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Comparison of Different Methods: Static and Dynamic Headspace and Solid-Phase Microextraction for the Measurement of Interactions between Milk Proteins and Flavor Compounds with an Application to Emulsions

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Interactions between 10 aroma compounds from different chemical classes and 5 mixtures of milk proteins have been studied using static or dynamic headspace gas chromatography and solid-phase microextraction (SPME). Static headspace analysis allows the quantification of the release of only the most abundant compounds. Dynamic headspace analysis does not allow the discrimination of flavor release from the different protein mixtures, probably due to a displacement of headspace equilibrium. By SPME analysis and quantification by GC-MS (SIM mode) all of the volatiles were quantified. This method was optimized to better discriminate aroma release from the different milk protein mixtures and then from oil/water emulsions made with these proteins. The highest difference between the release in different proteins was observed for ethyl hexanoate, which has a great affinity for β-lactoglobulin. Ethyl hexanoate is thus less released from models and emulsions containing this protein.

KEYWORDS: **Flavor compounds; milk proteins; interactions; headspace; solid-phase microextraction; emulsion**

INTRODUCTION

Flavor perception in foods, which is a high determinant of food acceptance, is influenced by interactions between flavor compounds and a variety of nonflavor matrix components. Interactions of flavor compounds with proteins are known to have a strong influence on the release of flavor from foods (*1*). Proteins often cause a decrease in the volatility of flavor compounds. It is well-known that proteins interact with volatiles both reversibly (*2*, *3*) and irreversibly (*4*, *5*). In general, proteins retain the volatiles by reversible hydrophobic interactions, adsorption or absorption, and by chemical bonds of various strengths. The strength of the interactions depends on the ability of flavor compounds to induce the unfolding of the protein. They may occur between all components and can be very different in nature. Interactions between volatile aroma substances and nonvolatile compounds are of two types: attractive (fixation of volatile compounds on nonvolatile substrate) or repulsive (release of the volatile compounds).

Interactions between proteins and volatiles greatly depend on the physical and chemical nature of the molecules involved. Of all the different proteins in food, β-lactoglobulin is one of the best known and most studied. This whey protein has been extensively characterized; it has good emulsifying properties (*6*) and is known to interact with many flavor compounds, such as aldehydes and ketones (*2*), ionones (*7*), and hydrocarbons (*8*).

However, aroma compounds do not have the same behavior according to chemical classes. For example, short acids and methylpyrazines (*9*) were found not to interact with β-lactoglobulin, whereas methoxypyrazines do interact with β-lactoglobulin (*10*). Hydrophobic interactions occurred between esters and β-lactoglobulin, because the global affinity increased when the length of one of the two hydrophobic chains increased (*9*).

The impact of a food component on the retention or release of a volatile compound usually implies the use of a headspace GC technique. Trapping involving headspace concentration, using porous polymer absorbents, has been widely used for the analysis of aroma release (*11*).

More recently, a technique called solid-phase microextraction has been developed by Pawliszyn and co-workers (*12*). The fused silica fiber used as adsorbent is introduced into the headspace above the sample. Several factors such as the need to be in the linear range of detection and competition effects on the fiber between volatiles can cause biases in the quantitative determination of compounds (*13*−*15*). Researchers have demonstrated many instances of competition. For example, ethanol replaced acetone and isoprene on PDMS/DVB (*15*, *16*).

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Table 1. Aroma Compounds by Concentration Order in the Blend: Odor Threshold, Binding Constant

aroma compound	odor threshold, mg/kg in water (lit. cited)	binding constant with β-lactoglobulin K_B, M^{-1} (lit. cited)
ethyl butanoate (fruity)	0.001 (*25*)	55 (*9*)
ethyl hexanoate (fruity)	0.003 (*25*)	543 (*9*)
mesifuran (burnt)	0.01 (*25*)	19 (*10*)
methyl hexanoate (fruity)	0.087 (*25*)	244 (*9*)
hexenol (green)	0.5 (*26*)	70 (*3*)
3-methylbutanoic acid (fruity)	0.18 (*25*)	ND
diacetyl (buttery)	0.003 (*27*)	ND
linalol (flower)	0.006 (*26*)	565 (*10*)
γ-octalactone (sweet, flower)	0.0017 (*28*)	450 (*3*)
methyl dihydrojasmonate (wild strawberry)	ND[a]	ND
propylene glycol = solvent (95%)		

[a] ND, not detected.

