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Screening Biogenic Volatile Organic Compounds from Common Portuguese Shrubs Using Headspace–Bar Adsorptive Microextraction (HS-BAμE)

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Abstract: In this study, headspace–bar adsorptive microextraction (HS-BAμE) combined with gas chromatography–mass spectrometry (GC-MS) was employed to screen the major biogenic volatile organic compounds (BVOCs) emitted by six different Portuguese shrub species (*Erica scoparia* L., *Cistus ladanifer* L., *Cistus monspeliensis* L., *Lavandula stoechas* L., *Thymus villosus* L., and *Thymus camphoratus*). The HS-BAμE/GC-MS methodology was developed, optimized, and validated using five common monoterpenoids (α-pinene, β-pinene, limonene, 1,8-cineole, and thymol) and one sesquiterpenoid (caryophyllene oxide). Under optimized experimental conditions (microextraction-sorbent phase: activated carbon (CN1), 3 h (35 °C); back-extraction: *n*-C_6 (1 h)), good efficiencies (>45%), low analytical thresholds (5.0–15.0 μg/L) and suitable linear dynamic ranges (20.0–120.0 μg/L, $r^2 > 0.9872$) were achieved, as well as acceptable intra and inter-day precisions (RSD ≤ 30.1%). Benchmarking the proposed methodology, HS-BAμE(CN1), against the reference methodology, HS-SPME(PDMS/DVB), revealed comparable analytical responses and demonstrated excellent reproducibility. Among the six shrub species studied, *Thymus camphoratus* exhibited the highest emissions of BVOCs from its leaves, notably, 1,8-cineole (4136.9 ± 6.3 μg/g), α-pinene (763.9 ± 0.5 μg/g), and β-pinene (259.3 ± 0.5 μg/g). It was also the only species found to release caryophyllene oxide (411.4 ± 0.3 μg/g). The observed levels suggest that these shrub species could potentially serve as fuel sources in the event of forest fires occurring under extreme conditions. In summary, the proposed methodology proved to be a favorable analytical alternative for screening BVOCs in plants. It not only exhibited remarkable performance but also demonstrated user- and eco-friendliness, cost-effectiveness, and ease of implementation.

Keywords: BVOCs; HS-BAμE; GC-MS; Portuguese shrubs; *Erica scoparia* L.; *Cistus ladanifer* L.; *Cistus monspeliensis* L.; *Lavandula stoechas* L.; *Thymus villosus* L.; *Thymus camphoratus*

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1. Introduction

Volatile organic compounds (VOCs) are defined as compounds containing 15 or fewer carbon atoms that are volatile or semi-volatile, characterized by high vapor pressures (above 10 Pa at 25 °C), a boiling point below 260 °C (1 atm), and low solubility in water. They can be categorized into two groups based on their emission sources; anthropogenic volatile organic compounds, emitted from fossil fuels and human activities, and biogenic volatile organic compounds (BVOCs), emitted from natural sources such as oceans, microorganisms present in the soil, and plants [1,2]. Some BVOCs, notably terpenoids emitted by plants, contribute to the aroma of many scents, and play a crucial role in the perfumery industry, constituting common essential oils. This aspect is particularly significant when determining the composition of certain plant species. BVOC emissions are influenced by various environmental factors, including sunlight, precipitation, temperature, wind strength, and moisture. Therefore, the time of year affects the quantity of BVOCs emitted by plants [1,3]. Recent studies suggest that when heated, most plants emit highly flammable BVOCs, which in the presence of an ignition source, can exacerbate the spread

of forest fires. This phenomenon has contributed to catastrophic events like the one in Pedrogão Grande, Portugal. Since these BVOCs have lower densities than air, they tend to accumulate at lower altitudes, underscoring the importance of studying shrubs, which are small-to-medium-sized perennial woody plants. Moreover, under specific weather conditions (temperature $\geq$ 30 °C, 30 consecutive days without rain, relative air humidity $\leq$ 30%, and wind speeds exceeding 30 km/h) known as the 'rule of 30', the terpenoids emitted can intensify ignition, accelerating the spread of fires. In recent years, Portugal has experienced a significant number of forest fires, with approximately 3 million hectares burned between 1980 and 2020, equivalent to around one-third of its territory. Consequently, it is imperative to study the characteristics of BVOCs emitted by some of the most common shrubs, especially in the Mediterranean region, which is marked as one of the most susceptible areas to forest fires due to its climatic conditions characterized by regular periods of high temperatures and drought [3].

The state-of-the-art analytical methodologies for monitoring BVOCs emitted by plants typically involve an enrichment step prior to analysis by gas chromatography–mass spectrometry (GC-MS) [4–8]. Currently, one of the most used non-exhaustive techniques for BVOC monitoring is solid phase microextraction (SPME), particularly in the headspace (HS) mode, which is applicable to liquid, solid, and *in situ*/*in vivo* matrices [4,9,10]. SPME uses selective and sensitive fibers and operates in a single step, meeting the principles of green analytical chemistry (GAC) by being a solvent-free approach [11]. However, there is a need for alternative analytical methods for HS monitoring of compounds that are user- and eco-friendly, cost-effective, and easy to implement. In recent years, bar adsorptive microextraction (BAμE) has emerged as a passive or static technique for various applications in the liquid phase (e.g., wastewater, urine, etc.) analysis. BAμE uses sorption-based coatings, including polymers and activated carbons (ACs), offering high selectivity and sensitivity for monitoring organic compounds at trace levels [12–15]. Additionally, BAμE devices can be easily prepared in any lab without relying on commercial suppliers, making them more accessible. Recently, BAμE has been successfully applied in the HS mode to monitor BVOCs emitted by tree leaves, demonstrating simplicity and comprehensiveness as an analytical alternative [7].

In this study, HS-BAμE followed by GC-MS analysis is employed to profile major BVOCs emitted by the leaves of common shrub species in Portugal (*Erica scoparia* L., *Cistus ladanifer* L., *Cistus monspeliensis* L., *Lavandula stoechas* L., *Thymus villosus* L., and *Thymus camphoratus*). The development, optimization, validation, and application to real matrices are discussed, along with a comparison to reference methodologies, and the influence they could have on forest fires under extreme conditions.

