Headspace Hanging Drop Liquid Phase Microextraction and GC-MS for the Determination of Linalool from Evening Primrose Flowers

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Headspace hanging drop liquid phase micro-extraction (HS-HD-LPME) is studied as a novel solvent-based sample pretreatment method for floral volatile aroma compounds. This paper reports on application of the HS-HD-LPME combined with GC-MS for the analysis of linalool component emitted from evening primrose flowers. The effect of several variables on the method performance was investigated. Additionally, the separation of enantiomers on a cyclodextrin capillary column was performed to identify chirality of (−)-linalool component. Since the unsurpassed volume of a few micro-liters of solvent is used, there is minimal waste or exposure to toxic organic solvents. This method enables to combine extraction, enrichment, clean-up, and sample introduction into a single step prior to the chromatographic process.

Key Words : Headspace hanging drop liquid phase microextraction (HS-HD-LPME), Evening primrose, Linalool, GC-MS

Introduction

The recent trend in analytical sample preparation methods is miniaturization of pretreatment method, which is fast, simple, inexpensive, requires little solvent and produces little waste. Considering the above requirements, headspace solid-phase microextraction (HS-SPME) has found wide acceptance, particularly for gas chromatography-mass spectrometry (GC-MS) of volatile aroma compounds.[1-8] More recently, liquid phase micro-extraction (LPME) was developed as an alternative to SPME.[9-16] This method has also been called solvent microextraction, single drop micro-extraction (SDME), or liquid-liquid microextraction.

This relatively new technique has been described in several papers. Liu and Dasgupta[9-11] were the first to report a novel drop-in-drop system where a microdrop of a water-immiscible organic solvent, suspended in a larger aqueous drop, extracted sodium dodecyl sulphate ion pairs. At the same time, Jeannot and Cantwell[12] introduced a new solvent microextraction technique, where a 8.0 μL micro-drop of organic solvent containing a fixed amount of internal standard was left suspended at the end of a Teflon rod immersed in a stirred aqueous solution containing 4-methyl-acetophenone. He and Lee[13] introduced for the first time the term LPME. They investigated the extraction of 1,2,3-trichlorobenzene using static and dynamic modes of LPME. Psillakis and Kalogerakis[14,15] applied solvent microextraction to the analysis of nitroaromatic explosives. They used a 1 μL drop suspended at the end of a microsyringe needle tip, immersed in a stirred aqueous solution. De Jager and Andrews used the same drop-based method for the analysis of cocaine, cocaethylene, ecgonine methyl ester and anhydroecgonine methyl ester in urine samples.[16]

Since only a few micro-liters of solvent are used, there is minimal waste or exposure to toxic organic solvents. In addition, this method enables to combine extraction, enrichment, clean-up, and sample introduction into a single step prior to the chromatographic process.

Static, dynamic, and headspace modes of LPME have been introduced, however, most methods were only applied for liquid samples.[9-20] A few reports can be found in which HS-LPME is applied to the extraction of volatile aromas from solid plant material. This paper reports on application of the headspace hanging drop liquid phase micro-extraction (HS-HD-LPME) technique coupled with GC or GC-MS for headspace analysis of solid floral aroma such as evening primrose (*Oenthera odorata* Jacqui) of the family oenotheraceae. The objectives of the present study are to study the applicability of HS-HD-LPME to determinate volatile compounds in natural aroma samples and to investigate the effect of several variables on the method performance. Additionally, separation of enantiomers on a cyclodextrin capillary column was performed to identify chirality of linalool component in evening primrose flower sample.

Experimental Section

Plant material and reagents. The freshly picked flower sample of evening primrose was collected in the early August, 2002 at Mt. Boolamsan located in Seoul, Korea. Samples were analyzed immediately after arrival. All working standards were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Tokyo Kasei (Nihonbashi, Tokyo, Japan). Organic solvents such as methylene chloride, chloroform, hexane, 1-octanol (99%, HPLC grade) and *n*-hexadecane (99%, anhydrous) of analytical reagent grade were obtained from Sigma-Aldrich.

