Determination of Essential Oils in Hops by Headspace Solid-Phase Microextraction†

Jennifer A. Field,* Gail Nickerson, Derryl D. James, and Chris Heider

Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331

A solid-phase microextraction (SPME) method is described for the determination of the essential oils, humulene and caryophyllene, in the headspace of female hop cones and male hop lupulin samples. Optimized SPME sampling parameters for the determination of the humulene to caryophyllene (H/C) ratio for female hops (50 mg) and male lupulin (25 mg) samples include a 100 μm poly(dimethylsiloxane) SPME fiber and a 4 h sample exposure period at a temperature of 50 °C. Increasing the temperature to 90 °C or increasing the length of sample exposure at 50 °C promoted caryophyllene oxidation. In a survey of five female hop varieties, good agreement was found between the H/C ratio obtained by headspace SPME and conventional steam distillation. The H/C ratio for 10 male lupulin varieties determined by headspace SPME and pentane extraction also indicated good agreement between the two extraction methods.

Keywords: *Hops; essential oils; SPME; headspace analysis*

INTRODUCTION

Essential oils are volatile compounds collected by steam distillation and other methods from plant material and are used to identify plant species and varieties. Traditional techniques for the separation and isolation of essential oils from plant tissues typically involve steam distillation and organic solvent extraction. However, steam distillation technology is time-consuming, requires large amounts (>100 g) of sample, is difficult to automate, and can not be directly interfaced with chromatographic methods such as gas chromatography. Rapid methods for collecting essential oils are needed especially in plant breeding where many samples must be tested and only small amounts of material are available (Haunold and Nickerson, 1987).

Selection of parents for hop breeding depends upon agronomic and chemical characteristics. Over 250 chemical constituents have been identified in the essential oil of hops (*Humulus lupulis*) (Neve, 1991). Two of the more abundant components in the essential oil of hops are the sequiterpene isomers α-humulene (2,6,6,9-tetramethyl-1,4,8-cycloundecatriene; bp 266−268 °C) and β-caryophyllene (4,11,11-trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene; bp 262−264 °C) with a molecular weight of 204.36 AMU (Windholz, 1983; Figure 1). Humulene is found mainly in hops, while caryophyllene occurs in many different plants, especially in cloves (*Caryophyllus aromaticus*). Hop varieties used for their aroma in beer production all have high (>2.5) humulene to caryophyllene (H/C) ratios (Kralj et al., 1991). Only the female cones are used to add flavor and aroma to beer, the most important commercial use of hops. Breeding hops for aroma requires selecting males with high (>2.5) H/C ratios. Typically H/C ratios in female hops range from 0.07 to 3.75 (Kenny, 1990; Haunold et al., 1993), and a similar H/C range is found in male lupulin (Nickerson et al., 1988).

While it is easy to measure the chemical characteristics of female hops, including the H/C ratio, it is more difficult to measure the H/C ratio in male lupulin. Lupulin may account for 4−33% of the female cone weight, but male flowers only have 10−50 lupulin glands/flower or less than 1% by weight (Brooks, 1962). Rapid methods have been proposed for the extraction of essential oils in hops including solid-phase extraction, vacuum steam distillation, and supercritical carbon dioxide extraction (Lam et al., 1986; Green and Osborne, 1993; Verschuere et al., 1992). However, the methods are for female genotypes and require a number of steps, specialized equipment, or the use of solvents. Solvent extraction with pentane is the conventional method used to determine the H/C ratio of male lupulin (Nickerson et al., 1988). Unfortunately, the technique is not satisfactory because direct injection of the male lupulin extract rapidly reduces gas chromatographic resolution due to build up of nonvolatile compounds on the column.

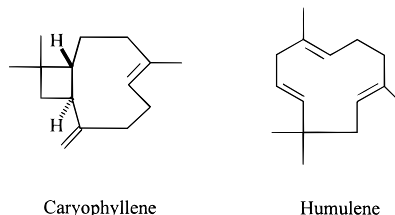

Figure 1. Structures for the sesquiterpene isomers humulene and caryophyllene.

