Simultaneous distillation–extraction of high-value volatile compounds from *Cistus ladanifer* L.

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Abstract

The present paper describes a procedure to isolate volatiles from rock-rose (*Cistus ladanifer* L.) using simultaneous distillation–extraction (SDE). High-value volatile compounds (HVVC) were selected and the influence of the extraction conditions investigated. The effect of the solvent nature and extraction time on SDE efficiency was studied. The best performance was achieved with pentane in 1 h operation. The extraction efficiencies ranged from 65% to 85% and the repeatability varied between 4% and 6% (as a CV%).

The *C. ladanifer* SDE extracts were analysed by headspace solid phase microextraction (HS-SPME) followed by gas chromatography with flame ionization detection (GC-FID). The HS-SPME sampling conditions such as fiber coating, temperature, ionic strength and exposure time were optimized. The best results were achieved with an 85 µm polyacrylate fiber for a 60 min headspace extraction at 40 °C with 20% (w/v) of NaCl. For optimized conditions the recovery was in average higher than 90% for all compounds and the intermediate precision ranged from 4 to 9% (as CV %). The volatiles α-pinene (22.2 mg g<sup>−1</sup> of extract), 2,2,6-trimethylcyclohexanone (6.1 mg g<sup>−1</sup> of extract), borneol (3.0 mg g<sup>−1</sup> of extract) and bornyl acetate (3.9 mg g<sup>−1</sup> of extract) were identified in the SDE extracts obtained from the fresh plant material.

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1. Introduction

*Cistus ladanifer* L. is a native and widespread species in the Mediterranean region [1,2]. In Portugal, *C. ladanifer* L. is widely distributed, being one of the most abundant species in the southern part of the country, occurring in large areas as pure dense stands [3]. This strong aromatic shrub secretes a gum resin, known as labdanum, which has been extracted in countries such as Spain, France and Portugal. Different odoriferous materials are obtained from the plant, such as Cistus oil, labdanum gum and labdanum oil. The “Cistus” products are particularly appreciated for their balsamic odour, as well as for their fixative properties [4,5]. Several research works have been reported in the literature on *C. ladanifer* L. volatile compounds, due to the great importance of this raw material for the fragrance industry (Table 1).
the first separator recovering the waxes and the second stage recovering the desired volatile oils. In addition, SFE is still an expensive extraction technique.

Among the several techniques that have been developed to isolate volatile compounds, simultaneous distillation–extraction (SDE), introduced in 1964 by Likens and Nickerson, is one of most widely employed. This method has been successfully applied in the extraction of essential oils [12–14], aroma compounds [15,16] and other volatile products [17–19] from numerous matrices. In the flavour area, this technique is usually considered to be superior to classical ones, such as distillation or solvent extraction. This technique combines steam distillation together with continuous extraction with a solvent or a mixture of solvents [20].

This one-step extraction technique is less time consuming and allows, due to the continuous recycling, a greater reduction of solvent volumes. Under certain conditions a higher concentration factor can be achieved and the direct analysis of the extract can be performed without further concentration. Moreover, given its particular characteristics, this technique allows to carry out the analysis without a sample clean-up step. The extracts obtained by SDE are free from non-volatile materials such as cuticular waxes or chlorophylls.

Because of the high extraction efficiencies associated to the high reproducibility, SDE has also been used to quantify volatile compounds in several matrices [20]. The recoveries are however dependent on the operation conditions and therefore the best experimental conditions should be investigated. As far as the authors knows, the use of this technique to isolate volatile compounds of C. ladanifer L., has not been reported before.