2. Materials and Methods

2.1. Chemical Standards, Materials, and Samples

Acetonitrile (ACN, 99.9%), *n*-hexane (*n*-C_6, 96.0%), dichloromethane (DCM, 99.9%), and *n*-heptane (*n*-C_7, 99.0%), all of HPLC-grade, were obtained from Carlo Erba (Val-de-Reuil, France). Methanol (MeOH, 99.9%), also of HPLC-grade, and isooctane (for analysis) were obtained from Honeywell (Issy-les-Moulineaux, France). (+) -α-pinene (98.0%, USA), (-) -β-pinene (99.0%, USA), 1,8-cineole (99%, USA), R-(+) -limonene (97.0%, Mexico) and bromopentafluorobenzene (99.0%, UK), used as internal standard (IS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thymol and (-) caryophyllene oxide (99%, Germany) were acquired from Sigma-Aldrich and José M. Vaz Pereira, Lda. (Lisbon, Portugal), respectively. A standard mixture of *n*-alkanes C_7–C_{30} (50.0 mg/L of each in *n*-C_7), of HPLC and GC-grade was obtained from Sigma-Aldrich. Ultra-pure water was obtained from a Milli-Q water purification system from Merck (Millipore, Burlington, MA, USA).

For the HS-BAμE assays, the sorbent coatings tested included reverse phase polymers such as *N*-vinylpirrolidone Strata^{TM} X (S-X; pore size: 85 Å, particle size: 33 μm, surface area: 800 m^2/g), styrene–divinylbenzene co-polymer Strata$^{®}$ SDB-L (S-DVB; pore size: 280 Å, particle size: 100 μm, surface area: 500 m^2/g), and the ciano-based co-polymer

Strata® CN (S-CN; pore size: 70 Å, particle size: 55 µm, surface area: 500 m^2/g) obtained from Phenomenex (Torrance, CA, USA), as well as *N*-vinylpirrolidone–divinylbenzene co-polymer Oasis® HLB (HLB; pore size: 80 Å, particle size: 30 µm, surface area: 800 m^2/g) obtained from Waters (Milford, MA, USA). The commercial ACs tested included CA1 (pH_{PZC} 2.2, surface area: 1043 m^2/g, $V_{mesopores}$: 0.66 cm^3/g, $V_{\alpha\text{-supermicropores}}$: 0.26 cm^3/g), CN1 (pH_{PZC} 5.1, surface area: 1179 m^2/g, $V_{mesopores}$: 0.68 cm^3/g, $V_{\alpha\text{-supermicropores}}$: 0.3 cm^3/g), and SX PLUS (pH_{PZC} 8.4, surface area: 833 m^2/g, $V_{mesopores}$: 0.44 cm^3/g, $V_{\alpha\text{-supermicropores}}$: 0.15 cm^3/g, $V_{\alpha\text{-ultramicropores}}$: 0.08 cm^3/g) obtained from Salmon & Cia (Lisboa, Portugal) and the R (pH_{PZC} 6.5, surface area: 964 m^2/g, $V_{mesopores}$: 0.40 cm^3/g, $V_{\alpha\text{-supermicropores}}$: 0.16 cm^3/g, $V_{\alpha\text{-ultramicropores}}$: 0.09 cm^3/g) obtained from Riëdel-de Haën (Hannover, Germany). For the HS-SPME assays, a manual holder and polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 µm) fibers were used, both purchased from Supelco® (Bellefonte, PA, USA).

The leaves from all shrub species were sampled only once; the *Cistus, Erica,* and *Thymus villosus* L. were sampled on 17 April 2023 at Parque Natural de Sintra-Cascais, and *Thymus camphoratus* was sampled on 6 May 2023 at Ribeira da Azenha-Vila Nova de Milfontes (37°48′17.6″ N 8°46′58.4″ W). *Cistus ladanifer* L. and *Cistus monspeliensis* L. were sampled at Abano (38°44′26.16″ N 09°28′10.10″ W), *Lavandula stoechas* L. and *Erica scoparia* L. at Biscaia (38°45′45.84″ N 09°28′30.68″ W), and *Thymus villosus* L. at Pedra Amarela (38°45′30.11″ N 09°26′01.94″ W). The collected fresh leaves were stored in sealed flasks in a thermal box and, upon arrival at the laboratory, were stored in the fridge until analysis.

2.2. Experimental Set-Up

2.2.1. Standard Solutions and Real Sample Preparation

The individual stock solutions for each BVOC (5000.0 mg/L) in *n*-C_6 were freshly prepared at the beginning of the study and renewed whenever necessary. The standard mixture (200.0 mg/L of α-pinene, β-pinene, limonene, 1,8-cineole, thymol, and caryophyllene oxide in *n*-C_6) for HS-BAµE assays was freshly prepared every 2 months and stored at −20 °C, and working standard mixtures (5.0 mg/L) were prepared daily. In the case of the IS, an individual standard solution was prepared in *n*-C_6. Subsequently, a solution with 20.0 mg/L of IS in *n*-C_6 was prepared every two weeks.

2.2.2. Preparation and Conditioning of BAµE Devices

The BAµE devices were *lab-made* and prepared using polypropylene hollow tubes (7.5 × 3.0 mm) coated with different powdered sorbents (polymers and ACs) fixed with an appropriate adhesive film [7]. The BAµE devices were then cleaned up with ultrapure water under agitation on a magnetic board with fifteen positions (Variomag H+P Labortechnik AG Multipoint 1,5 e Cimarec i Poly Komet, Oberschleißheim, Germany) for about 10 to 20 min. Prior to the water cleaning step, the devices were dried on paper.