Headspace hanging drop liquid phase microextraction (HS-HD-LPME). HS-HD-LPME was carried out using a 10 μL microsyringe (Hamilton #701, Reno, NV, USA). The microsyringe was pre-rinsed more than 10 times with the solvent. About 1 g of evening primrose flower sample or 1.0

mg linalool (liquid) working standard was placed in a 25 mL crimp top vial, and vial was hermetically sealed with a PTFE-silicon stopper and an aluminum cap. Once extraction equilibrium has been reached for 60 min, and the needle of 10 μL microsyringe was used to pierce the vial septum. Then the 0.5 μL organic solvent was extruded out of the needle and kept a single hanging drop at the needle tip to exposure in the headspace above the primrose sample or linalool working standard for 10-60 minutes at 40 °C. During extraction, the syringe was set in a fixed position relative to the vial to keep the position between the needle tip and the headspace. Schematic diagram of HS-HD-LPME apparatus is shown in Figure 1. After extraction, the drop was retracted back into the barrel of the microsyringe and injected into a GC.

Gas chromatography. GC analyses were carried out by using a HP 5890 series II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) with flame ionization detector (FID). Injector and FID temperature were 290 °C. Chromatographic separations were performed on a crossbond 5% phenyl poly (dimethylsiloxane) (Rtx-5MS, Restek, 30 m × 0.25 mm × 0.25 μm film thickness) column. The column oven temperature was held 70 °C for 3 min and then programmed to 280 °C at a rate 5 °C/min, and held at final temperature for 10 min. Gas flow-rates were kept as follows: nitrogen carrier gas, 1 mL/min; hydrogen, 30 mL/min; air, 300 mL/min. A split injection with a ratio of 1 : 10 was used. GC peak areas were integrated with a HP 3396A integrator (Hewlett-Packard).

Gas Chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed using a Trace GC 2000 gas chromatograph equipped with a GCQ Plus ion trap MS^n (Thermoquest-Finnigan, Austin, TX, USA) mass spectrometer. The columns used were a 5% phenyl poly dimethylsiloxane (SPB-5, Supelco, 60 m × 0.25 mm × 0.25 μm film thickness) fused silica column to characterize fragrances and a 30% hepatkis (2,3-di-O-methyl-6-O-*t*-butyldimethyl-silyl)-β-cyclodextrin (Cyclosil-B, J&W, 30 m × 0.25 mm × 0.25 μm film thickness) column to analyze enantiomers from evening primrose.

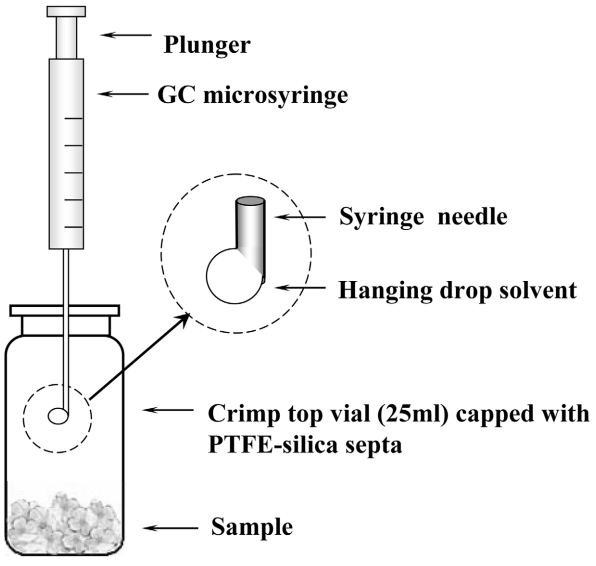

Figure 1. Schematic diagram of headspace hanging drop liquid phase microextraction (HS-HD-LPME).