The chromatographic analysis of essential oils is often a difficult task. Even the use of high-efficient capillary columns cannot avoid the possibility of the co-elution of components. The complexity of an aroma mixture makes it almost unavoidable. To overcome this problem, extracts are usually fractionated before analysis. The drawbacks of this procedure are well known. On the basis of these considerations and taking into account that C. ladanifer L. essential oil has been reported as having a complex chemical composition [7], with nearly 400 different components detected by GC [11], it seemed useful to develop a simple and accurate procedure to analyse the extracts of C. ladanifer L. obtained by SDE. To achieve this aim, we explored the possibility of using the solid phase microextraction (SPME) technique. This is a solvent-free sampling technique, low cost, reproducible, and easy to use. Headspace SPME (HS-SPME) in particular, has been widely applied to the aroma analysis of various matrices [21–24]. The ability to isolate and concentrate the volatile compounds, without interferences from the matrix is the most obvious advantage. Furthermore, the selectivity of the analysis can be enhanced by the selection of the stationary phase that best suits the analytes [25]. Two different HS-SPME fiber coatings were evaluated, aiming the microextraction of the target flavour components.

This paper focuses on the application of the SDE technique to isolate and quantify high commercial value volatile compounds of C. ladanifer L., as well as a methodology of analysis employing SPME–GC–FID.

2. Experimental

2.1. Reagents and standards

The analytical standards with purity higher than 99% were purchased from Sigma–Aldrich (Sintra, Portugal) and used without further purification. The aroma standards used are α-pinene (pin), 2,2,6-trimethylcyclohexanone (hex), linalool (lin), borneol (bor), α-terpineol (ter), citronellol (cit), geraniol (ger), eugenol (eug), bornyl acetate (bor-ac), and geranyl acetate (ger-ac).

Individual stock standard solutions of about 100 mg L\(^{-1}\) from each of the above compounds were prepared in ethanol (LiChrosolv, Merck, Darmstadt, Germany). A stock solution, containing all the above compounds, was prepared by appropriate dilutions in ethanol (LiChrosolv, Merck). Working standard solutions (1% (v/v) in ethanol), containing all compounds, were prepared daily by appropriate dilutions of the stock solution in

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Table 1
Summary of some studies on Cistus ladanifer reported in the literature

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction method</th>
<th>Analysis method</th>
<th>No. of detected compounds</th>
<th>Main constituents</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. ladanifer (commercial oil produced in Spain)</td>
<td>Steam distillation</td>
<td>GC/FID, GC/MS</td>
<td>85</td>
<td>α-Pinene, camphene, 2,2,6-trimethylcyclohexanone, p-cymene, bornyl acetate</td>
<td>[6]</td>
</tr>
<tr>
<td>C. ladanifer (Spanish origin but cultivate in Corsica)</td>
<td>Hydrodistillation</td>
<td>GC/FID, 13C-NMR</td>
<td>45</td>
<td>α-Pinene, viridiflorol ledol, bornyl acetate</td>
<td>[7]</td>
</tr>
<tr>
<td>C. ladanifer (Portuguese origin)</td>
<td>Supercritical-CO(_2)</td>
<td>GC/FID, GC/MS, GC/sniffing</td>
<td>18</td>
<td>Acetophenone, 2,2,6-trimethylcyclohexanone, 2-phenylethanol, borneol</td>
<td>[8]</td>
</tr>
<tr>
<td>C. ladanifer (Spanish origin)</td>
<td>Supercritical-CO(_2)</td>
<td>GC/FID</td>
<td>41</td>
<td>Champhor, α-pinene</td>
<td>[9]</td>
</tr>
<tr>
<td>C. ladanifer (var. maculates), C. ladanifer (var. albiflorus) (Spanish origin)</td>
<td>Steam distillation</td>
<td>GC/FID, GC/MS</td>
<td>22</td>
<td>α-Pinene, viridiflorol, 2,2,6-trimethylcyclohexanone, trans-pinocarveol, terpinen-4-ol</td>
<td>[10]</td>
</tr>
</tbody>
</table>

a No. of identified compounds.
water (LiChrosolv, Merck). Sodium chloride (PA grade) and anhydrous sulphate (PA grade) were purchased from Panreac (Barcelona, Spain). Pentane was purchased from Riedel-de Häen (Buchs, Switzerland), hexane and cyclohexane were purchased from Panreac (Barcelona, Spain); diethyl ether was purchased from Merck. All these solvents were analytical grade.