Elmore et al. (*17*) have compared dynamic headspace and solid-phase microextraction (SPME). The samples studied were cola and diet cola. The dynamic headspace method extracted a greater number of volatile compounds from both samples than did SPME. Reproducibilities were similar for both techniques.

In the present study, we have thus chosen to compare static and dynamic headspace with SPME, for the measurement of interactions between milk proteins and aroma compounds from different chemical classes present in a concentration range used for industrial purpose and their incidence on flavor release from emulsions.

MATERIALS AND METHODS

Materials. *Water.* Pure water was obtained from a Milli-Q system (Millipore, Bedford, MA).

Flavor Compounds. The flavor blend is composed of flavor compounds from different chemical classes and dissolved in propylene glycol; these compounds were obtained by flavor suppliers (FIS, York, U.K.). They appear by concentration order in the blend in **Table 1**. Final concentrations of flavor compounds varied between 0.16 and 10 ppm. They represent some major chemical functions found in food aromas: ketones, esters, alcohols, acids. etc.

Blank Solutions. The blank solutions were made by dissolving the flavor blend in water (1% w/w), 24 h before analysis.

Protein Solutions. Commercial protein powders (milk proteins) are described as follows: W, whey protein concentrate, LACPRODAN DI 9224, MD Foods; A, mixture of W and α-lactalbumin, LACPRODAN ALPHA 10, MD Foods; B, mixture of W and β-lactoglobulin, PROTARMOR 865, Armorproteine; L, milk powder (0% fat), Besnier-Bridel-Aliment; WL, mixture of W and L (50/50). The protein solutions were made by dissolving proteins in water (3% w/w). The flavor compounds (1%) were incorporated and mixed for 15 min, 24 h before analysis for maturation.

Emulsions. Emulsions were composed of 3% proteins, 9% fat, 0.5% emulsifier, and 1% flavor blend, in water. They were prepared 24 h before analysis for maturation.

Extraction Methods. *Static Headspace Analysis.* Analyses were done in triplicate in amber flasks (40 mL) closed with mininert valves (Supelco, Bellefonte, PA). Analyzed solutions (10 mL), with (protein solutions) or without protein (blanks), were stirred at 25 °C until equilibrium (1 h). Injections of vapor phase (1 mL) were realized on a GC 8000 series, Fisons Instruments gas chromatograph equipped with a DB-Wax column (J&W Scientific, i.d. = 0.32 mm, 30 m, film thickness = 0.5 μm). Injector and detector temperatures were, respectively, 250 and 260 °C. The H_2 carrier gas velocity was 1.9 mL min^{-1}.

Dynamic Headspace Analysis. Protein solutions or blank solutions (20 mL) were put in a 500 mL flask. The samples were purged with helium at a flow rate of 40 mL min^{-1}, at room temperature (25 °C). The volatiles were adsorbed on a Tenax trap (5 min). Water was eliminated with a back-flush of nitrogen at 100 mL min^{-1} for 1 min. The Tenax trap was thermally desorbed (240 °C for 20 min with a flow of 20 mL min^{-1} hydrogen), and the volatiles were cryofocused on a cold trap (−130 °C) and injected at 250 °C in a GC system (HP 6890 series, Hewlett-Packard, Palo Alto, CA). Temperature program: 40 °C (5 min) at 240 °C (5 min) at 3 °C min^{-1} rate.

