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# Comparison of different extraction methods for the determination of α- and β-thujone in sage (*Salvia officinalis* L.) herbal tea

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## Abstract

*Salvia officinalis* L. (sage) is an important industrial plant used both for food and pharmaceutical purposes. The terpene fraction of this plant is responsible for many of its therapeutic and culinary properties. We used different extraction methods Tenax TA® purge and trap, headspace (HS) solid-phase microextraction, HS sorptive extraction, and stir bar sorptive extraction to analyze the terpene fraction extracted from sage tea by GC–MS. Twenty compounds were identified, including α-, β-thujone, and several other oxygenated monoterpenes (1,8-cineole, linalool, camphor, boneol, and bornyl acetate) and oxygenated sesquiterpenes (caryophyllene oxide, viridiflorol, humulene epoxide I, II, and III). Tenax TA® and HS sorptive extraction extracted a lower number of identified compounds, whereas HS solid-phase microextraction allowed the complete extraction of volatiles with particular reference to α- and β-thujone. The importance of the determination of thujones content in sage herbal tea is also discussed.

Abbreviations: HS, headspace; HS-SPME, HS solid-phase mi- croextraction; HSSE, HS sorptive extraction; SBSE,stirbar sorptive extraction; TDU, Ger stel thermal desorption unit

## 1 Introduction

*Salvia officinalis* L. (sage) is an aromatic small perennial shrub native to the Mediterranean region and it is largely cultivated for culinary and medicinal purposes [1]. Terpenoids and phenolics have been identified as the two major typical sage secondary metabolites. Among the terpenoids, volatile oils have been largely investigated [2, 3]. Sage essential oils have been studied for their toxicity [4, 5] and antimutagenic [6], antimicrobial [7-9], antiviral [10], preservative [11, 12], immunomodulatory [13], antioxidant [1, 14], larvicidal [15], and anticancer [16] properties.

However, a significant amount of terpenoids is also present in sage teas, contributing to their antioxidant [17-20], hepatotoxic [21], antimutagenic [22], and antidiabetic [23] effects.

Thujone is a natural monoterpene also associated with sage. There is currently a heated debate on the toxicity of thujones [24]. On one hand, the specific limits for sage preparations were removed from the European regulation, so that *S. officinalis* and other thujone-containing flavoring plants can now be used in foods without restrictions [25]. On the other hand, the European Medicines Agency (EMA) has evaluated herbal medicinal products containing sage and set an acceptable daily intake of 5.0 mg/person in the *S. officinalis* monograph, because of the presence of thujones [26]. While this warning is obvious for alcohol-containing products, the scientific foundation of applying the warning for the ingestion of thujone in the form of aqueous extracts such as sage tea is questionable [25]. Currently, no risk appears to be associated with the occasional medicinal use of sage (especially in the traditional use as herbal tea); however, the data regarding thujone exposure through medicines are extremely limited.

From an analytical point of view, variations in the compositions of the sage thujone fraction are considerable depending on the quality of the plant material as well as the methods used for extraction. Besides solvent extraction or hydrodistillation, headspace (HS) analyses have been used to characterize volatile fractions from aromatic plants [27, 28] and are particularly suitable for herbal teas. Several adsorbing systems are available for the HS analysis of volatile terpenes. With thermal desorption, the most commonly used sorbent is Tenax TA®, a macroporous, semicrystalline polymer manufactured from 2,6-diphenyl-*p*-phenylene oxide [27]. HS analysis of sage has been performed on some *Salvia* species by using solid-phase microextraction (HS-SPME) [29], which is a powerful analytical tool for profiling the terpenoid metabolomic patterns [30]. Another HS technique is HS sorptive extraction (HSSE), which uses stir bars used for stir bar sorptive extraction (SBSE) followed by thermal desorption and GC–MS [31-33]. To the best of our knowledge, there are no reports on sage tea HS-SPME, HSSE, or Tenax TA® analyses.

Since one of the major dietary contributions of thujones appears to derive from sage infusion, the purpose of this research is the comparison of different extraction methods for thujone and other volatiles from sage, including HSSE, Tenax TA®, and HS-SPME, followed by GC–MS analysis. Owing to the wide application of sage components for food and pharmaceutical industries, our results give a new contribution in the field of volatilome analysis of this important industrial plant.