2.2.3. HS-BAµE Optimization and Validation Assays

To optimize the experimental conditions, a one-variable-at-a-time (OVAT) strategy was adopted, which means that while studying the behavior of one parameter, the remaining were kept constant. For HS-BAµE experimental optimization, the selected parameters were the sorbent coating phase (SX PLUS, R, CN1, CA1, HLB, S-X, S-DVB, and S-CN), the desorption solvent (MeOH, ACN, DCM, *n*-C_6, *n*-C_7, mixture of *n*-C_6 + *n*-C_7 (1:1%, *v/v*), and isooctane), desorption time (15, 30, 45, and 60 min), equilibrium time (1, 2, 3, 6, and 16 h) and temperature (15, 35, and 55 °C) [7,13–17]. For each assay, 100 µL of a standard mixture solution containing the six selected BVOCs (5.0 mg/L) was spiked at the bottom of a 5 mL vial. The previously prepared BAµE devices were then inserted into the HS vial and immediately closed and placed into a thermostatic sand bath with controlled temperature for BVOC microextraction. After the enrichment step, the BAµE devices were removed and transferred into 2 mL vials with inserts followed by 90 µL of an organic solvent. Then, the 2 mL vials were subjected to ultrasonic treatment (Branson 3510 E-DTH, 42 ± 2.5 kHz, 100 W, Zurich, Switzerland) for liquid desorption (LD) of the extracted BVOCs. Next, 10 µL of the

IS solution (20.0 mg/L) was added into each insert and agitated into a vortex (Velp, Itália) for 5 s. Finally, the BAμE devices were removed and the vials were sealed prior to GC-MS analysis. Each assay and blank analysis was performed in triplicate. The data obtained from the HS-BAμE/GC-MS assays were analyzed by comparing the abundance areas with control standard solutions of the target BVOCs. Figure 1 illustrates the experimental setup used.

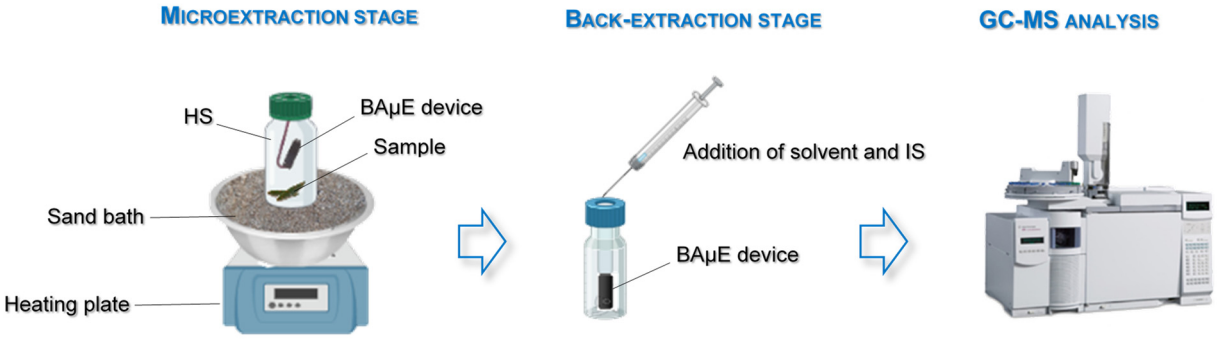

Figure 1. Experimental set-up for the two stages of the HS-BAμE/GC-MS assays proposed in the present study.

In order to validate the proposed methodology under optimized conditions, several parameters were evaluated, including selectivity, sensibility, linearity, and precision, according to previous studies [14–17], using the same procedure as described above. Initially, selectivity was assessed by verifying the absence of interfering compounds with identical retention times to the target BVOCs, performed using blank assays. Afterward, the sensibility of the HS-BAμE/GC-MS methodology was evaluated through the experimental determination of the limits of detection (LOD) and quantification (LOQ). Then, calibration curves were plotted by analyzing the calibration standards of the six BVOCs ranging from 0.75 mg/L to 6.0 mg/L (15.0 μg/L to 120.0 μg/L, respectively). The linearity was assessed using the determination coefficients (r^2), the residual dispersion, and the lack-of-fit test for each calibration plot. The last step of validation assays was dedicated to performing intra- (n = 6) and inter-day (n = 3, for 2 days) assays. To perform the precision assays for the HS-BAμE/GC-MS methodology, the IS concentration was maintained at 20.0 mg/L; the low, medium, and high concentrations of the BVOC mixtures corresponded to 1.0, 2.0, and 5.0 mg/L (20.0, 40.0, and 100.0 μg/L, respectively). The acceptance criterion was the relative standard deviation (RSD), which had to be lower or equal to 30% for all spiking levels [12]. All validation assays were performed in triplicate, except when indicated.

For comparison with a reference methodology, the validated HS-BAμE/GC-MS was compared with HS-SPME/GC-MS using three BVOCs (α-pinene, 1,8-cineole, and thymol), following a similar procedure as described in previous work [7]; optimized conditions: PDMS/DVB fiber, HS extraction for 35 min at 35 °C; however, the IS concentration was maintained at 40.0 mg/L and the BVOC mixture was used in three different concentration levels—low, medium, and high, corresponding to 25.0, 100.0, and 200.0 mg/L (0.3, 2.5 and 5.0 μg/L, respectively).

2.2.4. Application to Shrub Leaves

To address the performance of the methodology validated in real matrices, it was applied for the analysis of BVOCs in the leaves of the different shrubs. For HS-BAμE/GC-MS assays, fresh leaves were cut and weighed into 5 mL vials, using amounts of 15.0 mg for *Erica scoparia* L., 10.0 mg for *Lavandula stoechas* L. and *Cistus monspeliensis* L., and 2.0 mg for *Thymus villosus* L., *Thymus camphoratus*, and *Cistus ladanifer* L. Then, the remaining procedure described in Section 2.2.3 was followed. The amounts of the six BVOCs emitted by the shrub leaves of the species mentioned above were determined using the calibration plots obtained previously. For quantification purposes, fresh weight (FW) was used to

carry out the analysis; the fresh leaves were dried in a heated oven (80 °C) until their weight was constant to eliminate leaf moisture (volatilization gravimetry). This allowed the determination of the mass of each BVOC released by dry-weight (DW) leaves (μg/g).