2.2. Plant material and volatiles extraction

C. ladanifer L. branches were collected randomly, from wild growing plants in the Miranda region (Northern Portugal) in July. The samples were stored at room temperature prior to analysis for a period inferior to 48 h.

The essential oil samples were isolated from the fresh material (~15 g leaves plus 300 mL of distilled water) by simultaneous distillation–extraction (SDE) for 1 h, using a modified Likens–Nickerson apparatus [26] with organic solvent pentane (50 mL). These operating conditions were obtained after optimization. The extractions were carried out at atmospheric pressure. The sample was kept at 120 ± 2°C, the solvent at 50 ± 2°C and the condensation system at 5.0 ± 0.1°C.

The extracts were dried with anhydrous sulphate and concentrated at room temperature, under vacuum, by rotatory evaporator, until solvent evaporation. The collected oil was weighed (mean value of 88.5 mg for six replicate extractions), dissolved in 5 mL ethanol and stored at 4°C until analysis. The optimization of the SDE experimental conditions was carried out using standard solution mixtures. The influence of the solvent nature and the extraction time on the extraction efficiency of the target compounds was investigated.

2.3. SPME analytical procedure

A SPME fiber holder (manual) and 85 μm polyacrylate (PA) and 100 μm polydimethylsiloxane (PDMS) fibers were purchased from Supelco (Bellefonte, PA, USA). The fibers were conditioned before use in the GC injector according to the manufacturer’s recommendations. Blank runs were performed periodically to check possible fibers contamination or carry-over.

A Corning stirrer/hot plate (Supelco) was used to heat and agitate samples (4 mL vials filled with 2 mL sample). After extraction, the fibers were inserted into GC injector for 5 min, in splitless mode, at 260°C. This procedure was enough to ensure total desorption and no carry-over was observed. For the SPME optimization (fiber coating, temperature, ionic strength, exposure time), a standard solution containing all compounds, with an average concentration of 100 μg L⁻¹ per compound was used. All the studies were performed in duplicate and under constant stirring velocity.

2.4. Gas chromatographic analysis

The gas chromatographic analyses were carried out on a Finnigan 9000 (Austin, USA) equipped with a flame ionization detector (FID). The GC was equipped with a Varian (Walnut Creek, CA, USA) CP-Sil 5CB capillary column (30 m × 0.53 mm × 1.5 μm). The oven temperature was initially kept at 70°C for 1 min, then programmed at 2 ℃ min⁻¹ up to 150°C and held for 1 min and finally increased to 280°C at 15 ℃ min⁻¹ and kept for 15 min. The split/splitless injector and detector temperatures were set at 260 and 300°C, respectively. Both carrier and make-up gases were helium (99.999% purity) at 5 and 25 mL min⁻¹, respectively. The total run time was 66 min. The compounds were identified by comparison with the retention time of the standards and, to avoid any doubts, the standard addition method was employed for peak verification.

2.5. Quantification

Compounds were quantified by peak area using the external standard method. Individual calibration curves were obtained extracting the compounds by SPME from aqueous standards.

The SDE samples of C. ladanifer were extracted in the same conditions as standards (PA fiber, sample heated at 40°C, salt concentration of 20% (w/v) and extraction period of 60 min) after dilution (1:800). The ethanol content in the aqueous C. ladanifer samples was 1% (v/v).

3. Results and discussion

3.1. Optimization of SDE extraction

SDE exploits the differences in volatility and polarity among the analytes and other non-volatile components present in the matrix. The extracting solvent plays a key role in the process; it has to be chosen to facilitate the extraction of the components that are important as well as to exclude or limit the components that could interfere with the analysis. Another parameter of strong influence is the extraction time. It is known that a matrix containing lipids strongly increases the time required for an acceptable recovery. However, for a non-fat material, such as plants, a time of extraction of 1–2 h is generally enough [20].

For these reasons the influence of the quoted parameters (solvent nature and extraction time) on the SDE selectivity and extraction yield was investigated in order to find the best experimental conditions to recover the selected compounds.