# 2 Materials and methods

## 2.1 Plant material

*Salvia officinalis* L. dried leaves were purchased from the Pharmaceutical Laboratory Labofarm (Starogard Gdanski) in Poland. Samples were ground using an electrical blender and stored in closed containers at 5°C in the dark.

## 2.2 Preparation of sage tea

HS analyses were performed by following the guidelines suggested by Brevard et al. [34]. Sage tea infusions were prepared by immersing 4 g of sage dried leaves into 400 mL of boiling MilliQ water for 15 min. The herbal tea was then filtered through a Whatman No. 4 paper. The filtered sample (7 mL) was placed in a 20 mL HS-SPME Teflon-sealed vial (Supelco, Bellefonte, PA, USA) and left for 2 h at 45°C in a water bath for equilibration.

## 2.3 HS analysis using HS-SPME, HSSE, and Tenax TA®

Pulegone (Fluka, USA) was added to the sage tea as internal standard for HS-SPME, HSSE, SBSE, and Tenax TA® extractions. Before use, HS-SPME, Twisters, and Tenax TA® were conditioned at 250°C, according to the manufacturer's instructions. Temperature, equilibration time, and extraction time were selected after several experiments combining different temperatures (20, 40, 60, and 80°C), equilibration times (2, 5, 30, 90, 120, and 240 min), and extraction times (2, 5, 10, 20, and 30 min).

- *HS-SPME*: An HS-SPME fiber holder was clamped above the vial and a carboxen/polydimethylsiloxane HS-SPME fiber (model 57334-U, Supelco) was inserted into the HS of the infusion for a static extraction of 5 min, which was found to be the shortest time needed for exhaustive extraction. The fiber was then withdrawn into the needle of the holder and inserted into the injection port of the GC–MS system (see Section 'GC–MS').
- *HSSE*: Stir bars (Twister from Gerstel, Germany, 0.5 mm thick, 10 mm long, polydimethylsiloxane coating) were suspended in the HS-SPME Teflon-sealed vial HS for 5 min with a glass insert (Gerstel, Germany) and static extraction was performed as previously described [35]. The stir bar was then introduced in the Gerstel thermal desorption unit (TDU) connected to a Gerstel-cooled injection system 3 cryofocusing system as previously reported [35] and subjected to GC–MS analysis (see Section 'GC–MS').
- *Purge and Trap of Tenax TA*®: The sage tea HS was purged with a constant flow with a GC-grade air generator (HPZA-3500–220, Parker Balston, Cleveland, OH, USA) at 200 mL/min for 5 min. Clean glass TDU liners (Gerstel) were filled with 20 mg sorbent Tenax TA® 60/80 2,6-diphenyl-*p*-phenylene oxide (Supelco) as previously described [36]. Tenax TA® was desorbed in the TDU connected to a Gerstel-cooled injection system 3 cryofocusing system that uses liquid $CO_2$ as a cooling agent. Desorption was carried out as described earlier [36].

For semiquantitative analyses, calibration curves were prepared by using solutions of the compounds to be quantified exceeding 20% of the whole composition in the concentration range of the same compounds in the sample. For compounds representing <20% of the whole composition, semiquantification was performed using one pure standard for each class of similar components. The medium used for the calibration was the same as the sample matrix, but free of the target compounds.

## 2.4 Sage tea direct analysis by SBSE

- *SBSE*: A Twister stir bar (same as the one described above) was placed in the HS-SPME Teflon-sealed vial and stirred in the filtered sage tea for 5 min at 46°C. After extraction, the Twister was patted dry with a clean sterile cheesecloth. The stir bar was then introduced in the TDU as previously reported [35] and subjected to GC–MS analysis (see Section 'GC–MS').

## 2.5 GC–MS

HS-SPME, HSSE, Tenax TA®, and SBSE volatiles were desorbed as described above and analyzed by GC (Agilent Technologies, model 6890N) coupled with MS (Agilent Technologies, model 5973A). Compounds were separated on a Zebron ZB-5MS (model 7HG-G010-11, Phenomenex, USA) capillary column (stationary phase: 95% polydimethyl siloxane/5% diphenyl, 30 m length, 250 μm id, 0.25 μm film thickness) with the following temperature program: 60°C for 5 min

followed by a temperature rise at a 3°C/min rate to 270°C (held for 5 min). Twisters and Tenax TA® were exposed in the TDU port during the entire GC run. The HS-SPME fiber was inserted in the injection port and exposed during the entire GC run. Carrier gas was He with a constant flow of 1 mL/min, transfer line temperature to MSD was 280°C, ionization energy 70 eV, and full scan range 50–300 *m*/*z*. Quantitative analyses were confirmed by GC coupled to a flame ionization detector performed with the same column and GC conditions as above.