Solid-Phase Microextraction. Solid-phase microextraction (SPME) is a relatively inexpensive and solvent-free extraction technique that requires only small amounts of sample (Zhang et al., 1994). The SPME apparatus consists of a reusable fused silica fiber coated with an adsorbent organic phase that is attached to a stainless steel plunger mounted to a holder. The extraction procedure involves the direct exposure of the SPME fiber to the headspace of a sample or directly into an aqueous sample. Once equilibrium is attained between the SPME fiber coating, headspace, and sample, the fiber is retracted into the holder and introduced directly to the heated injection port of a gas chromatograph (GC) where the analytes are desorbed from the fiber and transferred to the GC column for separation (Zhang and Pawliszyn, 1993). While SPME has been

* Author to whom correspondence should be addressed.

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used for the detection of organic contaminants in water (Zhang et al., 1994; Zhang and Pawliszyn, 1993; Potter and Pawliszyn, 1992, 1994; Bucholz and Pawliszyn, 1994; Chen and Pawliszyn, 1995) and caffeine and flavors in beverages and food (Hawthorne et al., 1992; Yang and Peppard, 1994; Shirey et al., 1995), little attention has been given to the application of SPME for the determination of essential oils in plant materials.

The theory and practice of headspace SPME has been developed in full detail by Zhang and Pawliszyn (1993). The partition coefficient between the SPME fiber coating and sample headspace (K_1) and the partition coefficient between the headspace and the sample matrix (K_2) are expressed by the equations

$$K_1 = C_1/C_3 \quad (1)$$

$$K_2 = C_3/C_2 \quad (2)$$

where C_1 is the equilibrium concentration of the analyte in the SPME fiber coating, C_2 is the equilibrium concentration of analyte in the sample, and C_3 is the equilibrium concentration of the analyte in the headspace. The overall equilibrium constant describing analyte partitioning between the SPME fiber coating and sample is described by the expression

$$K = K_1K_2 \quad (3)$$

However, because SPME is an equilibrium sampling method, the kinetics of mass transport also must be understood in order to establish the time required to reach equilibrium (Zhang and Pawliszyn, 1993).

In this study we report the development of a headspace SPME as a rapid and nondestructive method for monitoring the H/C ratio in female hops and male lupulin samples. The optimum headspace SPME sampling parameters and the factors controlling the time required to reach equilibrium are discussed. The H/C ratio for female hops obtained by headspace SPME is compared to conventional steam distillation and direct headspace analysis by gastight syringe. In addition, the H/C ratios for male lupulin determined by headspace SPME are compared to that determined by conventional pentane extraction.

EXPERIMENTAL SECTION

Samples. A pelletized hop sample (Cascade variety), obtained from John I. Haas Inc. (Yakima, WA), was pulverized and sieved to obtain a homogeneous sample for the purpose of optimizing the headspace SPME sampling parameters. Hop varieties including First Choice, Harleys Fulbright, New Zealand Hallertauer, Southern Cross, and Experimental Variety 67-11-58 and 85-6-23 were obtained from the 1995 fall harvest through Ron Beaston of the Riwaka Research Center, Horticulture and Food Research Institute of New Zealand. At harvest, the hops were machine picked, dried 14 h at 50 °C, and rehumidified to an 8% moisture content. Samples were compressed into miniature bales and stored at −4 °C until ground for analysis in a food chopper (American Society of Brewing Chemists, 1992). All hop samples (50 mg) were placed in 1.8 mL GC autosampler vials and sealed with a Teflon-lined crimp-top cap.

Male lupulin samples were prepared by slurrying dried male flowers in water and pouring the slurry sequentially through 28, 80, and 200 mesh screens (Nickerson et al., 1988). The lupulin was washed off the 200 mesh screen with water and dried on filter paper at room temperature. Male lupulin samples (25 mg) were placed in a 0.1 mL vial, and a conical glass rod was used to squash the lupulin glands against the side of the vial, after which the vial was sealed with a Teflon-lined crimp-top cap.