Ten target volatile compounds were chosen, α-pinene, 2,2,6-trimethylcyclohexanone, linalool, borneol, α-terpineol, citronellol, geraniol, eugenol, bornyl and geranyl acetate. All of them have been described as constituents of the C. ladanifer essential oil [6–11]. The compounds α-pinene and 2,2,6-trimethylcyclohexanone have been referred in the literature as major constituents of the essential oil and were therefore included in this study. The other selected compounds are considered high-value substances for the perfume industry and cosmetics and can be found in the composition of numerous personal care products and fragrances.

The runs necessary for the SDE optimization were carried out in duplicate, with aqueous samples spiked with a standard mixture containing the selected compounds on a concentration of ~100 mg L⁻¹ each. The sample was heated at 120°C and the tested solvents at a temperature 20°C above the respective boiling point.
Table 2
Influence of solvent on the SDE extraction efficiency

<table>
<thead>
<tr>
<th></th>
<th>Pentane</th>
<th>Hexane</th>
<th>Cyclohexane</th>
<th>Diethyl ether</th>
<th>Pentane + diethyl ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>66</td>
<td>57</td>
<td>52</td>
<td>61</td>
<td>52</td>
</tr>
<tr>
<td>2,2,6-Trimethylcyclohexanone</td>
<td>75</td>
<td>69</td>
<td>64</td>
<td>72</td>
<td>70</td>
</tr>
<tr>
<td>Linalool</td>
<td>84</td>
<td>78</td>
<td>75</td>
<td>84</td>
<td>80</td>
</tr>
<tr>
<td>Borneol</td>
<td>83</td>
<td>74</td>
<td>69</td>
<td>85</td>
<td>77</td>
</tr>
<tr>
<td>α-Terpinol</td>
<td>81</td>
<td>77</td>
<td>74</td>
<td>80</td>
<td>81</td>
</tr>
<tr>
<td>Citronellol</td>
<td>82</td>
<td>73</td>
<td>68</td>
<td>82</td>
<td>84</td>
</tr>
<tr>
<td>Geraniol</td>
<td>82</td>
<td>73</td>
<td>69</td>
<td>80</td>
<td>83</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>83</td>
<td>77</td>
<td>70</td>
<td>83</td>
<td>78</td>
</tr>
<tr>
<td>Eugenol</td>
<td>71</td>
<td>66</td>
<td>66</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td>Geranyl acetate</td>
<td>78</td>
<td>76</td>
<td>70</td>
<td>82</td>
<td>76</td>
</tr>
</tbody>
</table>

Extraction of aqueous samples spiked with a standard mixture, with an average concentration of ~100 mg L⁻¹, for 60 min. Sample heated at 120 °C and solvent heated at 20 °C above the boiling point.

Four solvents (pentane, cyclohexane, hexane and diethyl ether) and a solvent mixture (pentane/diethyl ether, 1:1 v/v) were used in order to evaluate different extractive selectivities (Table 2). In these studies the extraction time was set to 60 min.

The best results were obtained with solvents pentane, diethyl ether and solvent mixture (pentane/diethyl ether) with average extraction efficiencies of 79 ± 6%, 78 ± 8% and 76 ± 9%, respectively. The difference between these solvents, under the experimental conditions described above, either in what concerns extraction yields or selectivity towards the compounds extracted, was slight.

Higher differences, especially when compared to pentane, were observed for the extractions with hexane and cyclohexane, where extraction efficiencies achieved were in average 72 ± 6% and 68 ± 7%, respectively. Based on these results, pentane was the selected solvent for the studies carried out subsequently.

The effect of extraction time on the SDE efficiency was the last parameter being evaluated. Four different extraction times (30, 60, 90 and 120 min) were tested and the results showed that, for most of the compounds, an increase in the time of extraction above 60 min has a small effect in the yield or no effect at all (Fig. 1). However, for the compounds α-pinene and eugenol the extraction time seems to have a more pronounced effect in the yield.

In the first case, a decrease in the extraction efficiency was noticeable after 60 min, probably due to analyte losses. On the other hand, the recovery of eugenol seems to have reached a maximum after 90 min. These compounds are, respectively, the most and less volatile compound among the studied compounds which may justify the observed results.