### 2.6 Component identification

Separated compounds were identified by pure standard comparison, by comparison of their mass spectra and linear retention indices (Kováts index) with those of reference substances and by comparison with the NIST mass spectral search software v2.0 using the libraries NIST 98 library. Linear retention indices were calculated against a mixture of $C_8$–$C_{20}$ *n*-alkanes. Literature indices were taken from Adams [37]. External calibration curves were made with standard solutions of α-thujone, camphor, and borneol (99%, Fluka) for quantitative measurements as previously described [38]. Pulegone (99%, Fluka) was used as internal standard in all sage tea extractions.

### 2.7 Statistical analysis

The overall data sets are expressed as mean values of at least five replicates. Three technical replicates were run for each biological replicate. Analysis of variants (ANOVA) and Tukey–Kramer's Honestly Significant Difference (HSD) test ($P < 0.05$) were used to determine significant differences among extractions using the SYSTAT 10 software.

## 3 Results and discussion

This study aims to compare different extraction methods for α- and β-thujone analysis in the food and medicinal plant *S. officinalis* L. Since sage tea is reported to possess biological activity, we used different HS extraction methods, HS-SPME, HSSE, Tenax TA®, and a liquid sorption method, as well as SBSE, to characterize the terpene volatile fraction in this herbal tea.

### 3.1 HS analysis of sage tea

Sage is used as a herbal medicinal product, with the most typical form of application as an infusion with boiling water. Most of the literature has focused on phenolic antioxidant compounds [17, 20, 39]; however, components of the essential oil fraction present in sage tea, particularly thujones, may contribute to its biological activity [18, 21, 23]. Furthermore, since most of the literature refers to solvent extraction of sage tea terpenes, there is a lack of information on HS composition. In general, all HS methods used were able to extract both α- and β-thujone (Supporting Information Fig. S1).

Among HS methods, HSSE and Tenax TA® showed the lowest performance to extract volatiles from sage tea. In fact, of over 20 identified terpenes, only nine and 11 compounds were detected from HSSE and Tenax TA®, respectively (Table 1 and Supporting Information Fig. S1). However, HSSE was found to extract significantly ($P < 0.05$) higher amounts of β-thujone, 1,8-cineole, and bornyl acetate compared to the other extraction methods.

Table 1. Comparative chemical analysis among sage (*Salvia officinalis* L.) tea HS analyses by using HSSE, Tenax-TA® purge and trap extraction, HS-SPME, and terpene sorption by SBSE

