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Headspace Solid-Phase Microextraction Method for the Study of the Volatility of Selected Flavor Compounds

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Changes in the volatility of selected flavor compounds in the presence of nonvolatile food matrix components were studied using headspace solid-phase microextraction (HS-SPME) combined with GC-MS quantification. Time-dependent adsorption profiles to the SPME fiber and the partition coefficients between different phases were obtained for several individual volatiles, showing that HS-SPME analysis with a short sampling time can be used to determine the "true" headspace concentration at equilibrium between the headspace and a sample matrix. Equilibrium dialysis followed by HS-SPME/GC-MS was carried out to confirm the ability of HS-SPME extraction for monitoring the free volatile compounds in the presence of proteins. In particular, a short sampling time (1 min) avoided additional extraction of volatiles bound to the protein. Interactions between several selected flavor compounds and nonvolatile food matrix components [β-lactoglobulin or (+)-catechin] were also studied by means of HS-SPME/GC-MS analysis. The volatility of ethyl hexanoate, heptanone, and hexanal was significantly decreased by the addition of β-lactoglobulin compared to that of isoamyl acetate. Catechin decreased the volatility of ethyl hexanoate and hexanal by 10−20% and increased that of 2-heptanone by ~15%. This study indicates that HS-SPME can be a useful tool for the study of the interactions between volatile compounds and nonvolatile matrix components provided the kinetic and thermodynamic behavior of the volatiles in relation to the fiber chosen for the studies is carefully considered.

KEYWORDS: HS-SPME/GC-MS; "true" headspace; partition coefficients; volatility; equilibrium dialysis; flavor/matrix interactions

INTRODUCTION

Headspace solid-phase microextraction (HS-SPME) is a solvent-free sampling technique based on the sorption characteristics (adsorption or absorption) of fiber coating materials (*1*). The analytes (volatiles or semivolatiles) from gaseous, liquid, or solid matrices are first released from the matrices and sorbed onto a fiber coated with an ad(ab)sorbent polymer introduced into the headspace. Following sorption, analytes are either thermally desorbed onto a gas chromatographic (GC) inlet or solvent desorbed into a high-performance liquid chromatographic (HPLC) inlet (*2*, *3*). Originally developed by Pawliszyn and co-workers in the early 1990s (*2*), applications for SPME have extended from environmental analyses to food/beverage analyses. This technique has been utilized in various areas of food/flavor chemistry, for example, the quantification and/or qualification of volatile compounds found in beers (*4*, *5*), vodkas (*6*), coffees (*7*, *8*), colas (*9*), and wines (*10*, *1*). It has also been used to analyze volatile organic metabolites produced by microorganisms (*11*, *12*). HS-SPME sampling has several advantages compared to other conventional headspace techniques including ease of use and sensitivity (*13*, *14*). The different types of fibers commercially available make it possible to selectively analyze a variety of analytes from various sample matrices.

It is well-known that addition of a flavor compound to different food matrices results in different sensorial responses either quantitatively or qualitatively (*15*). Analytical measurement of flavor volatility in the presence of different food components has been used to provide important information on interactions between flavors and food matrices. In particular, the headspace concentration above a food directly represents the availability of volatiles to aroma perception (*16*, *17*). Although the measurement conditions are usually controlled thermodynamically and/or kinetically, conditions that rarely occur in the mouth during eating, the knowledge obtained from the well-defined variables provides insights into the flavor behavior in multicomponent and complex food systems.

Equilibrium dialysis and several headspace analysis techniques (e.g., static headspace and dynamic headspace) have been widely used to study the volatility of flavors in the presence of nonvolatile food components. However, there are several drawbacks raised with these procedures, including degradation or loss of some volatiles during equilibrium dialysis and dynamic

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headspace sampling (*18, 19*) and poor sensitivity and selectivity associated with static headspace analysis (*20*).

Because of its simplicity, sensitivity, selectivity, and ease of automation, we evaluated the use of HS-SPME for studying the effect of nonvolatile matrix components on flavor volatility. In the present work, HS-SPME analysis with different sampling times was performed to show the different kinetics of volatile adsorption onto the fiber. Partition coefficients between headspace and water were measured with several selected flavor compounds using static headspace analysis and then evaluated in relation to the SPME sampling time. HS-SPME was used to study volatile/protein binding interactions, and results were validated by equilibrium dialysis. Finally, the HS-SPME method was also employed with flavored solutions containing β-lactoglobulin or catechin in order to show application of the method for evaluating the effects of nonvolatile food constituents on flavor release (or retention).