SPME. Solid-phase microextraction fibers (100 and 30 μm film thicknesses) coated with poly(dimethylsiloxane) were generously donated by Supelco (Bellefonte, PA). Unless otherwise noted, the following conditions were used for headspace SPME of female hops and male lupulin samples. Autosampler vials containing either hops or male lupulin were placed in a heated block (50 °C), and the SPME fiber was inserted through the septa for a period of 4 h. The SPME fiber was then withdrawn from the vial and inserted manually into the inlet of the GC and desorbed for 3 min under split (1:67) injection conditions.

Headspace SPME Optimization. The time required for complete desorption of analytes from the SPME fiber in the heated GC inlet was investigated by varying the desorption time from 3 to 30 min. Following the first desorption event, the SPME fiber was re-exposed to the GC inlet for a second 3 min desorption period to constitute a blank run. The time required for complete desorption was determined by the absence of humulene and caryophyllene in a subsequent blank run.

Experiments were performed to determine the length of time needed to reach equilibrium at 50 °C. Individual (50 mg) samples of the pelletized hop sample were sealed in 1.8 mL autosampler vials and placed in a heated block (50 °C), after which the SPME fiber was immediately introduced to the headspace of the vial. The SPME fiber was exposed to individual samples for time periods from 5 min to 24 h. The area counts recorded for humulene and caryophyllene were normalized to the mass of the sample.

A second set of experiments was conducted using pelletized hop samples that were preheated at 50 °C for a period of 12 h in the absence of the SPME fiber. Following the period of preheating, the SPME fiber was introduced into the headspace of each vial for periods of time from 5 min to 24 h.

Hop and Male Lupulin Survey by Headspace SPME. Duplicate samples of five ground hop varieties were analyzed by headspace SPME at 50 °C with a 100 μm fiber and a fiber exposure time of 4 h. Single samples of each variety were analyzed by conventional steam distillation. Ten samples of male lupulin were analyzed in duplicate by headspace SPME using the same SPME sampling parameters as that for hops samples. Duplicate samples of the male lupulin also were analyzed by pentane extraction. To determine the precision of headspace SPME, six replicate samples of the pelletized Cascade hop sample were analyzed by headspace SPME at 50 °C with a 100 μm fiber and a fiber exposure time of 4 h.

Steam Distillation. The pelletized and ground hop samples were analyzed by conventional steam distillation in order to validate headspace SPME as an alternative extraction method. For steam distillation analyses, a modified Dean−Stark receiver filled with 5 L of water that was buffered to pH 7 with KH_2PO_4 (34 g) and 25 mL of 1.26 N NaOH was used to collect and separate the distillate from 200 g samples of female hops (American Society of Brewing Chemists, 1992). After 6 h the oil was measured volumetrically upon collection, diluted to a 10% (v/v) solution with pentane, and sealed in glass ampules. A 1.0 μL injection volume of the 10% hop extract was analyzed by GC.

Direct Headspace Analysis. Portions (50 mg) of the pelletized hop sample were placed in 1.8 mL autosampler vials with crimp-top caps and stored at −10 °C until analysis. Direct headspace analyses were performed after equilibrating the vials in a heated block (50 °C) for 4 h. Prior to sampling the headspace of the vial, 0.5 mL of air was pulled into the needle of a 5 mL SGE gastight syringe (Supelco, Bellefonte, PA). With the valve closed, the syringe was heated to 50 °C. The 0.5 mL of heated air was then introduced into the headspace of the vial and retracted into the needle two times in order to avoid placing the vial under vacuum and to mix the headspace contents of the vial. Before removing the needle, 0.5 mL of the vial headspace was withdrawn into the syringe and the valve closed. The needle was then immediately introduced into the GC inlet, the valve opened, and the sample injected under splitless conditions with a 0.5 min purge time.