Table 2. Kinetics of the Adsorption of Flavor Compounds on the SPME Fiber (Chromatographic Peak Area)

	45 min	60 min	80 min	100 min
diacetyl	318690	671940	671820	613985
ethyl butanoate	28644941	66637586	66487464	63793679
methyl hexanoate	11610211	25862526	24379659	25481588
ethyl hexanoate	7233585	16387290	15845741	15499538
hexenol	570671	1104420	1034985	1178690
linalol	292783	530216	536283	532265
mesifuran	45516	94950	91550	94784
lactone	84780	174717	169076	1733530

SPME. Different parameters were tested to optimize this method: similarity of adsorption of three fibers from the same batch; influence of the quantity in the flask (1, 5, and 10 g); equilibrium time (45, 60. and 80 min at 30 °C); trapping time of flavor compounds on the fiber (1 and 2 min); linearity of detection (several quantities of flavor blend were tested: 0.1, 0.3, 0.5, 1, and 2%).

Samples were placed in a 20 mL vial and allowed to equilibrate for different times. An SPME fiber, polydimethylsiloxane/divinylbenzene (PDMS/DVB), 65 μm (Supelco), was used for sampling volatile compounds.

Volatile compounds were desorbed by inserting the fiber into the GC injector set at 250 °C for 10 min, 1 min for desorption (purge off) and 9 min for cleaning (purge on).

All of the SPME operations were automated using an MPS2 multipurpose sampler (Gerstel Applications, Brielle, The Netherlands).

GC-MS Analysis. An HP 5890 equipped with a split/splitless injector coupled with a mass selective detector 5970 (Hewlett-Packard, Palo Alto, CA) was used. A fused-silica capillary column, DB-Wax, 50 m, 0.32 mm i.d., 1 μm film thickness (J&W Scientific), was employed. The carrier gas was helium (35 cm s^{-1}).

The GC oven heating was started at 50 °C and then increased to 220 °C at a rate of 5 °C min^{-1}. The total time of analysis was 39 min. The injector was maintained at 250 °C.

The mass spectrometer was operated in the mass range from 29 to 300 at a scan rate of 1.89 s/scan. The quantification was realized by selective ion monitoring (SIM) mode. The selected and specific ions are 43 for diacetyl, 88 for ethyl butanoate, 87 for methyl hexanoate, 101 for ethyl hexanoate, 82 for hexenol, 93 for linalol, 142 for mesifuran, 60 and 87 for 3-methylbutanoic acid, 85 for γ-octalactone, and 83 for methyl dihydrojasmonate.

Statistical Analysis. Results obtained are given in percents (peak area in protein solutions divided by the same peak area in blank solutions). Experiments were done in triplicate. For each flavor compound a one-way analysis of variance (protein effect) was performed with Statbox software (Grimmer Logiciel, France). Mean comparison was made by a Newman−Keuls test at 5%.

RESULTS AND DISCUSSION

Static Headspace Measurement. This method allowed us to detect and quantify only 3 of the 10 aroma compounds in the protein solutions: the three esters, ethyl butanoate, methyl hexanoate, and ethyl hexanoate, which are the most abundant and present at a concentration >0.16 ppm and close to 10 ppm in the final concentration (**Table 3**).

For protein solutions that contained caseins (L and WL), the release of ethyl hexanoate was higher than the release in protein solutions which were exclusively composed of whey proteins (A, B, and W). The release observed with ethyl hexanoate was smaller than the release with ethyl butanoate or the release with methyl hexanoate; this lower release was mostly explained by