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA, purity = 96%) and β-lactoglobulin (BLG, mixture of variants A and B, purity = 90%) were purchased from Sigma (St. Louis, MO) and used without further purification. (+)-Catechin hydrate was from Sigma-Aldrich Chemical Co. (Milwaukee, WI). The flavor compounds (purity > 98%) used in this study, ethyl hexanoate, isoamyl acetate, ethyl acetate, hexanal, 2-heptanone, and 2-nonanone, were purchased from Aldrich (Milwaukee, WI). Dialysis membrane was obtained from Pierce (Rockford, IL) with a 7000 molecular weight cutoff.

HS-SPME Measurement as a Function of Fiber Sampling Time. HS-SPME sampling was performed at various adsorption times ranging from 1 to 60 min to determine the minimum time required for establishing the equilibrium. Each flavor compound (ethyl hexanoate, 2-nonanone, ethyl acetate, 2-heptanone, isoamyl acetate, and hexanal) was solubilized in water to give a concentration of 1 mg/L, and then the flavor solution (0.8 mL) was placed in a 2 mL headspace sampling vial with a septum seal after 30 min of sonication at room temperature (27 ± 1 °C). The samples were equilibrated at room temperature for 1 h before SPME sampling. All analyses were performed on an HP 5890GC/5970MS equipped with a Varian 8200 CX autosampler (Walnut Creek, CA). Polydimethylsiloxane fibers (100 μm thickness) were purchased from Supelco (Bellefonte, PA) and conditioned for 1 h in an injection port at 250 °C before first use. A DB-5 MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (J&W Scientific, Folsom, CA) was used for all analyses. The oven was kept at 50 °C for 3 min and raised at 10 °C/min to 200 °C. The injector and interface temperatures were kept at 230 and 280 °C, respectively. The carrier gas was helium with a flow rate of 27 cm/s. The injector was purge-off for 3 min, during which time thermal desorption of analytes from the fiber occurred. There was no carry-over between samples observed with a 3 min desorption time. Following desorption, the inlet was switched to purge-on for the remainder of the run, and at the same time the SPME fiber was removed from the injector. The MS detector was operated in the scan mode with 70 eV electron impact.

Determination of Headspace/Water Partition Coefficients. The equilibrium concentration of the volatile compounds in the headspace was measured using static headspace sampling (*21*). The flavor solutions for the measurement of the headspace/water partition coefficient ($K_{HS/water}$) were prepared by adding 10 mg of each volatile compound (ethyl acetate, isoamyl acetate, ethyl hexanoate, hexanal, 2-heptanone, and 2-nonanone) to 1 L of distilled water. The individual solutions were sonicated at room temperature (27 ± 1 °C) for 30 min to homogeneously solubilize the flavors. The sample solution (3 mL) was transferred into a 10 mL vial, then capped, and equilibrated at room temperature for 1 h. After the equilibration time, 1 mL of the headspace sample was injected onto an HP 6890 GC-FID by a 1 mL gastight syringe (Hamilton Co., Reno, NV) in splitless mode. The GC was equipped with a DB-5 MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The initial oven temperature was 40 °C for 1 min, ramped to 100 °C at 10 °C/min, and then further to 200 °C at 30 °C/min. The injector and detector temperatures were maintained at 210 and 250 °C, respectively. The syringe was thoroughly flushed with inert nitrogen gas before and after injection. One measurement consisted of at least four repetitions of this method.

Peak areas determined by static headspace were converted to concentrations using calibration curves. For the calibration curves, 1 mg/mL of a volatile compound in methanol was serially diluted in methanol to give concentrations of 10, 100, 500, and 1000 mg/L, and then 1 μL of the liquid sample was injected directly onto the GC-FID and analyzed using the same conditions as described previously for the samples. Each standard was analyzed in triplicte. Linear correlation coefficients ranged from 0.94 to 0.98 for all analytes.