| Compound | LRI | HSSE | Tenax-TA | SPME | SBSE |
|---|---|---|---|---|---|
| 1,8-Cineole | 1031 | 18.60 (1.65)[b)] | 1.65 (0.30)[c)] | 10.45 (2.30)[a)] | 9.25 (3.45)[a)] |
| Linalool | 1097 | 3.30 (0.01)[b)] | tr | 7.92 (0.90)[c)] | tr |
| α-Thujone | 1103 | 18.45 (8.75)[a)] | 12.65 (3.01)[a)] | 26.95 (9.70)[a)] | 17.90 (3.65)[a)] |
| β-Thujone | 1114 | 29.50 (3.41)[c)] | 9.20 (2.70)[b)] | 10.75 (3.15)[b)] | 11.65 (1.10)[b)] |
| Camphor | 1146 | 22.40 (3.65)[a)] | 15.40 (1.60)[a)] | 21.50 (5.70)[a)] | 25.05 (2.02)[a)] |
| Pinocamphone | 1163 | 3.00 (0.95)[a)] | 0.30 (0.05)[b)] | 2.65 (0.90)[a)] | 1.80 (0.80)[a)] |
| Isomenthone | 1163 | tr | 0.15 (0.09)[a)] | 1.35 (0.50)[a)] | 0.85 (0.20)[a)] |
| Borneol | 1169 | 22.60 (1.52)[a)] | 4.60 (1.00)[c)] | 15.40 (0.80)[b)] | 34.15 (1.65)[c)] |
| Terpinen-4-ol | 1177 | tr | 0.40 (0.10)[a)] | 7.20 (1.60)[c)] | 3.00 (0.40)[b)] |
| α-Terpineol | 1189 | tr | 0.70 (0.35)[a)] | tr | 4.30 (0.75)[b)] |
| Carvone | 1243 | tr | 0.15 (0.05)[b)] | 2.00 (0.35)[a)] | 1.65 (0.10)[a)] |
| Bornyl acetate | 1289 | 25.90 (2.95)[c)] | 2.10 (0.80)[b)] | 21.05 (2.20)[c)] | 8.45 (1.04)[a)] |
| Thymol | 1290 | tr | tr | 1.10 (0.25)[a)] | 0.93 (0.42)[a)] |
| Carvacrol | 1299 | tr | tr | 1.35 (0.20)[a)] | 1.30 (0.13)[a)] |
| Caryophyllene oxide | 1583 | tr | tr | 4.75 (0.15)[b)] | 1.80 (0.35)[a)] |
| Viridiflorol | 1593 | 2.75 (0.35)[b)] | tr | 11.55 (3.65)[a)] | 13.20 (0.15)[a)] |
| Humulene epoxide I | 1604 | tr | tr | 5.25 (1.11)[a)] | 2.20 (0.01)[b)] |
| Humulene epoxide II | 1608 | tr | tr | 10.53 (1.25)[a)] | 4.35 (0.10)[b)] |
| Humulene epoxide III | 1615 | tr | tr | tr | 0.65 (0.04)[a)] |
| Unknown sesquiterpene alcohol | 1648 | tr | tr | 6.85 (2.95)[a)] | 5.74 (1.74)[a)] |
| Total | | 146.50 (8.17)[a)] | 47.30 (2.58)[b)] | 168.60 (7.96)[a)] | 148.22 (7.77)[a)] |

1. Values are expressed as milligram per kilograms dry weight. SD is shown in parentheses. In the same row, different letters indicate significant ($P < 0.05$) differences. LRI, linear retention index calculated against a $C_8$–$C_{20}$ *n*-alkanes mixture; tr, traces (<0.1 mg/kg dry weight).

HS-SPME showed a higher ability to extract volatiles from sage tea HS, when compared to HSSE and Tenax TA®. In particular, HS-SPME extracted both α- and β-thujone and possessed a significantly higher extraction capacity for linalool, terpinen-4-ol, caryophyllene oxide, and humulene epoxide II. Since HS-SPME fibers have shown reliable performance in terms of both repeatability and intermediate precision of analytes recovery and consistency over time [29], we suggest the use of this method when routine control analysis of HS sage tea α- and β-thujone are required. Furthermore, this method is also suitable to characterize the total sage tea volatile fraction perceived by the olfactory system during drinking.

## 3.2 Sage tea terpene sorption by SBSE

SBSE has been applied successfully to trace analysis from liquid samples and has the analytical reproducibility needed in recording the analytical profiles of volatile and semivolatile components of biological mixtures [31]. Therefore, we used this method to evaluate the terpene fraction dissolved in the sage tea and as a further indicator of the amount of thujones that are potentially ingested during sage tea drinking. SBSE showed a good sorption ability for both α- and β-thujone.

When compared to HS extraction methods, SBSE achieved a significantly higher ($P < 0.05$) extraction of some terpenoids including borneol, α-terpineol, and humulene epoxide III (Table 1 and Supporting Information Fig. S1).

# 4 Concluding remarks

The results reported here show that HS-SPME allows the complete extraction of sage tea volatiles, with particular reference to α- and β-thujone. From a quantitative point of view, HSSE showed a significantly higher ability to extract β-thujone. Between these two methods, HS-SPME is the most economical and does not require a thermal desorption unit, as desorption is carried out directly by the GC–MS injector [27, 29, 40]. Furthermore, this method has been successfully applied for multiresidue analysis of pharmaceutical drugs [41] and chemotherapy controls [42]. Therefore, owing to the need to establish cheap, reliable, and sensitive methods for the detection of α- and β-thujone in sage tea, we suggest the use of HS-SPME for qualitative and semiquantitative determination.

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