Pentane Extraction of Male Lupulin. In order to validate the H/C ratios obtained by headspace SPME, male

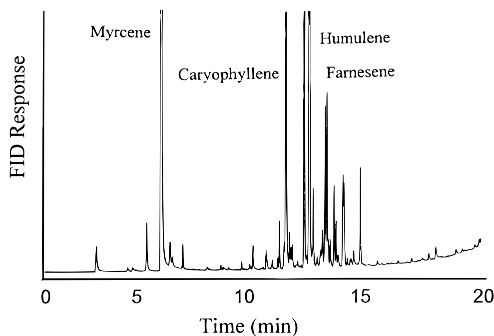

Figure 2. Gas chromatogram of a hop sample (New Zealand Hallertauer variety) obtained at 50 °C by headspace SPME.

lupulin samples were extracted with pentane. Lupulin (25 mg) was placed in a 0.1 mL vial together with 0.1 mL of pentane and sealed with a crimp-top cap. The sample was then sonicated 20 min to rupture the male lupulin glands. A 5 μL injection volume was used to inject the pentane extracts of male lupulin into the GC.

Gas Chromatography. All samples were analyzed using a Hewlett Packard 5890A gas chromatograph equipped with a flame ionization detector (Hewlett Packard, Wilmington, DE) and a Supelcowax 10 column (30 m × 0.25 mm × 25 μm film thickness; Supelco, Bellefonte, PA). The injection port was maintained at 220 °C, and the detector was operated at 250 °C. Helium was used as carrier gas to give a 1.0 mL/min column flow. The initial oven temperature of 40 °C was held for 2 min and then ramped to 90 °C at 25 °C/min followed by a second ramp (7.5 °C/min) to a final temperature of 240 °C, which was held for 1 min. For headspace SPME analyses, the fiber was inserted manually and desorbed under split (1:67) injection conditions.

RESULTS AND DISCUSSION

Desorption of humulene and caryophyllene from the SPME fiber in the GC injection port was completed within 45 s. Even so, a desorption time of 3 min was selected in order to ensure that compounds with boiling points higher than humulene or caryophyllene also were completely desorbed.

A typical GC/FID chromatogram for a female hops sample (New Zealand Hallertauer variety) obtained by means of headspace SPME is shown in Figure 2. Unlike chromatograms for extracts obtained by steam distillation, chromatograms obtained by solvent-free SPME sampling show no solvent peak. In addition to humulene and caryophyllene, other compounds commonly found in hops are observed in the chromatogram, including myrcene and farnesene (Figure 2).

Initial experiments were performed to determine the sampling time required to reach equilibrium at 50 °C. Over a period of 6 h, the area counts for humulene and caryophyllene increased; however, they do not exhibit identical behavior (Figure 3a). Caryophyllene reaches equilibrium faster than humulene presumably because caryophyllene has a higher vapor pressure than humulene as indicated by the shorter retention time of caryophyllene in the GC chromatogram. At 4 h, the H/C ratio reaches equilibrium (Figure 3b) with a value of 2.75 that is nearly equivalent to the value of 2.73 obtained by conventional steam distillation. The precision of headspace SPME, indicated by the relative standard deviation, is 2.3%, as determined from six replicate analyses of the pelletized Cascade hop sample performed at a temperature of 50 °C with a 100 μm SPME fiber and a 4 h fiber exposure time.

Experiments were performed to investigate the possibility of reducing the 4 h period required to reach