The headspace/water partition coefficient, $K_{HS/water}$, is given by the equation

$$K_{HS/water} = \frac{C^{\infty}_{HS}}{C^{\infty}_{water}} \quad (1)$$

where C^{∞}_{HS} and C^{∞}_{water} are the concentrations of the volatile compound in the air and water phase at equilibrium, respectively. As the volatile compound in the system distributes between the air phase and the water until equilibrium has been reached, the concentration in the solution can be calculated from the initial volatile concentration, C'_{water}, the volume of the headspace, V_{HS}, and the volume of the solution, V_{water} (eq 2).

$$C^{\infty}_{water} = C'_{water} - C^{\infty}_{HS}\left(\frac{V_{HS}}{V_{water}}\right) \quad (2)$$

Using this procedure, the partition coefficients between headspace and water ($K_{HS/water}$) measured in this study were comparable to those obtained in previous studies (*21*−*26*).

Calculation of Fiber/Headspace Partition Coefficients. Partition coefficients between the SPME fiber and headspace were estimated according to eq 3

$$\log K_{fiber/HS} = 0.005622(\text{LTPRI}) - 0.64845 \quad (3)$$

which has been derived by Liu and Wene (*27*) on the basis of the known chromatographic theory (*28*). The equation has been proven to be applicable to alcohols, aldehydes, esters, and ketones, with a direct correlation between an SPME fiber coating (PDMS) and a GC column. However, as discussed by Liu and Wene, the column they used to generate the LTPRI was 5% phenyl polydimethylsiloxane, which is slightly different from the stationary phase on the PDMS SPME fiber. Using identical stationary phases and more compounds in the standard mixture would provide the most accurate correlation between $K_{fiber/HS}$ and LTPRI.

Equilibrium Dialysis Experiment. Dialysis membrane tubing was cut and soaked in a 10% $NaHCO_3$ solution (90 °C) for 20 min followed by rinsing thoroughly with distilled water. BSA was dissolved in phosphate buffer (pH 7.3 at 24 °C) at a concentration of 10 μM, and then 10 mL of the BSA solution was transferred into the dialysis membrane and the tube sealed shut. Each ketone compound (2-heptanone or 2-nonanone) was solubilized in the buffer to give a concentration of 15 mg/L, and then 40 mL of the solution was transferred into a 120 mL wide-mouth sample jar (amber) along with the dialysis bag filled with the buffered protein solution. The sample container was tightly closed with a Teflon−PTFE-lined screw cap (Cole Parmer Inc. Co., Vernon Hills, IL). The samples were equilibrated at room temperature for 24 h with magnetic stirring before SPME analysis. Aliquots (0.8 mL) were then removed from each compartment (inside and outside membrane) and placed in 2 mL sample vials. The free concentration of each ketone was measured for each compartment by headspace SPME combined with GC-MS analysis.

Standard curves were constructed for each ketone in order to convert the ion response obtained from HS-SPME to molar concentration. A stock solution (1 mg/mL) in methanol was used to prepare 0.5, 1, 3, 5, and 10 μg/mL standard solutions, and the methanol content in the

standard solutions was maintained at <1%. The GC-MS conditions for both the equilibrium dialysis and the standard analysis were the same as used for the SPME sampling time measurement with 1 min of adsorption time. Each sample was analyzed five times, and the data were pooled for linear regression analyses. Excellent correlation coefficients (≥0.97) were obtained for each ketone.

Headspace Concentration of Flavors as a Function of Nonvolatile Matrix Components. BLG (5 g/L) or (+)-catechin (10 g/L) was dissolved in ~500 mL of water in a 1 L volumetric flask, and 10 mg of the flavor compound was directly added. The flask was then filled with water up to 1 L, and the sample solution was sonicated for 30 min. To aid solubilization, the catechin solution was heated to 50 °C as described by King and Solms (*29*). The conditions for equilibration and HS-SPME/GC-MS analysis were the same as the measurement of sampling time using 1 min of adsorption time. Experiments were done in triplicate, and comparison of means was made by a two-population (independent) *t* test at a 5% significance level.

RESULTS AND DISCUSSION

Headspace SPME Measurement of Flavor Volatility and Relationships to Partition Coefficients. The partition coefficient for a volatile compound between headspace and water ($K_{HS/water}$) can be related to its hydrophobicity, volatility, and solubility, and the presence of nonvolatile constituents in solution can subsequently change the thermodynamic behavior of the volatile compound.