The oven temperature program of the SPB-5 column was 50 °C (3 min)-5 °C/min -280 °C (10 min). Injector and transfer line temperatures were 290 °C. Flow rate of carrier gas (He, 99.9995%) was 1.0 mL/min, a split injection with a ratio of 1 : 30 was used, and the sample volume injected was 0.5 μL. The electron impact (EI) ionization mass spectrometer was operated as follows: ionization voltage, 70 eV; ion source temperature, 200 °C; scan mode, scan time, 0.75 sec; mass range, 50.0-400.0. The oven temperature program of the Cyclosil-B column was 50 °C (1 min)-5 °C/min-200 °C (5 min); injector, 200 °C; transfer line, 230 °C; the other conditions were the same as those of the SPB-5 column. Volatile compounds were identified by comparing the retention time and comparison of the obtained mass spectra of the relevant chromatographic peaks with those of authentic standards and with spectra of the NIST and Wiley libraries.

Results and Discussions

Solvent selection for HS-HD-LPME. In general, for LPME, the choice of organic solvent should be based on a comparison of selectivity, extraction efficiency, incident of drop loss and drop dislodgement, rate of drop dissolution, as well as level of toxicity.[21] Therefore, extraction solvent should be considered physical properties of a low vapor pressure and high boiling point, in order to reduce vaporization of the solvent drop during the extraction process. Also, it had fewer impurities, which interfered with the determination of sample matrix, under very low concentration.[18] Moreover, there is also a limit in detecting analytes when using a GC system due to the solvent peak, which may obscure early-eluting analytes.[15]

In this study, methylene chloride, chloroform, hexane, 1-octanol, toluene and hexadecane were compared in the extraction of linalool (liquid) standard. Methylene chloride, chloroform, and hexane were well known as solvent having good solubility for numerous organic compounds. However, those were not suitable for HS-HD-LPME because of the difficulty of holding their respective hanging microdrop during extraction process. Although 1-octanol was a solvent having very low vapor pressure and good solubility, however, it was co-eluted with linalool peak during the GC-FID analysis in the present study. Several researchers were reported toluene as suitable extraction solvent, since it had good selectivity and showed no significant solvent loss during extraction.[14,21,22] However, according to our experimental observation, the 0.5 μL hanging drop of toluene was evaporated completely into headspace within 2 min. In this study, hexadecane was selected as the extracting solvent because of its very low vapor pressure (0.00143 mmHg at 25 °C), high boiling point (287 °C), and good solubility of a large number of organic compounds. Moreover, it had lower

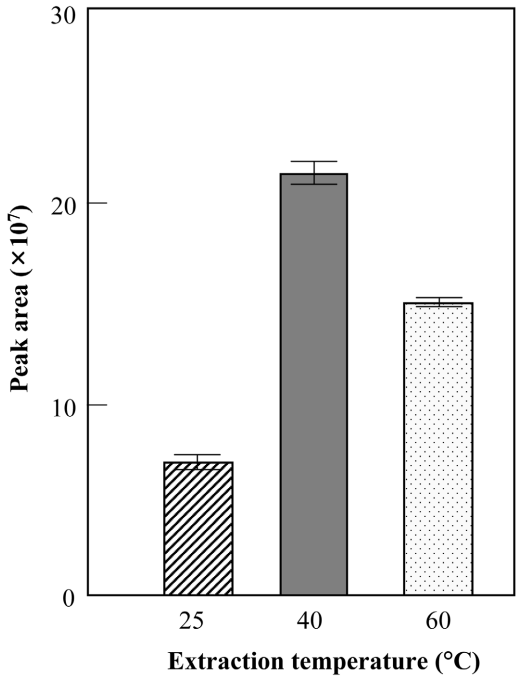

Figure 2. Effect of extraction temperature on the analytical signal for linalool in HS-HD-LPME.