2.3. Instrumental Set-Up

GC-MS analysis was performed on an Agilent 6890 series gas chromatograph equipped with an Agilent 7683 automatic liquid sampler and interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Little Falls, NY, USA). A fused silica capillary column (30 m length × 0.25 mm I.D. × 0.25 μm film thickness; Zebron ZB-5; 5% diphenyl, 95% dimethyl polysiloxane; Phenomenex, Torrance, CA, USA) was used. Helium was used as the carrier gas in the constant pressure mode (14.3 psi). For all analyses, a solvent delay of 3.2 min was used. The transfer line, ion source, and quadrupole temperatures were maintained at 280 °C, 230 °C, and 150 °C, respectively. In the full-scan mode, electron ionization mass spectra in the range of 45–550 Da were recorded at 70 eV of energy. Data acquisition and instrument control were performed by the MSD ChemStation software (G1701CA, version E.02.02.1431; Agilent Technologies, USA). The identification of each compound was addressed through the comparison of their retention indices (RIs) relative to a standard mixture of C_7-C_{30} *n*-alkanes [18] and the characteristic features of their mass spectra in comparison with Wiley's library spectral data bank (G1035B, Rev D.02.00; Agilent Technologies, USA). The calculation of each assay was performed by comparing the average peak areas of the extracted compounds with the IS peak area. A split/splitless (S/SL) injector operating at 270 °C was used in the SL mode (injection volume of 1 μL) for the HS-BAμE/GC-MS assays. The oven temperature was programmed to start at 54 °C (held isothermally for 2 min) then heated up to 170 °C at 20 °C/min (held isothermally for 2 min) and, finally heated up to 250 °C at 3 °C/min (held isothermally for 5 min), resulting in a run time of 42.17 min. For the HS-SPME/GC-MS assays, the injector (thermal desorption (TD) at 270 °C for 5 min) and oven temperature were programmed according to previous work [7].

3. Results and Discussion

3.1. GC-MS Conditions

From the outset, we initiated the optimization of instrumental conditions using a standard mixture (2.0 mg/L) comprising five monoterpenoids (α-pinene, β-pinene, limonene, 1,8-cineole, and thymol) and one sesquiterpenoid (caryophyllene oxide). These compounds, commonly found in the six Portuguese shrubs under study (*Erica scoparia* L., *Cistus ladanifer* L., *Cistus monspeliensis* L., *Lavandula stoechas* L., *Thymus villosus* L., and *Thymus camphoratus*), were selected based on several reports [4,5,8,19]. Figure 2 illustrates the chemical structures of the target BVOCs. The standard mixture containing the six terpenoids underwent analysis by GC-MS using the SL injection mode and conventional instrumental conditions (see Section 2.3). The total ion chromatograms obtained exhibited adequate selectivity and sensitivity, with symmetrical peak shapes and resolution achieved for each compound within an acceptable analytical time (<12 min). Instrumental sensitivity was evaluated by determining the LOD and LOQ for each target compound, with signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively. The values obtained were 0.20 and 0.50 mg/L for α- and β-pinene, 0.05 and 0.20 mg/L for limonene and 1,8-cineole, 0.01 and 0.03 mg/L for thymol, and 0.10 and 0.30 mg/L for caryophyllene oxide, respectively. Subsequently, instrumental calibration was performed by determining linear regressions for each target analyte within a concentration ranging from 0.25 to 6.00 mg/L. Suitable linearity was achieved, with all r^2 values exceeding 0.9831. Finally, instrumental precision was assessed by conducting six consecutive injections of the same standard mixture, yielding relative standard deviations (RSDs) of less than 11.2%.

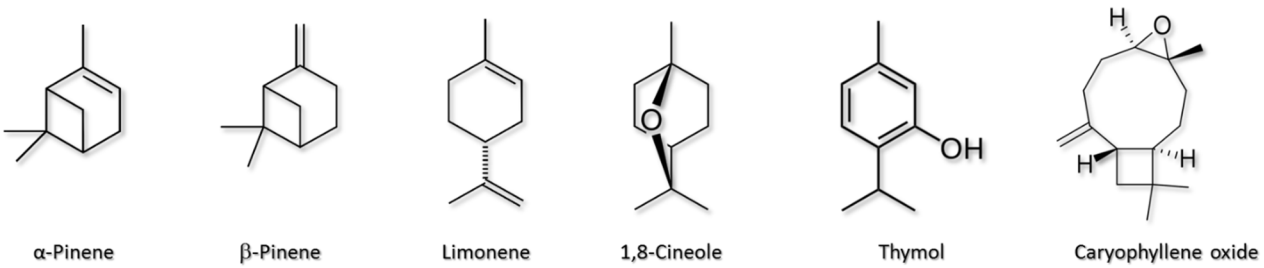

Figure 2. Chemical structures of the target BVOCs studied in the present work, including five monoterpenoids (α-pinene, β-pinene, limonene, 1,8-cineole, and thymol) and one sesquiterpenoid (caryophyllene oxide).

3.2. Evaluation of the HS-BAμE/GC-MS Methodology

3.2.1. Optimization Assays

After optimizing the instrumental system, experimental conditions for the HS-BAμE/GC-MS methodology were established (Figure 1). The assays were conducted via HS in 5 mL vials containing a standard mix of six target BVOCs: α-pinene, β-pinene, limonene, 1,8-cineole, thymol, and caryophyllene oxide. As the HS-BAμE technology operates in two stages, i.e., microextraction in the gaseous phase followed by back-extraction in the liquid phase, an OVAT strategy was adopted to optimize parameters such as sorbent coating phase, equilibrium time and temperature, as well as solvent type and desorption time. One significant advantage of this methodology compared to others considered as reference methods (e.g., SPME) is the ease of selecting the best coating phase from a variety of available materials tailored to the target compounds involved [7]. For this study, four polymers (S-CN, S-DVB, HLB, and S-X) and four ACs (R, SX PLUS, CA1, and CN1) were tested. Figure 3a illustrates the comparison of efficiency achieved for all sorbent phases tested under standard experimental conditions, indicating that the ACs generally exhibit greater selectivity than the tested polymers, resulting in higher recoveries. Additionally, it can be observed from the same figure that while thymol is poorly recovered, caryophyllene oxide consistently shows a very good response. Based on the data obtained from the sorbent phase assays, three AC phases (R, CA1, and CN1) appear to offer the best selectivity for the six target BVOCs. These sorbents are nanoporous solids capable of efficiently retaining analytes in the gaseous phase through dispersive and/or electrostatic interactions. The R phase, characterized by ultramicropores ($\leq$0.7 nm) and supermicropores (0.7–2.0 nm), demonstrates the ability to retain molecules of various sizes. However, the CA1 and CN1 phases, containing only supermicropores, exhibit a preference for smaller-sized molecules such as the six target BVOCs under study. Furthermore, the results indicate that the chemical surface characteristics of the ACs play a crucial role. The R phase, with a neutral surface (pH_{PZC} 6.50) and smaller pore size, appears to be ideal for retaining α- and β-pinene. Conversely, CA1 and CN1 phases, with acidic surfaces (pH_{PZC} 2.20 and 5.10, respectively) and larger pore sizes, exhibit enhanced retention of limonene and 1,8-cineole. On the other hand, the polymers studied can also retain the six target BVOCs, primarily through π–π, dipole–dipole, hydrogen bonds, and ionic interactions, depending on particle size and surface area. Nevertheless, based on the results depicted in Figure 3a, since the CN1 phase exhibited the most acceptable RSDs and greater recovery efficiencies, it was selected to optimize the following parameters.