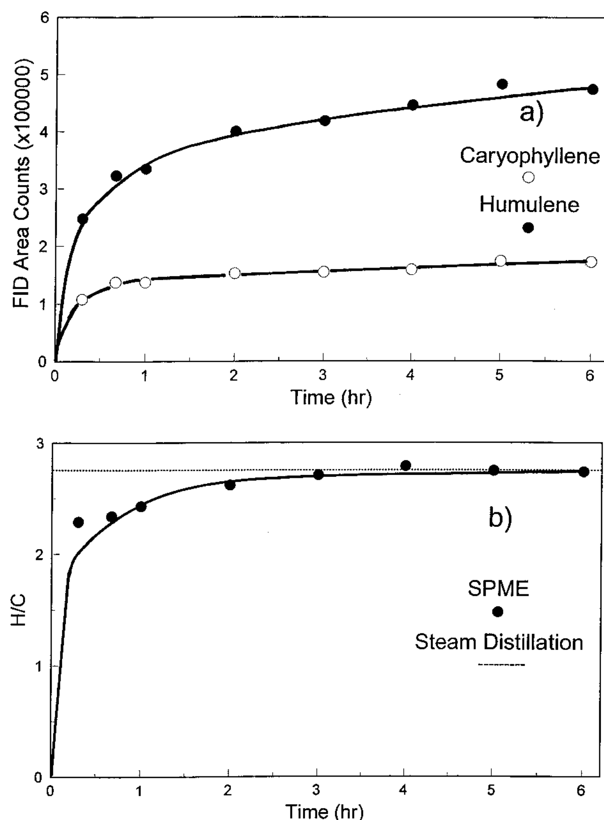

Figure 3. Effect of time on (a) the flame ionization detector area counts for humulene and caryophyllene determined by headspace SPME and (b) the humulene/caryophyllene ratio obtained by headspace SPME and conventional steam distillation.

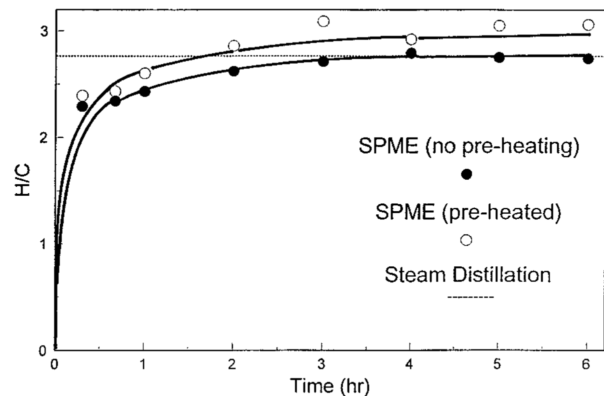

Figure 4. Humulene/caryophyllene ratio for a hop sample heated to 50 °C exposed to the SPME fiber with and without a 12 h period of preheating.

equilibrium. Hop samples were allowed to preheat at 50 °C for a period of 12 h prior to introducing the SPME fiber. The H/C ratio for preheated samples follows the same trend with time as that observed for the samples that were not preheated (Figure 4). Therefore, preheating samples does not shorten the time to reach equilibrium. Semivolatile compounds such as humulene and caryophyllene are expected to have large SPME fiber/headspace equilibrium constants (K_1) such that the SPME fiber depletes the amount of humulene and caryophyllene in the sample headspace. Therefore, upon introduction of the SPME fiber to the headspace of the preheated sample, the sample must re-equilibrate so that the same amount of time is required to reach equilibrium for both preheated and nonpreheated samples.

The H/C ratio for preheated samples at equilibrium was 3.0, which is greater than the H/C ratio obtained for the same hop sample analyzed by steam distillation (2.73) and headspace SPME (2.75) without a period of preheating. The higher H/C ratio is consistent with the loss of caryophyllene to oxidation over the 12 h preheating time as evidenced by the presence of caryophyllene oxidation products in chromatograms for preheated samples. No oxidation products of humulene were observed. The preferential oxidation of caryophyllene is consistent with reports that caryophyllene forms oxidation products more readily than humulene (Yang et al., 1993). In contrast, caryophyllene oxidation products were not observed in chromatograms for samples that received no preheating. Because caryophyllene oxidizes during preheating and because preheating does not reduce the time required to reach equilibrium, all samples were subsequently analyzed by exposing the sample to the SPME fiber without a period of preheating.