Average extraction efficiencies were of 75%, 79%, 80% and 78% at 30, 60, and 90 and 120 min, respectively and therefore a 60-min extraction time was selected as the best compromise between extraction efficiency and time. Moreover, a longer extraction time could lead to thermal degradation or interactions with consequent artefact formation.

As a result of the optimization studies an operation time of 60 min with the solvent pentane was selected for the extraction of the *C. ladanifer* leaves.

### 3.2. Optimization of SPME operating conditions

The sensitivity of the SPME technique depends on several factors such as the coating material of the fiber, temperature of the sample, ionic strength of the matrix, extraction time and fiber exposure time. Each of these factors was investigated to determine the optimum material and operating conditions for the analysis of the volatiles of the *C. ladanifer* extracts. A standard solution containing all compounds, with a concentration of ~100 µg L⁻¹ of each compound was used for the SPME optimization. All runs were made on duplicate and performed under constant stirring velocity.

Two fiber coatings were investigated in this study: poly(dimethylsiloxane) (PDMS) and poly(acrylate) (PA). PDMS is a nonpolar rubbery coating that is known to work very effectively for a wide range of analytes, both polar and nonpolar [21,25]. On the other hand, the PA coating offers a vitreous (the glass transition temperature \( T_g \) is 44 °C) and more polar membrane.

For most of the studied compounds, the PA coated fiber showed higher extraction efficiency than the PDMS fiber (Fig. 2). With the exception of the α-pinene, 2,2,6-
trimethylcyclohexanone, bornyl and geranyl acetates, which are less polar compounds, and were extracted to a higher degree into the nonpolar PDMS coating, the remaining analytes were extracted proportionally more into the polar PA fiber. In fact these compounds have alcohol functional groups (hydroxyl groups), which provides them with higher polarity and consequently more affinity to the fiber PA.

As a result, the PA coated fiber was selected for the following experiments mainly due to the higher affinity (than the PDMS fiber) to the monoterpenic alcohols, which are the most valuable flavours.

The influence of the sample temperature on the analysis of headspace volatiles was studied for 40, 50 and 60 °C. For most of the compounds considered, the extraction efficiencies decreased while the heating temperature increased (Fig. 3). The only exceptions to this behaviour were eugenol and geranyl acetate, for which the best temperature was 50 °C, probably because these analytes have the lowest vapor pressure.

As previously pointed out, the concentration in the headspace of the analytes normally increases with the temperature, enhancing their extraction kinetics, but it should be balanced by the fiber/headspace partition coefficients that decrease with the temperature increase [25]. The amount of the volatile compounds absorbed on the SPME coating can be determined from the equation $n = C_0 V_1 V_2 K_1 K_2/(K_1 K_2 V_1 + K_2 V_3 + V_2)$, where $n$ is the mass of the volatile compound absorbed by the SPME coating, $C_0$ the initial concentration of the volatile compound in the sample; $V_1$, $V_2$ and $V_3$ the volumes of SPME coating, sample and headspace, respectively, $K_1$ the partition coefficient of the volatile compounds between the SPME coating and the headspace, and $K_2$ is the partition coefficient between the headspace and the sample [25]. The $n$ value depends on the partition coefficient ($K$) of the volatile compounds between the SPME coating and the sample. Since the partition coefficient $K$ is equal to $K_1 K_2$ it is controlled by both the partition coefficient $K_1$, between the SPME coating and the headspace, and the partition coefficient $K_2$, between the headspace and the sample [25].

As the temperature of the sample increased, more molecules of the compounds are usually released to the headspace so that $K_2$ increased. However, the absorption is an exothermic process; more molecules absorbed on the SPME coating diffused to the headspace with increasing temperature, which inversely decreased the partition coefficient $K_1$. Since $K_1 \gg K_2$ for most organic compounds [25], the net effect of the increased temperature decreases $K$. This may explain the optimized extraction temperature observed of 40 °C. A lower extraction temperature is also preferable because the probability of thermal decomposition occurrence is also lower.

