 2.

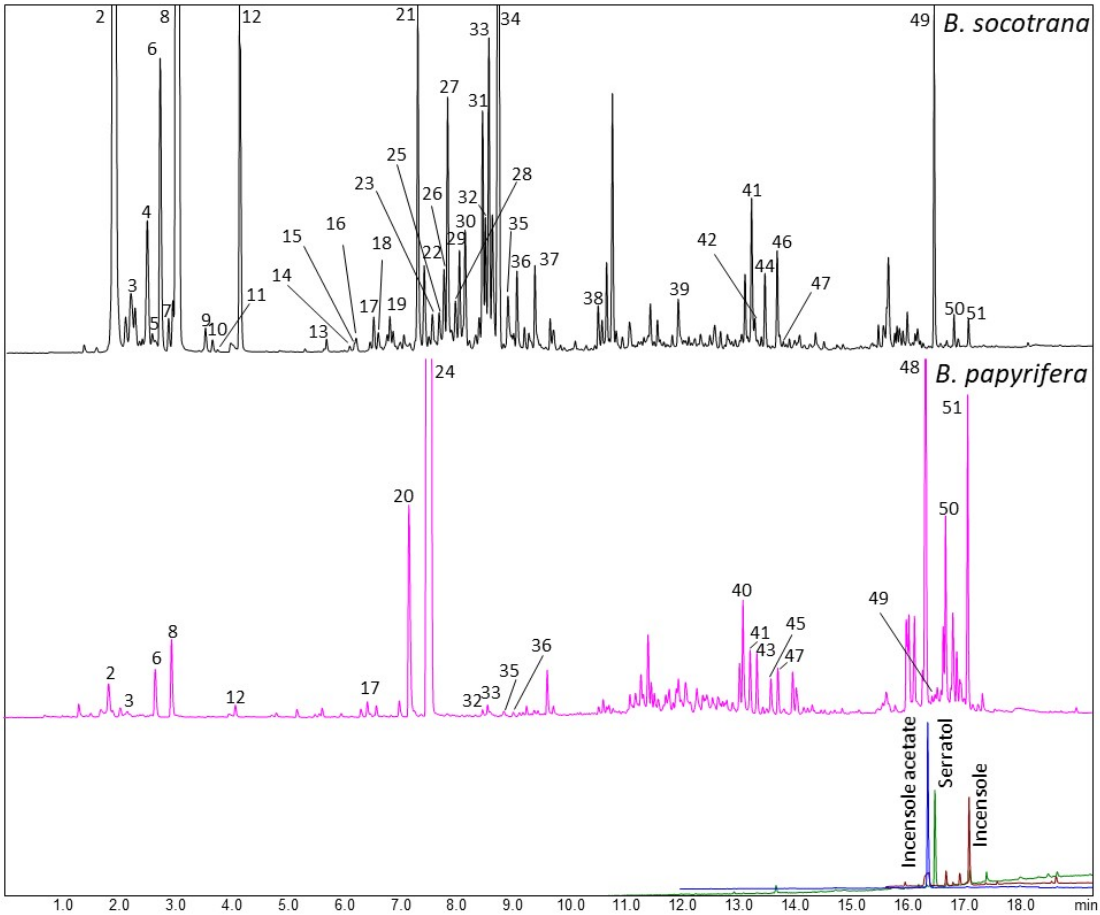
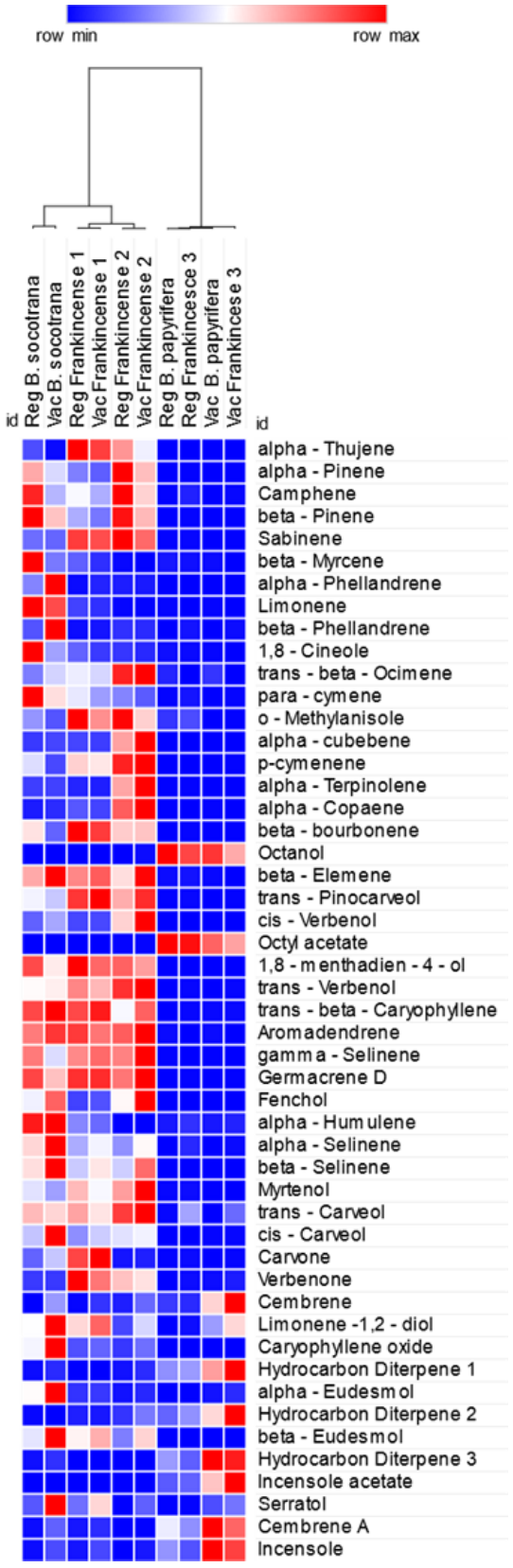