HS-SPME measurements of volatile compounds are influenced by the partition coefficient of the analyte between the headspace and the matrix as well as between the fiber and the headspace. Once equilibrium of a volatile between the headspace and matrix is obtained, an SPME fiber inserted in the headspace may cause a fluctuation in the equilibrium by extracting analytes from the headspace onto the fiber, resulting in reequilibration between the three phases. However, the length of the sampling time will influence the extent of the reequilibration. For example, if HS-SPME is used for quantification of analytes, the fiber is held in the headspace until the steady state between the three phases is reached and a maximum amount of analyte is adsorbed ("extracted headspace") (*30*). Conversely, Roberts and co-workers (*30*) proposed that HS-SPME with a short sampling time (<1 min) can determine the "true headspace" concentration at equilibrium between headspace and water, which can minimize the disruption caused by the fiber/headspace partition. The "true headspace" discussed by Roberts and co-workers (*30*) reflects the volatile compounds in the air space at equilibrium between the headspace and the sample solution.

In the present study, an SPME fiber was introduced into a sample headspace and held for different time periods (1−60 min) before analysis by GC-MS. To clearly illustrate the kinetic profiles of individual volatiles, changes in response with different sampling times are shown using a logarithmic scale on both the *x* and *y* axes. Clearly, the time to reach the steady state varies for different volatile compounds. For example, 2-nonanone required ≥60 min to reach the steady state, whereas for ethyl acetate 1 min was enough to reach the steady state.

These results are closely related to the partition coefficients of the compounds (**Table 1**). For example, comparing the kinetics of 2-heptanone and 2-nonanone to the fiber adsorption (2-nonanone has a larger $K_{fiber/HS}$ value than 2-heptanone but a similar $K_{HS/water}$), 2-nonanone required a longer equilibration time when the SPME fiber was introduced into the headspace. The longer equilibration time for the compounds with high $K_{fiber/HS}$ is caused by slow diffusion of analyte molecules from the aqueous phase to the headspace when the fiber is introduced into the headspace (*14*). Although the diffusion coefficient in the fiber coating is actually smaller than that in the aqueous phase, the thickness of the coating is usually so small that diffusion coefficients in the fiber are negligible (*14*). However, compounds with relatively high $K_{fiber/HS}$ values and small $K_{HS/water}$ values are present at low concentration in the headspace. For these compounds, even relatively short SPME sampling times may change the headspace concentration and, subsequently, influence the equilibrium between the headspace and water ($K_{HS/water}$).

Table 1. Calculated Partition Coefficients of Flavor Compounds

	$K_{fiber/water}$[a]	$K_{fiber/HS}$[b]	$K_{HS/water} \times 10^3$	K_H[d]
ethyl acetate	5.7	617	9.3 ± 0.4[c]	0.23
isoamyl acetate	124.7	21877	5.7 ± 0.2	0.14
ethyl hexanoate	1997.2	93325	21.4 ± 5.0	0.52
hexanal	133.4	19055	7.0 ± 0.6	0.17
2-heptanone	136.6	22387	6.1 ± 0.3	0.15
2-nonanone	2070.5	309030	6.7 ± 0.1	0.16

[a] Partition coefficient between fiber and water; $K_{fiber/water} = K_{fiber/HS}K_{HS/water}$. [b] Partition coefficient between fiber and air; calculated by linear temperature-programmed retention index (LTPRI) (*27*): log $K_{fiber/HS}$ = 0.005622(LTPRI) − 0.64845. [c] Mean of four (at least) determinations ± standard deviation; experimentally obtained from static HS measurement. [d] Henry's constant in atm·L·mol^{-1}: calculated by $K_{HS/water}RT$, where R is a gas constant (= 0.082 L·atm·K^{-1}·mol^{-1}) and T is the temperature in Kelvin.

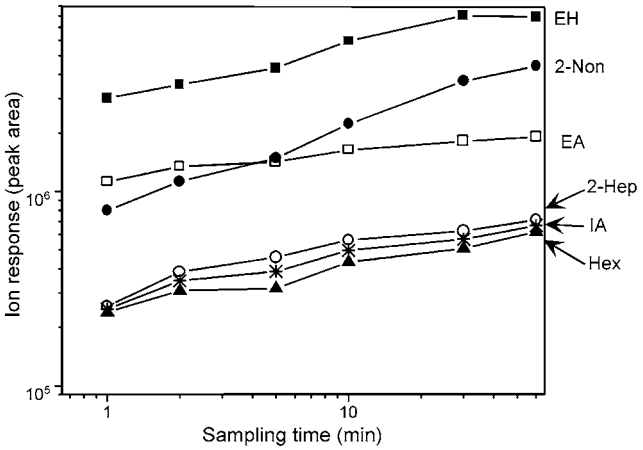

Figure 1. Changes in ion response of (■, EH) ethyl hexanoate, (●, 2-Non) 2-nonanone, (□, EA) ethyl acetate, (○, 2-Hep) 2-heptanone, (∗, IA) isoamyl acetate, and (▲, Hex) hexanal as a function of adsorption time (SPME sampling time) in headspace measured by HS-SPME/GC-MS analysis.