Table 3. Classification of Flavored Protein Solutions and Emulsions (Newman–Keuls Test at the Risk Level of 5%) According to Aroma Release (Higher to Smaller Values in Percents, Peak Area in Protein Solutions Divided by the Same Peak Area in Blank Solutions) by Static, Dynamic, and SPME Headspace Analysis[a]

	protein solutions						emulsions	
aroma compound	static headspace analysis		dynamic headspace analysis		SPME analysis		SPME analysis	
ethyl butanoate	a	WL = 102 ± 5			a	B = 98 ± 1		B = 97 ± 2
	ab	W = 99 ± 1			b	L = 93 ± 2		A = 96 ± 2
	b	B = 95 ± 1		NQ[a]	b	A = 92 ± 3.5	NS	W = 96 ± 2
	b	L = 94 ± 3			c	W = 84 ± 3		L = 90 ± 7
	b	A = 93 ± 1			d	WL = 79 ± 3		WL = 86 ± 2
methyl hexanoate	a	WL = 93 ± 5			a	L = 89 ± 2	a	W = 22 ± 1
	a	L = 92 ± 7			b	A = 84 ± 4	ab	A = 21 ± 1
	ab	W = 82 ± 2		NQ	bc	W = 82 ± 2	bc	B = 20 ± 1
	ab	A = 80 ± 2			c	B = 79 ± 2	c	L = 19 ± 1
	b	B = 71 ± 2			d	WL = 75 ± 5	c	WL = 18 ± 1
ethyl hexanoate	a	L = 59 ± 2			a	L = 78 ± 3	a	W = 12 ± 1
	a	WL = 58 ± 3			b	W = 76 ± 2	b	A = 11 ± 1
	b	A = 50 ± 1		NQ	c	A = 73 ± 3	c	WL = 10 ± 1
	b	W = 48 ± 1			d	WL = 69 ± 4	c	L = 9.8 ± 1
	c	B = 37 ± 2			e	B = 64 ± 1	c	B = 9 ± 1
diacetyl				A = 73 ± 11		A = 102 ± 2		
				L = 67 ± 6		B = 100 ± 2		
		NQ	NS	W = 66 ± 2	NS	W = 87 ± 2		NQ
				WL = 66 ± 1		L = 82 ± 3		
				B = 63 ± 2		WL = 78 ± 1		
linalol				WL = 72 ± 1	a	W = 92 ± 2		L = 164 ± 12
				L = 67 ± 5	ab	L = 855 ± 2		A = 154 ± 8
		NQ	NS	W = 65 ± 5	abc	A = 82 ± 4	NS	WL = 150 ± 4
				B = 55 ± 1	bc	WL = 79 ± 9		B = 149 ± 3
				A = 54 ± 13	c	B = 74 ± 1		W = 143 ± 6
mesifuran				W = 72 ± 5	a	A = 84 ± 8	a	W = 10 ± 1
				L = 56 ± 2	a	W = 81 ± 3	b	L = 9 ± 1
		NQ	NS	A = 55 ± 17	a	WL = 77 ± 7	b	WL = 9 ± 1
				B = 48 ± 9	a	L = 73 ± 9	b	B = 8 ± 1
				WL = 46 ± 2	b	B = 49 ± 2	b	A = 8 ± 1
hexenol					a	L = 84 ± 2		L = 126 ± 16
					a	B = 82 ± 2		WL = 106 ± 8
		NQ		NQ	ab	WL = 77 ± 6	NS	B = 95 ± 5
					ab	A = 76 ± 4		A = 90 ± 18
					b	W = 72 ± 1		W = 85 ± 9
γ-octalactone					a	A = 88 ± 2		W = 63 ± 2
					a	L = 84 ± 5		A = 62 ± 13
		NQ		NQ	ab	W = 79 ± 3	NS	B = 54 ± 3
					ab	WL = 78 ± 7		L = 53 ± 6
					b	B = 72 ± 6		WL = 51 ± 4
methyl dihydrojasmonate					a	W = 53 ± 9		
					ab	A = 40 ± 4		
		NQ		NQ	b	L = 38 ± 10		NQ
					b	WL = 32 ± 9		
					b	B = 22 ± 1		

[a] W, whey protein concentrate; A, mixture of W and α-lactalbumin; B, mixture of W and β-lactoglobulin; L, milk powder (0% fat); WL, mixture of W and L; NS, not significant at the risk level of 5%; NQ, not quantified. Means within a row with different letters (a–e) are significantly different ($P = 0.05$).

a greater retention by the proteins due to a greater affinity of ethyl hexanoate than the other esters, confirming the hydrophobic nature of the interactions already mentioned by Damodaran and Kinsella (*18*) and Pelletier (*9*).