Figure 6 – Heat map on the investigated samples sampled by Reg-HS-SPME (Reg) and Vac-HS-SPME (Vac). Samples are hierarchically clustered by applying the one minus Pearson correlation.



425 **Table 1** – Relative analyte abundance (RAA) obtained by sampling 5, 40, 100mg of *B. socotrana*
426 with Vac-HS-SPME *vs*. Reg-HS-SPME for 15 min at 80°C. Legend: red triangle (▼) RAA < 0.75,
427 yellow line (▬) 0.75 < RAA < 1.25, green triangle (▲) RAA > 1.25

Compound Name	5 mg	40 mg	100 mg
alpha-Tujene	▼0.66	▼0.60	▬0.76
alpha-Pinene	▼0.68	▼0.69	▬0.79
Camphene	▼0.70	▼0.54	▬0.76
beta-Pinene	▼0.50	▼0.53	▬0.82
Sabinene	▼0.41	▼0.50	▬0.81
beta-Myrcene	▼0.60	▼0.59	▬0.84
alpha-Phellandrene	▼0.59	▼0.67	▬0.91
Limonene	▼0.50	▼0.60	▬0.84
beta-Phellandrene	▼0.49	▼0.55	▬0.90
1,8-Cineole	▼0.39	▼0.53	▬1.02
p-Cymene	▼0.49	▼0.56	▬0.84
o-Methyl-anisole	▼0.47	▼0.56	▬0.77
p-Cymenene	▼0.54	▼0.67	▬0.99
alpha-Terpinolene	▼0.37	▼0.67	▬1.02
alpha-Copaene	▼0.33	▬0.96	▲1.47
beta-Elemene	▼0.36	▬1.01	▲1.43
trans-Pinocarveol	▼0.30	▼0.65	▬1.00
trans-Caryophyllene	▼0.35	▬1.00	▲1.37
Germacrene D	▼0.42	▬1.01	▲1.40
alfa-Fenchol	▼0.37	▬0.83	▬1.04
alpha-Humulene	▼0.36	▬1.12	▲1.34
alpha-Selinene	▼0.42	▲1.25	▲1.42
beta-Selinene	▼0.43	▬1.21	▲1.41
trans-Carveol	▼0.43	▬0.94	▬1.12
cis-Carveol	▼0.43	▬0.98	▬1.16
Carvone	▼0.43	▬0.83	▬1.11
Limonene-1,2-diol	▼0.64	▲1.28	▬1.10
Caryophyllene oxide	▼0.33	▲1.51	▲1.48
Cembrene	▬1.19	▲3.31	▲2.92
beta-Eudesmol	▬0.83	▲2.19	▲1.85
Serratol	▲2.32	▲3.07	▲3.66
Cembrene A	▲2.50	▲2.99	▲3.71
Benzylbenzoate	▲1.53	▲2.32	▲2.29
Incensole	▲2.06	▲2.70	▲3.93

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