For volatile compounds, such as ethyl acetate, which have a relatively small $K_{fiber/HS}$ and large $K_{HS/water}$ values, the sampling time is determined largely by diffusion in the vapor phase; the sampling time is quite short as shown in **Figure 1**. In addition, at these relatively high concentrations of 1 mg/L, the amount of analyte extracted by the fiber coating is small compared to the amount of analyte existing in the headspace, and the concentration in the aqueous solution is virtually unchanged during the extraction.

These results are consistent with those reported by Roberts et al. (*30*) and Fabre (*20*) and indicate that it may be necessary to carefully consider the kinetic profiles of headspace concentrations when volatile compounds are extracted from the headspace. This is particularly true when the experimental objectives are to measure true headspace and short sampling times (e.g., ~1 min) are used.

Binding Studies Using Equilibrium Dialysis Combined with HS-SPME Extraction. The application of HS-SPME for

Table 2. Headspace Concentrations and Relative Bindings for 2-Heptanone and 2-Nonanone in the Presence or Absence of BSA

	free concentration[a] (mg/L)		
	in[c]	out	binding[b] (%)
2-heptanone w/BSA	1.32 ± 0.08	1.38 ± 0.14	29 ± 9
2-heptanone w/o BSA (control)	1.86 ± 0.11	1.92 ± 0.10	
2-nonanone w/BSA	3.74 ± 0.15	3.60 ± 0.09	45 ± 10
2-nonanone w/o BSA (control)	6.90 ± 0.13	6.55 ± 0.11	

[a] Mean of five repetitions ± standard deviation. [b] (Mean of control − mean of headspace concentration for each ketone w/BSA) × 100/mean of control. [c] BSA concentration inside membrane = 0.67 g/L.

quantifying flavor/matrix interactions was initially evaluated in combination with an equilibrium dialysis technique similar to that described by Vaes et al. (*31*). In equilibrium dialysis, ligands bind to a protein (or other binding substrate) contained on one side of a dialysis membrane, and at equilibrium the free ligand concentration will be the same on both sides of the membrane. The ligands chosen for the present study were ketones, which have been previously shown to significantly bind to BSA using a liquid−liquid partition equilibrium method (*32*). Using 1 min of HS-SPME sampling as previously described, we measured the concentration of free ligand (2-heptanone or 2-nonanone) from the respective compartments of an equilibrium dialysis cell with or without added BSA (**Table 2**). To exclude the possibility of nonselective binding of volatiles to the membrane, or the sample container, control samples without BSA added were also analyzed.

The results indicate that the free headspace concentration inside the membrane (containing BSA) as measured by HS-SPME was very close to the free concentration outside (no BSA). The protein itself did not influence the amount of volatiles adsorbed by the fiber or interfere with measurement. In addition, the sampling procedure was able to measure the headspace concentration without inducing additional extraction of volatiles bound to BSA. The relative binding for the individual ketones was also calculated relative to the corresponding controls (**Table 2**). Binding to BSA was greater (~15%) for 2-nonanone compared to 2-heptanone, consistent with previous studies (*32*). This study shows an example of the suitability of HS-SPME to analyze the free volatile concentration of compounds in the headspace of a heterogeneous solution.

Binding of Volatiles to β-Lactoglobulin and Catechin. The HS-SPME technique was further used to study flavor binding to food proteins and polyphenolics. BLG, one of the most important milk proteins, has been extensively studied in terms of structural characteristics and binding phenomena with small ligands, particularly with many volatile compounds (*33*−*35*). Catechin, which is a well-known polyphenolic compound, has attracted much attention in relation to its biological function and sensorial effects and has been shown in some studies to interact selectively with flavor compounds (*36*). The possible binding of volatile compounds to β-lactoglobulin or (+)-catechin has been studied by means of various experimental techniques, but not by the HS-SPME method. For ease of comparison, the extent of retention for the individual volatiles in the presence of the nonvolatile constituents was expressed in a normalized ion response related to the response for water (100%) and was statistically compared using a *t* test (**Figure 2**).