Temperature was investigated as an alternative means for increasing the vapor pressure of humulene and caryophyllene and thereby potentially decreasing the time required to reach equilibrium. Samples heated to 90 °C were sampled by headspace SPME over a period of 6 h, and the H/C ratios were compared to that obtained by headspace SPME at 50 °C. The H/C ratios obtained from samples heated to 90 °C were significantly above the H/C ratio obtained by either headspace SPME at 50 °C or steam distillation. Caryophyllene oxidation products also were evident in chromatograms from hop samples heated to 90 °C, indicating that elevated temperatures lead to caryophyllene oxidation and erroneously high H/C ratios.

To determine if analyte diffusion through the SPME fiber coating contributed significantly to the time required to reach equilibrium, the extraction kinetics of two fiber thicknesses were investigated. A 30 and 100 μm SPME fiber gave similar extraction profiles, indicating that the thinner (30 μm) fiber coating does not significantly decrease the time required to reach equilibrium. On the other hand, the area counts for humulene and caryophyllene were 10−20 times higher for the 100 μm fiber compared to the 30 μm fiber. Because the 30 μm SPME fiber gave H/C values below that obtained for the 100 μm fiber as well as by steam distillation, a 100 μm SPME fiber was used for all subsequent extractions.

Direct headspace measurements were performed with a gastight syringe to independently assess the kinetics of humulene and caryophyllene diffusion from the hop sample into the sample headspace. The area counts obtained for humulene and caryophyllene by direct headspace analysis were a factor of 15−20 lower than those determined by headspace SPME and clearly illustrate the decreased sensitivity of direct headspace sampling compared to headspace SPME. The extraction profiles for direct headspace analysis by gastight syringe and headspace SPME measurements were similar in shape, indicating that the kinetics of humulene and caryophyllene diffusion into the headspace are comparable in the presence and absence of the SPME fiber (Figure 5). Similar extraction kinetics for headspace SPME and direct headspace analysis suggest that the amount of time required for the analytes to diffuse into the SPME fiber coating is negligible relative to the slow diffusion of analytes from the hop sample into the headspace. Furthermore, headspace measurements underestimated the H/C ratio (1.82) for the hop sample compared to the H/C ratios obtained by headspace SPME (2.75) and steam distillation (2.73). Lower H/C ratios are consistent with reports of poor humulene recovery by direct headspace analysis, presumably due to the lower vapor pressure of humulene compared to caryophyllene (Murakami et al., 1987).

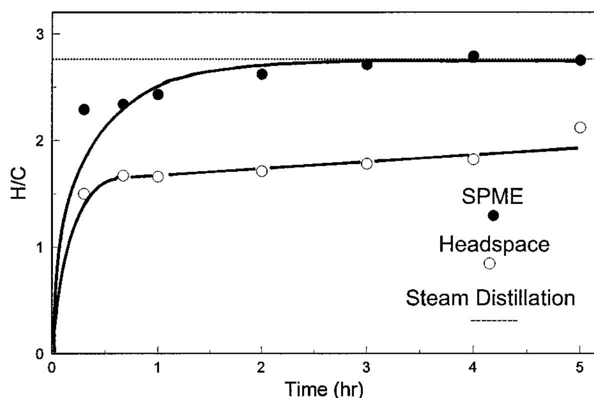

Figure 5. Humulene/caryophyllene ratios obtained by headspace SPME, direct headspace analysis by gastight syringe, and conventional steam distillation.

The partition coefficient between the SPME fiber and sample headspace (K_1) for humulene and caryophyllene were experimentally determined from area counts acquired by headspace SPME and direct headspace analysis by gastight syringe. Measurements used to determine K_1 were determined at equilibrium, which corresponded to a 4 h sample equilibration time at 50 °C. Equation 4 was used to calculate K_1 for each analyte i as presented in Zhang and Pawliszyn (1993):