Salt addition to aqueous samples generally increases the amount of analytes extracted [25]. This approach is widely used to enhance the sensitivity of analytical methods by lowering the solubility of compounds in the aqueous phase and is commonly referred to as “salting out”. For most of the analysed compounds, peak areas increased significantly with the salt concentration (0, 10, 20, 30% (w/v) NaCl) (Fig. 4). A salt content of 20% (w/v) had the effect of improving the amount extracted of $\alpha$-terpineol by a factor of 4.9 while with a salt concentration of 30% (w/v) the extracted amount was almost 10 times higher. Similar results were obtained for the other analytes. The salt addition decreased the solubility of the compounds in water, especially the most polar ones, increasing the extraction efficiency.

Two exceptions were observed to this behaviour, $\alpha$-pinene and geranyl acetate. For $\alpha$-pinene the chromatographic signal decreased as the amount of salt increased. A slight decrease was observed for a salt amount of 10% (w/v) but a decrease of about 27% in the chromatographic signal was observed for a 30% (w/v)
salt amount. Other authors have reported similar results. Steffen and Pawliszyn [21] observed that for α-pinene the solution with no salt was the most effective for the extraction. Yang and Peppard [27] observed that the extraction efficiency of limonene, also a terpene hydrocarbon such as α-pinene, decreased with increasing salt concentration.

For geranyl acetate the maximum extraction yield was observed for a 20% (w/v) salt concentration while the other compounds were extracted to a higher degree from a 30% (w/v) NaCl solution.

Although the best extraction efficiencies were achieved with the highest salt concentration, the reproducibility of the analysis was lower (CV ~ 9.4% on average). So a 20% (w/v) NaCl solution was selected as a compromise between a small CV (~5% on average) and the higher extraction. Other researchers have reported similar findings when using HS-SPME for the analysis of flavour compounds in wines [23].

The results obtained when studying the effect of exposure time on the extraction efficiency of the HS-SPME shows it increases with the exposure time (30, 60, 120 and 180 min). As it can be seen in Fig. 5, the amount extracted increases rapidly during the first 30 min. For most of the analytes, the sorption equilibrium seems to be reached within the time range considered. Longer exposure times did not increase considerably the total amount of volatile compounds extracted. An increase of about 6% was observed when the extraction time was raised from 120 to 180 min. However, the effect of the extraction time was more effective for the geranyl acetate and eugenol compounds. The data suggest that these compounds need a longer exposure time to achieve equilibrium. Eugenol is the less volatile compound and the most soluble one, therefore the concentration of this analyte in the headspace is lower than the other studied compounds. In this case the headspace extraction may affect the concentration of the aqueous phase and the extraction takes more time to reach equilibrium. The geranyl acetate behaviour may be explained by its relatively strong affinity to the fiber coating. As a higher amount of geranyl acetate is extracted by the PA coating, due to a higher partition coefficient between the fiber coating and the headspace, more time is needed to reach equilibrium.

Although SPME has a maximum sensitivity at the partition equilibrium, full equilibration is not necessary for quantitative analysis. A proportional relationship is obtained between the amount of analyte extracted by the fiber and its initial concentration in the sample matrix before reaching partition equilibrium.

Therefore, an extraction time of 60 min was selected. It seems to represent the best compromise between the extraction yield and the time required for the analysis without affecting the sensitivity or the reproducibility.

The effect of the fiber desorption time was also evaluated. A period of 5 min was found to be enough to desorb the analytes from the PA fiber at 260 °C. This procedure allowed reproducible and quantitative transfer of target analytes into the injector port. Intermediate blank runs were performed and no remaining compounds at the fiber were detected.

3.3. Performance evaluation of the analytical method

The validation of the proposed HS-SPME–GC/FID method included investigation of precision, estimation of the linear range and recovery studies.