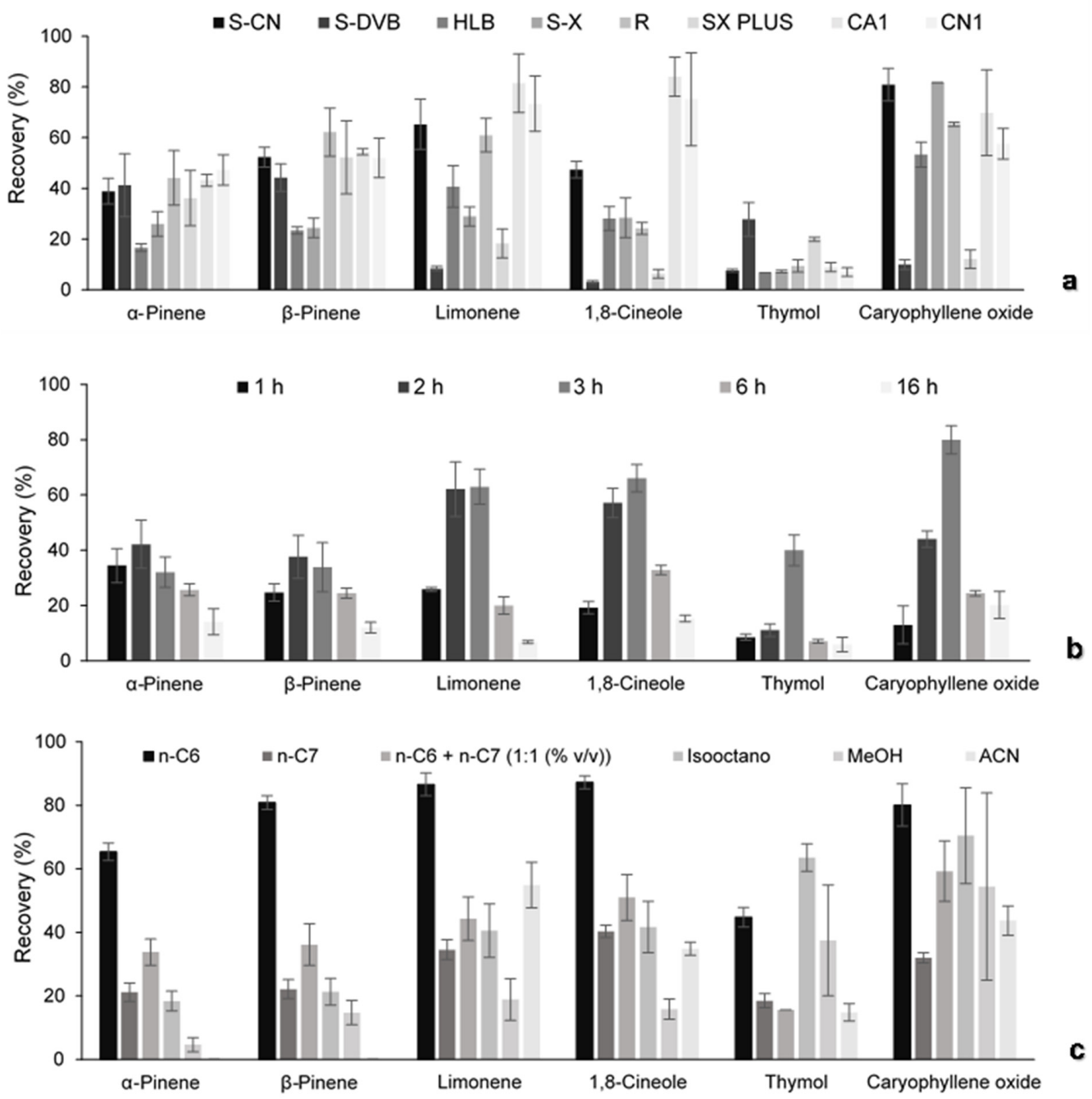

Figure 3. Effect of sorbent phase selectivity (**a**), equilibrium time (**b**), and back-extraction solvent (**c**) on the efficiency of the six target BVOCs obtained by the HS-BAμE/GC-MS methodology. The error bars represent the standard deviations of three replicates.

The study subsequently evaluated the key parameters for microextraction in the gaseous phase, focusing on equilibrium time and temperature. Initially, five equilibrium times (1, 2, 3, 6, and 16 h) were tested to assess the retention of the six BVOCs, as depicted in Figure 3b. Analysis of the data revealed that the recovery performance increased up to 3 h, after which a significant decrease in recovery was observed. Extending the equilibrium time to 6 and 16 h resulted in a considerable decrease in recovery efficiencies due to the occurrence of the *wall-effect* phenomenon. This phenomenon involves the adsorption of target compounds onto the inner walls of the glass vials over extended periods of time [7]. Furthermore, evaluation of thermostat effects (25, 35, and 55 °C) demonstrated that a temperature of 35 °C gave higher recovery yields for the target BVOCs. Consequently, the

combination of 3 h of equilibrium time at 35 °C appeared to promote greater recoveries with acceptable RSDs ranging from 6.4% to 26.2% and thus was selected for the remaining assays.