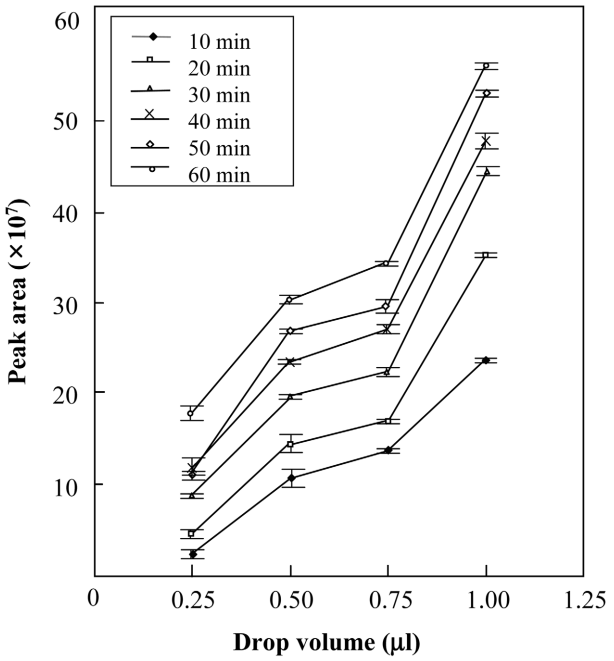

Figure 3. Effect of drop volume and extraction time on the analytical signal in HS-HD-LPME.

impurities by GC-FID with trace level linalool. Hexadecane was suitable as drop solvent for HS-HD-LPME to characterize volatile component of evening primrose flowers. However, it was found that the shortcoming of hexadecane is strong retaining in a GC column after run.

Extraction condition for HS-HD-LPME. The effect of the extraction temperature on the HS-HD-LPME efficiency was investigated at 25 °C, 40 °C, and 60 °C, respectively. The peak areas for linalool obtained at 40 °C were a little higher than those at 60 °C (Fig. 2).

The profile of extraction time and drop size on HS-HD-LPME was investigated by detecting FID signals with variation of those. In the preparation of reference solution, when linalool solution was diluted with hexane relatively lower intensity of linalool was detected resulting from good solubility between hexadecane and hexane. While linalool solution was diluted with methanol that has reverse polarity with hexadecane, nearly nothing was detected. Therefore, linalool reference in this study was used directly, without any dilution. The 0.25, 0.5, 0.75, and 1.00 μL hexadecane drop was exposed to headspace over 1.0 mg of a reference linalool standard in a 25 mL crimp top vial for every 10 min from 10 to 60 min at 40 °C. As shown in Figure 3, the intensities of FID increased with extraction time up to 60 min gradually. Therefore, the choice of optimal condition for the HS-HD-LPME method cannot be made based on the data in the Figure 3, because the magnitude of each peak area did not yet reach to the plateau. It was temporarily decided to perform all sample extractions at 40 °C for 30 min.

Also, when the drop volume was increased, the intensity of signal was increased, as shown in Figure 3. Even though a larger drop size gave an improved signal, drop size more than 4 μL are not favored.[22] Use of large drop volume dislodges drop from the microsyringe needle. Also, it results in extensive band broadening and long conditioning time in GC column. Therefore, 0.5 μL drop volume was used to study the performance of HS-HD-LPME.

In this study, sample vial was fixed in a 25 mL vial. The sample vial, which contains the HS portion, should have a volume about 10^5 times larger than the volume of the microdrop, to ensure that the concentration of the solute in the vial does not change during the measurement.[23,24] A. Przyjazny and J. M. Kokosa reported that using large vials result in higher sensitivity and shorter equilibrium time.[18]

It is important to make sure that volume of liquid phase of hexadecane selected is maintained constant through the experiment. Considering the vapor pressure of liquid phase of hexadecane, (1.88×10^{-6} atm), evaporating from the hexadecane drop may be negligible under given experimental condition. Even if the headspace is saturated with hexadecane vapor, the change in volume of the solvent drop may be negligible. It was explained by calculating the number of moles needed to saturate HS and moles used in the experiment. Under our experimental condition of 25 mL vial volume and 0.5 μL hexadecane solvent drop, corresponding number of moles (n) in the state of vapor pressure saturated was computed at 1.83×10^{-9} mol from the following perfect gas equation of state: $PV=nRT$, where P is vapor pressure of hexadecane (1.88×10^{-6} atm at 40 °C); V is headspace volume occupied (0.025 L); R is gas constant (8.20578×10^{-2} L·atm·K^{-1}·mol^{-1}); T is temperature (313K).