$$K_1{}^i = (A_f V_g)/(A_g V_f) \quad (4)$$

where A_f is the area counts for analyte i on the SPME fiber, V_g is the approximate volume of the vapor phase (1.8 mL), A_g is the area counts for analyte i in the headspace, and V_f is the volume of the SPME 100 μm fiber (0.007 759 cm^3). The area counts for humulene and caryophyllene determined by direct headspace analysis were corrected by 20% to account for dilution upon the introduction of 0.5 mL of air into the headspace of the vial. The values of K_1 calculated for humulene and caryophyllene are 14 084 and 9444, respectively, indicating a high affinity of humulene and caryophyllene for the SPME fiber, which results in the increased sensitivity of headspace SPME in comparison to direct headspace analysis by gastight syringe. Semivolatile compounds that have large K_1 values and small values for the partition coefficient between sample and headspace (K_2) require longer periods of time to reach equilibrium because the amount of mass in the headspace at any time is small (Zhang and Pawliszyn, 1993).

Hop and Male Lupulin Survey. A survey of five hop varieties was conducted to determine the agreement between headspace SPME and conventional steam distillation. The H/C ratios determined for the five hop varieties (0.36−2.83) fell within the expected range for hops. The H/C values obtained by headspace SPME are in acceptable agreement with the H/C ratios obtained by conventional steam distillation with a correlation coefficient (r^2) of 0.962 (Figure 6), indicating that headspace SPME can be used as a relatively rapid and inexpensive technique for determining the aroma quality of female hops.

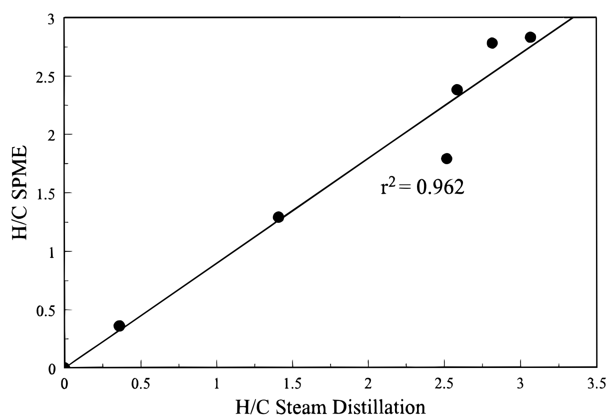

Figure 6. Humulene/caryophyllene ratios for female hop determined by headspace SPME and conventional steam distillation.

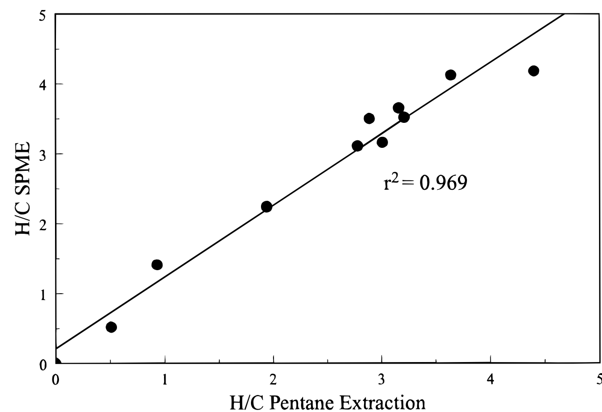

Figure 7. Humulene/caryophyllene ratios for male lupulin samples determined by headspace SPME and conventional pentane extraction.

The H/C ratios for male lupulin samples determined by headspace SPME were in good agreement with those obtained by conventional pentane extraction (Figure 7). The correlation of H/C ratios obtained by headspace SPME and pentane extraction was linear with a r^2 of 0.969, indicating that headspace SPME is a good alternative method for determining the essential oils in samples of plant tissue where only very small amounts of sample are available.

CONCLUSIONS

Headspace SPME provided measurements of humulene and caryophyllene ratios in hops and male lupulin samples that were in good agreement with those obtained by conventional steam distillation and pentane extraction, respectively. The advantages of headspace SPME sampling over conventional steam distillation of hops and the pentane extraction of male lupulin samples are small sample sizes, increased sensitivity, no sample cleanup, solvent-free extraction, and relatively low cost. Because SPME provides accurate and precise measurements of essential oil ratios in hops, headspace SPME is a good alternative screening tool for use in selecting female hops and male lupulin for breeding purposes.

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