The main parameters for back-extraction in the liquid phase were investigated, focusing on the choice of solvent and desorption time. Seven organic solvents, namely ACN, MeOH, DCM, *n*-C6, *n*-C7, isooctane, and a mixture of *n*-C6 + *n*-C7 (1:1, *v*/*v*), covering a wide range of polarities, were evaluated to determine the optimal back-extraction conditions. Analysis of the data presented in Figure 3c reveals that highly polar solvents like ACN and MeOH did not facilitate complete back-extraction of all six BVOCs, yielding either incomplete recovery or very low yields. Among the nonpolar solvents tested, *n*-C6 demonstrated the highest recoveries for all six BVOCs, whereas DCM showed a negligible response. Isooctane emerged as a viable alternative among the hydrocarbons tested, particularly effective for thymol and caryophyllene oxide. Furthermore, the impact of sonication time (15, 30, 45, and 60 min.) was assessed, with the results indicating that a sonication period of 60 min resulted in optimal desorption efficiencies, accompanied by acceptable RSDs ranging from 0.4% to 20.4% for the six target BVOCs. Consequently, based on these achievements, *n*-C6 under sonication for 60 min was identified as the preferred back-extraction condition for the study.

3.2.2. Validation Parameters

After optimizing the crucial experimental parameters—microextraction stage employing CN1 phase (3 h) at 35 °C and back-extraction stage utilizing *n*-C6 (100 µL) for 60 min—we proceeded with validation assays. These encompassed the assessment of analytical thresholds, linear dynamic ranges, as well as both intra- and inter-day precisions. Table 1 summarizes the validation data obtained from the proposed methodology (HS-BAµE/GC-MS) under optimized experimental conditions.

Table 1. LOD, LOQ, linear dynamic range, slope (a), x-intercept (b), and determination coefficient (r^2) values from the calibration plots obtained for the six target BVOCs under study and intra and inter-day precisions for HS-BAµE(CN1)/GC-MS assays under optimized experimental conditions.

BVOCs	LOD (µg/L)	LOQ (µg/L)	Linear Range (µg/L)	a	b	r^2	Intra-Day Precision (n = 5; RSD,%)	Inter-Day Precision (n = 3; RSD,%)
α-Pinene	5.0	15.0	20.0–100.0	0.0090	0.0614	0.9951	6.3–22.8	10.4–28.7
β-Pinene				0.0123	−0.0563	0.9912	8.2–22.1	12.9–26.9
Limonene			20.0–120.0	0.0199	0.0846	0.9879	13.8–18.5	13.4–26.7
1,8-Cineole				0.0250	0.0557	0.9895	4.0–11.3	13.4–15.8
Thymol	15.0	49.5	50.0–100.0	0.0102	0.0195	0.9872	17.5–28.4	18.0–26.4
Caryophyllene oxide				0.0347	0.1738	0.9947	10.2–16.5	18.8–30.1

Analytical thresholds were determined via LOD and LOQ, falling within the range of 5.0 to 49.5 µg/L. Generally, the LODs and LOQs align with values previously reported in other studies [7,20,21], except for those of thymol and caryophyllene oxide, where the values slightly exceeded expectations (0.1 µg/L ≤ LOD ≤ 0.5 µg/L).

The linear dynamic ranges were established by analyzing multi-standard solutions of the six BVOCs, spanning from 20.0 to 100.0 µg/L for α- and β-pinene, from 20.0 to 120.0 µg/L for limonene and 1,8-cineole, and from 50.0 to 100.0 µg/L for thymol and caryophyllene oxide. Calibration plots exhibited high determination coefficients ($r^2 \geq 0.9816$), indicating strong linearity. However, assessing linearity based solely on the r^2 parameter is insufficient, as it does not address data heteroscedasticity. Therefore, homoscedasticity was evaluated through lack-of-fit testing and residue dispersion [22]. The lack-of-fit test indicated a good fit for all calibration plots, suggesting homoscedastic data. Furthermore, residue plots displayed an unsystematic distribution of residues, confirming

the homoscedasticity of the experimental data and validating the suitability of the linear regressions plotted for the analysis of the six BVOCs.

The precision of the proposed methodology was evaluated by determining the RSDs using intra-day (n = 5) and inter-day (n = 3, over 2 consecutive days) assays. Three different spiking levels were assessed, including low, medium, and high contents, i.e., 20.0, 40.0, and 100.0 μg/L, respectively. In both intra-day and inter-day assays, the methodology demonstrated favorable RSDs across all studied spiking levels (4.0% $\leq$ RSD $\leq$ 30.1%), indicating its suitability for assessing the six BVOCs under investigation. Additionally, it was observed that intra-day precision yielded lower RSD values compared to inter-day precision, suggesting better repeatability in the obtained data than intermediate precision.

3.3. Comparison with Reference Methodologies

In this study, our focus was also on comparing the primary advantages and drawbacks of our proposed methodology with the SPME technique, which stands out as the most widely used technology for HS analysis and serves as a suitable benchmark; additionally, the HS-SPME technique is renowned for its outstanding selectivity, sensitivity, ease of handling, and versatility, particularly in the analysis of volatile and semi-volatile compounds by GC-MS. Figure 4 depicts the comparison of the response obtained from the HS-BAμE(CN1) against HS-SPME(PDMS/DVB) methodologies, followed by GC-MS analysis of three prevalent BVOCs (α-pinene, 1,8-cineole, and thymol), all conducted under similar experimental conditions. This comparison aims to provide a visual representation of how the two methodologies perform relative to each other in terms of detecting and quantifying the selected BVOCs. Hence, if the responses obtained in the analysis of BVOCs are consistent between the two methodologies, it can be inferred that both are equivalent, generating similar and proportionate results. However, by observing the figure, it becomes apparent that the HS-SPME(PDMS/DVB) methodology consistently demonstrates an average order of magnitude approximately fifteen times higher than that of the HS-BAμE(CN1) methodology, as evidenced by the slopes achieved. Apart from the various factors such as interaction and specific affinity, as well as potential synergistic effects between the chosen sorbent phases and the target BVOCs, the differences in response observed for each monoterpene under study may be attributed to the distinct sample introduction approaches by GC-MS for both methodologies, as was discussed in previous work [7]. While the HS-SPME(PDMS/DVB) methodology relies on TD, wherein all the microextracted sample is analyzed, the HS-BAμE(CN1) methodology is based on LD, where only a small portion (1/100) of the sample is analyzed. This difference influences the response of each methodology. Moreover, assuming that both analytical technologies align with GAC principles or that any inherent differences are negligible, we can assert that the HS-BAμE methodology is significantly more cost-effective and comprehensive; this last aspect is due to the ease of interfacing with various instrumental systems. From a practical standpoint, the HS-SPME methodology, based on TD, appears to be more sensitive and user-friendly, although more energy-consuming, while the HS-BAμE methodology, relying on LD, offers the distinct advantage of enabling sample reanalysis, which is often crucial whenever repetition and data confirmation are mandatory. Finally, the BAμE methodology proves to be more suitable for routine analysis compared to SPME; while the former merely necessitates a straightforward magnetic plate with multiple positions, the latter demands a costly CombiPAL autosampler.