On the other hand, the number of moles (n) of 0.5 μL hexadecane is 1.70×10^{-6} mol from following calculation: (5

$\times 10^{-4}$ mL) $\times$ (0.77 g·mL^{-1})/(226.44 g·mL^{-1}). The amount of 0.5 μL hexadecane used was very greater than the least number of moles of the vapor pressure needed to saturate headspace. Therefore, the vaporization of hexadecane of liquid state can theoretically be ignored under the given condition in our study.

The equilibrium of an analyte is reached when the concentration differences between two neighboring phases have been satisfied with the values of their partition coefficient (K). The overall partition equilibrium constant (K) is given by the relationship:

$$K = K_{gs}\, K_{lg}$$

where K_{gs} is defined as the analyte partition coefficient between the headspace gaseous phase and sample matrix; K_{lg} is the analyte partition coefficient between micro-drop solvent and the headspace gaseous phase; and K is the overall partition equilibrium constant. Since K_{gs} is constant, under standardized equilibrium condition, K_{lg} can be calculated from the following expression. The K_{lg} values can be calculated by the following forms:

$$K_{lg} = (A_l V_g) / (A_g V_l)$$

where A_l refers to the peak area of analyte on HS-HD-LPME micro-drop solvent, 701610 ± 5.04 (± RSD); V_g is the volume of the gas sample injected by static-HS-GC using gas tight syringe, 5000 μL; A_g is the peak area of analyte in the headspace, 179088 ± 10.55 (± RSD); and V_l is the volume of micro-drop solvent, 0.5 μL. HS-HD-LPME followed by static-HS-GC was applied successively to the same sample.

The K_{lg} values and the relative concentration factors (CF) of linalool were measured for relative evaluation of the efficiency of HS-HD-LPME. And the CF of an analyte achieved by drop solvent *vs* the corresponding static-HS-GC sampling is the ratio between the analyte peak area obtained by HS-HD-LPME-GC and the corresponding area obtained by static-HS-GC. The results of experimental K_{lg} values and relative CF for linalool of evening primrose samples are 3.92 $\times 10^4$ and 3.92.

Calibration curve, precision and accuracy. Under the optimized conditions described previously, a linear calibration curve for the peak area as a function of (−)-linalool concentration was obtained in the range of 0.1-100.0 μg with a slope value of 7 $\times 10^7$ and an intercept of 1 $\times 10^8$ (r^2 = 0.9971, n=3). The precision of the method has been evaluated by replicate determination of different concentrations. The relative standard deviation (RSD) of (−)-linalool peak area was 1.1%-9.8% for triplicate measurements. The accuracy defined as the deviation between added and found concentration less than 13.5%.

Application of the HS-HD-LPME technique to real samples for characterization of evening primrose aroma. Living flowers of wild evening primrose were analyzed by HS-HD-LPME. Linalool was identified as dominant component from the fragrances of evening primrose flowers. The total ion chromatogram and mass spectra of linalool obtained by HS-HD-LPME from evening primrose flowers was shown in Figure 4.