Ethyl hexanoate appeared to be more retained (less volatile) in the presence of BLG compared to isoamyl acetate. The

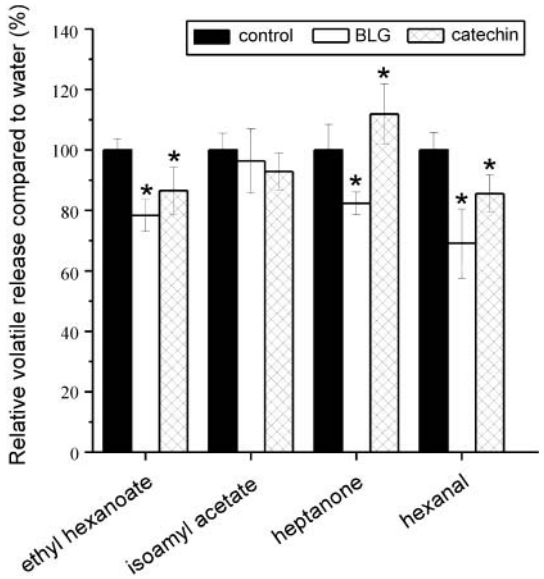

Figure 2. Changes in ion response of flavor headspace concentration with the addition of BSA (5 g/L) and catechin (10 g/L) analyzed by SPME/GC-MS. Asterisks (*) on the tops of the bars indicate the significant difference (*t* test, $p < 0.05$) for each treatment relative to the control. For each compound results are normalized to the response for water (control).

volatility of ethyl hexanoate was reduced by 20% in the presence of BLG when compared to the value for water, whereas isoamyl acetate volatility was decreased only by 5%. The result is comparable with other studies, in which the extent of interactions between esters (methyl esters and ethyl esters) and BLG increased with the hydrophobicity of the esters (*18*, *20*). BLG addition also influenced the volatility of 2-heptanone and hexanal in aqueous solution. 2-Heptanone and hexanal showed, respectively, 20 and 30% reduced release into the headspace. Ketones and aldehydes are well-known ligands which bind to number of proteins including BLG (*37*, *38*). In particular, aldehydes can tightly bind to proteins with partially irreversible chemical bonds (*39*).

The greatest effect of (+)-catechin was observed for the esters (especially ethyl hexanoate) and the aldehyde, hexanal, for which the volatility decreased by 10−20%, whereas the volatility of 2-heptanone increased 15%. Dufour and Bayonove (*36*) showed that catechin influenced the release behavior of several wine volatiles using an exponential dilution technique. In this previous study, isoamyl acetate, ethyl hexanoate, and benzaldehyde were found to be more retained than limonene in a hydroalcoholic solution with catechin concentrations ranging from 0 to 5 g/L. No increase in the solubility of ethyl benzoate was observed in a 20 mM (5.8 g/L) catechin solution (*29*). Catechin may affect the thermodynamic behavior of volatiles selectively according to the chemical class of the volatile.

In this study, HS-SPME analysis combined with GC-MS was used to evaluate the binding between several selected flavor compounds and two nonvolatile matrix constituents. The evolution of ion responses and the partition coefficients for individual flavor compounds clearly indicated that the SPME sampling time should be carefully chosen to be consistent with the objectives of the analysis (e.g., equilibrium headspace or "extracted" headspace) and the thermodynamic characteristics of the volatiles. Equilibrium dialysis combined with HS-SPME analysis validated the assumption that HS-SPME with a short sampling time can successfully extract free volatiles from the headspace without interference from matrix components such as proteins. In addition, this technique was applied to the simple

flavored aqueous solutions containing either BLG or (+)-catechin to further show the capability of HS-SPME for quantifying flavor−matrix interactions. The volatility of each flavor compound in the presence of the nonvolatile constituents was dependent on the chemical and physiochemical characteristics of the flavors with relation to the nonvolatiles

The amounts of BLG and (+)-catechin as well as the volatile compounds used in this study are higher than those occurring naturally in foods and beverages. These high amounts (concentrations) have the advantages of facilitating the instrumental analysis and showing the application of the HS-SPME technique for the characterization of interactions between aroma compounds and nonvolatile food matrix components. However, these model systems would not completely explain the phenomena occurring in a real food system. To understand the interactions in a real food matrix, it is necessary to study lower concentrations as well as more complex model systems.

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