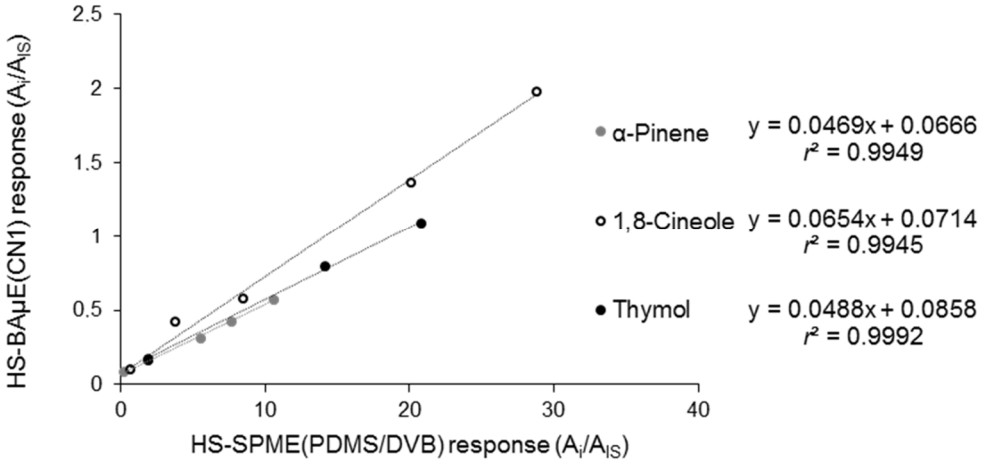

Figure 4. Comparison of the response from HS-BAμE(CN1) against HS-SPME(PDMS/DVB) methodologies followed by GC-MS analysis obtained for three common BVOCs (α-pinene, 1,8-cineole, and thymol) under similar optimized experimental conditions.

3.4. Application to Shrub Leaves

After optimizing and validating the proposed methodology, we proceeded with the assays using shrub leaf samples, as outlined in Section 2.2.4. It is crucial to note that field sampling, especially with live species like shrubs, is always subject to availability and prevailing atmospheric conditions. During the period under study (April–May 2023), there was abundant rainfall. It is widely recognized that excessive rainwater can lead to a significant decrease in the content of essential oils in plant leaves; consequently, this affects the type and amount of BVOCs released. Therefore, it is not our intention to systematically evaluate in depth the profiles of all BVOCs, including the minor ones emitted by the shrub species under study, as this requires a much more demanding and detailed sampling program. From a different perspective, the goal of the application to real matrices is to demonstrate, in addition to the levels found, that we have an effective and alternative analytical technology that can be regularly used for monitoring the main BVOCs emitted by plants. Table 2 presents the amounts of the six target monoterpenoids, expressed on a DW basis, emitted by the leaves of the six Portuguese shrubs under study using the HS-BAμE(CN1)/GC-MS methodology, where good reproducibility is observed. Based on the results in Table 2, it is evident that *Thymus camphoratus* exhibits the highest emissions of BVOCs among all six shrub leaves; notably, it releases significant amounts of 1,8-cineole (4136.9 ± 6.3 μg/g) as well as α- and β-pinene (763.9 ± 0.5 and 259.3 ± 0.5 μg/g, respectively). Moreover, it is the only species among the six to emit caryophyllene oxide (411.4 ± 0.3 μg/g). Conversely, *Thymus villosus* L. leaves demonstrated higher emissions of limonene (138.8 ± 0.3 μg/g) and substantial amounts of 1,8-cineole (104.1 ± 0.3 μg/g) and α-pinene (69.1 ± 0.1 μg/g). Figure 5b illustrates a total ion chromatogram profile obtained from application of the HS-BAμE(CN1)/GC-MS methodology to *Thymus camphoratus* leaves. Under optimized experimental conditions, a significant response to the main BVOCs emitted by this species is observed, comparable to the total ion chromatogram obtained by the injection of the standard solution itself (Figure 5a). Additionally, other BVOCs have been identified, demonstrating the effectiveness of the proposed methodology for BVOC monitoring. *Cistus ladanifer* L. emits moderate contents of α- and β-pinene (325.1 ± 0.2 and 148.5 ± 0.5 μg/g, respectively), whereas *Cistus monspeliensis* L. and *Lavandula stoechas* L. species present lower amounts (<30 μg/g) of both as well as limonene. None of the six target monoterpenoids evaluated was detected in *Erica scoparia L.* leaves, and thymol was not observed in any leaves of the six species under study. This absence may be due to thymol emissions being below the LODs used for the present methodology, or the prevailing conditions may have caused a decrease in its emission. In line with the methodology proposed in this paper, Gonçalves et al. [7] reported BVOC amounts from tree leaves that align well with our

findings. For example, α-pinene content in *Cistus ladanifer* L. and *Thymus camphoratus* fell within the expected range (95.2–3183.3 μg/g), as did β-pinene, limonene, and 1,8-cineole (10.2–3503.8, 9.2–883.0, and 110.8–7828.0 μg/g, respectively). This consistency confirms the robustness and reproducibility of our methodology, as demonstrated before [7]. Comparing our findings in six analyzed shrubs with prior studies [19,23] on the same or similar species, we observed higher maximum contents of α- and β-pinene, limonene, and 1,8-cineole (763.9, 259.3, 138.8, and 4136.9 μg/g, respectively) than previously reported (0.6–5.2, 0.1–2.0, 0.5–28.3, and 14.6–19.2 μg/g, respectively). This variance may stem from differences in sampling times or extraction techniques.