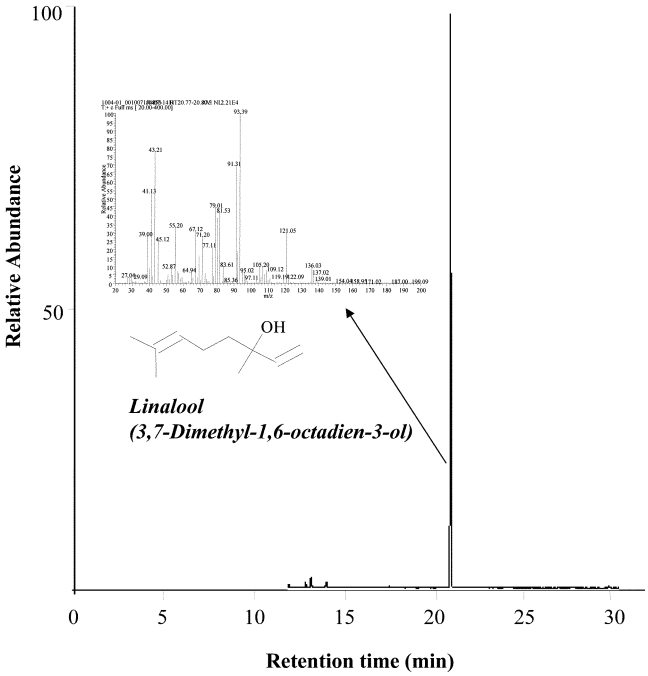

Figure 4. Total ion chromatogram and mass spectra (inset) of linalool obtained by HS-HD-LPME from evening primrose flowers sample.

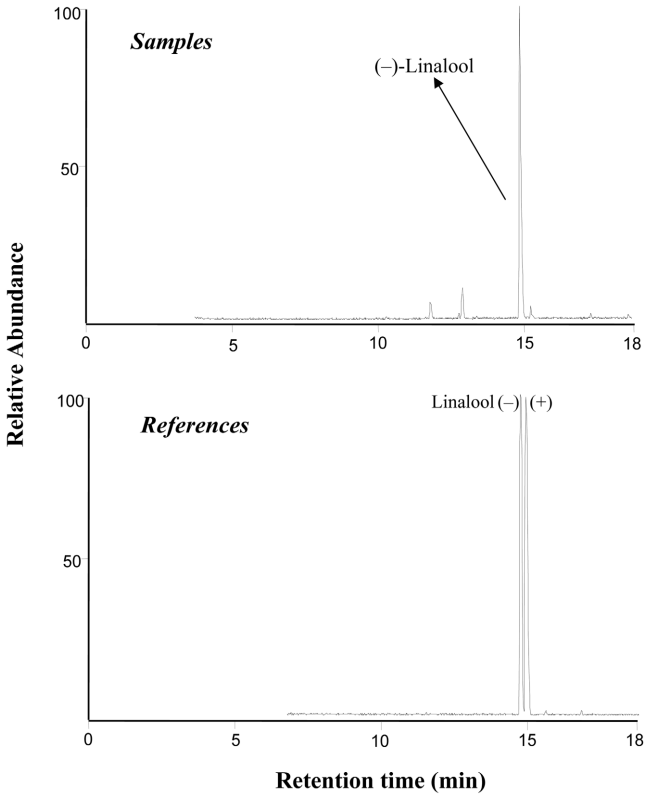

Figure 5. Enantiomeric separations of linalool isolated from evening primrose flowers using a 30% hepatkis (2,3-di-O-methyl-6-O-*t*-butyldimethyl-silyl)-β-cyclodextrin (Cyclosil-B, J&W, 30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness) column.

Linalool is well known as enantiomeric fragrance component. The enantiomeric analysis of linalool in evening primrose was performed by a 30% Hepatkis (2,3-di-O-methyl-6-O-*t*-butyldimethyl-silyl)-β-cyclodextrin (Cyclosil-B, J&W) column with HS-HD-LPME using (+)-linalool and (−)-linalool. As shown in Figure 5, the two peaks of (+)-linalool and (−)-linalool references were well resolved. Linalool peak of the fragrances emitted from evening primrose flowers was found in the form of (−) configuration by comparing retention times with two references. Enantiomers show different odor properties. There have been previous reviews on the enantiomeric separation and enantioselective perception of chiral odorants.[2,25,26]

Conclusions

This paper reports on the application of the HS-HD-LPME technique with GC or GC-MS for hedspace analysis of solid natural aroma from evening primrose. The HS-HD-LPME technique, which has rapidity, simplicity, inexpensiveness, requirement of little solvent and production of little waste, should be potential for the HS-extraction of volatile component. Linalool in the fragrances emitted from evening primrose flowers was found in the form of (−).

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