Standard curves were obtained using HS-SPME extraction at optimal sampling conditions (PA fiber, 60 min extraction time, sample kept at 40 °C, continuously agitated and 20% (w/v) in NaCl). The standard mixtures were diluted in water (1% (v/v) in ethanol), from the working individual standards. Six levels of concentration (ranges shown in Table 3) were tested in duplicate and regression lines were calculated for each compound (plotting the areas versus the concentration).

In order to evaluate precision, repeatability and intermediate precision were investigated. For all the compounds the coefficients of variation (CV) obtained fell below 10%.

Repeatability values ranged from 3% to 9%, obtained from six replicate extractions of a standard solution. Intermediate precision values were slightly higher, ranging from 4% to 9%, determined from ten extractions of a standard solution (Table 3).

Accuracy was estimated through recovery tests, C. ladanifer extracts were spiked (standard additions method) at four levels of concentration and extracted in duplicate.

Recoveries were on average higher than 90% for all compounds (Table 3).

3.4. Performance evaluation of the SDE method

After the preliminary tests carried out to optimize the working parameters of SDE a set of extractions was performed in order to confirm the method efficiency and precision.

The results showed that the SDE process adopted is suitable for the extraction and quantitative analysis of the target volatiles (Table 4). The average extraction efficiency achieved for six extractions of aqueous samples spiked with the selected compounds was 79% ranging from 65% to 85%. SDE reproducibility (CV), of the standard mixture of all compounds, was 5% on average, ranging from 4% to 6%.

The influence of the matrix characteristics on the extraction efficiency was evaluated by the addition of a standard solution, containing all compounds, to the aqueous phase in the SDE extractor containing the sample (C. ladanifer leaves). Recovery assays were replicated three times. The average values obtained
Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration range (µg L⁻¹)</th>
<th>R²</th>
<th>Repeatabilityᵃ</th>
<th>Intermediateᵇ precision</th>
<th>Recovery (%) ± CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CV (%) (n=6)</td>
<td>CV (%) (n=10)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>100.9–1008.8</td>
<td>0.9982</td>
<td>4</td>
<td>8</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>2,2,6-Trimethylcyclohexanone</td>
<td>25.0–250.4</td>
<td>0.9944</td>
<td>9</td>
<td>8</td>
<td>94 ± 6</td>
</tr>
<tr>
<td>Linalool</td>
<td>153.3–153.4</td>
<td>0.9974</td>
<td>6</td>
<td>8</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>Borneol</td>
<td>24.9–249.2</td>
<td>0.9998</td>
<td>3</td>
<td>4</td>
<td>102 ± 6</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>25.2–251.6</td>
<td>0.9995</td>
<td>5</td>
<td>5</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>Citronellol</td>
<td>10.0–100.1</td>
<td>0.9991</td>
<td>4</td>
<td>6</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Geraniol</td>
<td>9.9–99.1</td>
<td>0.9981</td>
<td>6</td>
<td>9</td>
<td>94 ± 6</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>10.0–100.1</td>
<td>0.9987</td>
<td>3</td>
<td>5</td>
<td>99 ± 4</td>
</tr>
<tr>
<td>Eugenol</td>
<td>50.3–502.8</td>
<td>0.9976</td>
<td>5</td>
<td>7</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>Geranyl acetate</td>
<td>10.0–100.1</td>
<td>0.9989</td>
<td>5</td>
<td>8</td>
<td>105 ± 3</td>
</tr>
</tbody>
</table>

ᵃ Obtained for six extractions of a standard solution corresponding to the lowest level of the concentration range.
ᵇ Obtained for 10 extractions of a standard solution corresponding to the lowest level of the concentration range.

Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>65</td>
<td>5</td>
<td>73</td>
<td>12</td>
</tr>
<tr>
<td>2,2,6-Trimethylcyclohexanone</td>
<td>76</td>
<td>5</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Linalool</td>
<td>84</td>
<td>4</td>
<td>87</td>
<td>8</td>
</tr>
<tr>
<td>Borneol</td>
<td>85</td>
<td>5</td>
<td>90</td>
<td>14</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>82</td>
<td>4</td>
<td>78</td>
<td>7</td>
</tr>
<tr>
<td>Citronellol</td>
<td>81</td>
<td>4</td>
<td>78</td>
<td>5</td>
</tr>
<tr>
<td>Geraniol</td>
<td>82</td>
<td>4</td>
<td>76</td>
<td>7</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>82</td>
<td>5</td>
<td>87</td>
<td>9</td>
</tr>
<tr>
<td>Eugenol</td>
<td>71</td>
<td>6</td>
<td>75</td>
<td>7</td>
</tr>
<tr>
<td>Geranyl acetate</td>
<td>79</td>
<td>5</td>
<td>82</td>
<td>12</td>
</tr>
</tbody>
</table>

³ Obtained for six extractions of an aqueous sample spiked with a standard mixture ~100 mg L⁻¹ in all compounds. Extracts were analysed by GC–FID.

The following compounds were found in the extracts, α-pinene, 2,2,6-trimethylcyclohexanone, borneol and bornyl acetate (Fig. 6). None of the other target volatiles were detected in the extracts. The average concentration values for the volatiles determined in the extracts are shown in Table 5. The predominant compound is α-pinene (22.2 mg g⁻¹ of extract), followed by, 2,2,6-trimethylcyclohexanone (6.1 mg g⁻¹ of extract), bornyl acetate (3.9 mg g⁻¹ of extract) and borneol (3.0 mg g⁻¹ of extract). All these compounds have been previously identified as volatile constituents of C. ladanifer [6–11]. The α-pinene concentration represents approximately 25% (w/w) of the whole SDE extract. High α-pinene content has been also reported in the literature as characteristic of some C. ladanifer essential oils [6,7,9,10]. It should be pointed out that the chemical composition of the SDE extracts was not studied in detail. Thus, it is possible to suggest that the SDE process may be further optimized for the isolation of the volatiles of C. ladanifer.

Table 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentrationᵃ (mg g⁻¹)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>22.2</td>
<td>11.0</td>
</tr>
<tr>
<td>2,2,6-Trimethylcyclohexanone</td>
<td>6.1</td>
<td>7.0</td>
</tr>
<tr>
<td>Borneol</td>
<td>3.0</td>
<td>13.2</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>3.9</td>
<td>8.7</td>
</tr>
</tbody>
</table>

ᵃ Values are expressed as mg g⁻¹ of extract. Results are mean of six analyses of C. ladanifer volatiles by SDE/HS-SPME/GC–FID.
sible that other volatiles, besides α-pinene, can be present in significant amounts. The 2,2,6-trimethylcyclohexanone was identified, in a previous work [8], as the main compound responsible for the *C. ladanifer* leaf odour. The presence of this compound in the extracts was thus expected. From a sensorial point of view the occurrence in the extracts of compounds such as bornyl acetate and borneol is desirable due to their odoriferous proprieties.

4. Conclusions

In this study the SDE technique was used to extract volatile compounds from the leaves of the *C. ladanifer* L. It was concluded that SDE provides good extraction yields and a clean extract with a small solvent consumption. The best extraction performance was achieved for an operation time of 60 min with solvent pentane. The extraction efficiencies ranged from 65% to 85% and the reproducibility was 5% in average.

The application of HS-SPME combined with GC-FID used to quantify the composition of the SDE extract proved to be suitable to achieve an accurate and reproducible analysis despite of the complexity of the matrix. The best results were obtained with an 85 μm PA fiber, sampling at 40 °C for 60 min with a 20% (w/v) NaCl content. The repeatability of the method ranged from 3% to 9% and the intermediate precision from 4% to 9%. Recovery was in average higher than 90%. Four target compounds were detected and quantified in the extracts, α-pinene (22.2 mg g⁻¹ of extract), 2,2,6-trimethylcyclohexanone (6.1 mg g⁻¹ of extract), borneol (3.0 mg g⁻¹ of extract) and bornyl acetate (3.9 mg g⁻¹ of extract).

The SDE extract has a similar aroma to the one released by the plant. This result is relevant and requires further research due to the potential interest for the perfume industry.

References