Table 2. Contents (DW basis) of the six target monoterpenoids obtained from the leaves of six Portuguese shrub species using in vitro HS-BAμE(CN1)/GC-MS assays conducted under optimized experimental conditions.

	Shrub Species					
	***Lavandula stoechas* L.**	***Cistus ladanifer* L.**	***Cistus monspeliensis* L.**	***Thymus villosus* L.**	***Thymus camphoratus***	***Erica scoparia* L.**
BVOCs	**Concentration (μg/g)**					
α-Pinene	29.6 ± 0.2	325.1 ± 0.2	22.6 ± 0.1	69.1 ± 0.1	763.9 ± 0.5	*d*
β-Pinene	-	148.5 ± 0.5	27.5 ± 0.2	-	259.3 ± 0.5	-
Limonene	23.6 ± 0.5	*d*	*d*	138.8 ± 0.3	-	*d*
1,8-Cineole	-	*d*	-	104.1 ± 0.3	4136.9 ± 6.3	-
Thymol	-	-	-	-	-	-
Caryophyllene oxide	-	-	-	-	411.4 ± 0.3	-

d—detected but not quantified.

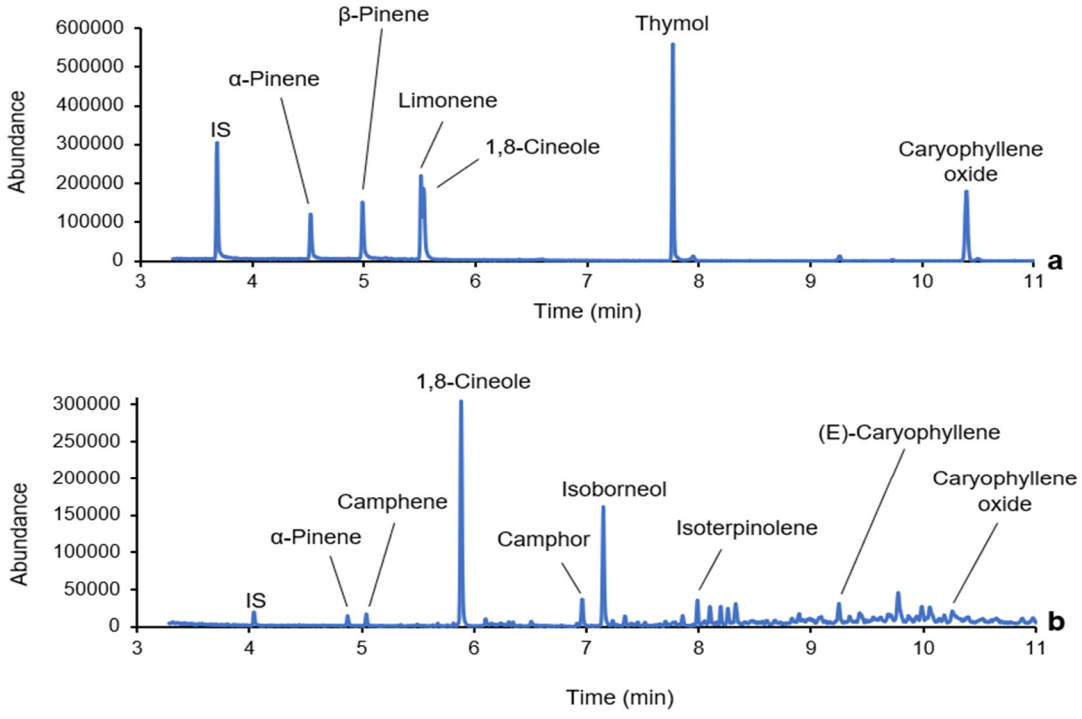

Figure 5. Example of total ion chromatograms obtained from a standard mix (**a**) using GC-MS and the leaves of *Thymus camphoratus* (**b**) using HS-BAμE(CN1)/GC-MS under optimized conditions.

Our results suggest that BVOC emission levels from the studied species vary based on factors such as chemotypes, sampling location, weather conditions (e.g., humidity, precipitation, temperature, wind strength), and anthropogenic activities. While the emitted volatiles from shrub leaves are significant, they are not as substantial as those from tree species [7]. Nevertheless, under certain atmospheric conditions (such as the 'rule of 30'),

they could contribute to the accumulation of BVOCs in forest environments, potentially leading to forest fire ignition due to the considerable BVOC emission levels. This study marks the first application of the proposed methodology to sesquiterpenoid monitoring and proves to be an effective approach for analyzing both monoterpene and sesquiterpene compounds in shrub leaves. It offers selectivity, cost-effectiveness, reproducibility, and robustness, enhancing our understanding of BVOC dynamics in ecosystems.

4. Conclusions

A novel analytical approach (HS-BAμE(CN1)/GC-MS) is proposed for monitoring BVOCs, particularly terpenoids, from six different Portuguese shrub species (*Erica scoparia* L., *Cistus ladanifer* L., *Cistus monspeliensis* L., *Lavandula stoechas* L., *Thymus villosus* L., and *Thymus camphoratus*). The methodology underwent optimization and validation and demonstrated robustness, sensitivity, selectivity, and excellent performance. Comparison with the reference methodology, HS-SPME(PDMS/DVB), showed comparable analytical responses and excellent reproducibility.

Among the six shrub species investigated, *Thymus camphoratus* exhibited the highest emissions of BVOCs from its leaves, notably 1,8-cineole (4136.9 ± 6.3 μg/g), α-pinene (763.9 ± 0.5 μg/g), and β-pinene (259.3 ± 0.5 μg/g). It was also the only species found to release caryophyllene oxide (411.4 ± 0.3 μg/g). These observed levels suggest that the shrub species could potentially contribute to fuel sources in the event of forest fires under extreme conditions.

In summary, the proposed methodology emerged as a favorable analytical alternative for BVOC screening in plants. It not only demonstrated outstanding performance but also exhibited user- and eco-friendliness, cost-effectiveness, and ease of implementation.

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