The release of ethyl hexanoate was less important in B protein solutions (β-lactoglobulin) than the release in A protein solutions (α-lactalbumin). This is due to a greater affinity of β-lactoglobulin ($K_B = 950 \pm 0.7$) for this flavor compound than that of α-lactalbumin ($K_B = 472 \pm 1.2$) (*19*).

Dynamic Headspace Measurement. Six of the 10 volatile compounds were detected by dynamic headspace in the protein solutions (**Table 3**). The first three, the esters (ethyl butanoate, methyl hexanoate, and ethyl hexanoate), could not be quantified, because their concentrations were too high and thus were not in the linear range of quantification by the detector (due to their concentration close to 10 ppm in the final concentration). For the three following compounds (diacetyl, mesifuran, and linalol), no significant effect of the nature of the protein was observed.

γ-Octalactone and methyl dihydrojasmonate were not detected due to their concentration close to 0.16 ppm in the final solution. 3-Methylbutanoic acid was not detected due to low concentration and high solubility in water.

As the flavor blend was formulated for an industrial application, it was not possible to change the concentration range of the different compounds. To quantify the compounds present at the lower concentration, a longer trapping time, a higher flow, or a higher temperature would be needed (*20*). In these conditions the esters could not be quantified due to their high

concentration and their high volatility, and the equilibrium would be more displaced.

In conclusion, for these two techniques static headspace analysis allows us to discriminate the release of the most volatile compounds from the different protein solutions. By using dynamic headspace, the differences observed were not significant, probably due to a displacement of the equilibrium, which reduces the retention effect of the protein. For this reason we performed SPME in conditions close to a "true" headspace (at equilibrium).

SPME Analysis. SPME, coupled with GC-MS (SIM mode), was used to quantify aroma release in the vapor phase.

It has been reported that the extraction efficiency of the SPME method depends not only on the nature of the coating of the SPME fiber but also on the temperature of sampling, time of extraction, etc. Thus, PDMS/DVB-coated fiber was chosen because its sensitivity was adapted to the 10 aroma compounds (*15*). Two other fibers, Carboxen/poly(dimethylsiloxane) (CAR/PDMS, 75 μm) and Carbowax/divinylbenzene (CW/DVB, 65 μm), were also tested. The aim was to evaluate the possibility to improve the volatile isolation by favoring the affinity of molecules such as acids for the stationary phase of the fiber. No real improvement was noticed using these two different SPME fibers (*21*). Ten flavor compounds were detected, and eight were quantified by this method.

An optimization of the experimental conditions was realized with the blank solutions.

(*a*) Three different fibers with the same coating (PDMS/DVB) were tested, but they did not give the same results.

The peak areas obtained with one fiber were twice the peak areas obtained with the two others for all of the compounds, due to repeatability of fabrication. Thus, it is important to use the same fiber for comparative analysis.

(*b*) Different quantities in the flask (20 mL) were tested: 1, 5, and 10 g.

No significant differences between the samples were found, which was expected for the vapor liquid equilibrium, and thus we may conclude that the presence of the fiber does not modify this equilibrium. Thus, we kept 5 g as the amount used in previous experiments.

(*c*) Different times of equilibrium were tested: 45, 60, 80, and 120 min.

After 1 h, peak areas of flavor compounds over the protein solution remain stable. Thus, 1 h was chosen for equilibration time (**Table 2**).

The same time was chosen in the presence of emulsions because peaks areas of flavor compounds remain stable too.

(*d*) The extraction time chosen was 60 s.

Our goal is not to obtain a complete extraction of the food matrix but results closer to a "true" headspace. For this reason we chose the conditions optimized by Roberts et al. (*15*).

(*e*) SPME was applied to the quantification of flavor release from protein solutions.

Diacetyl was better released from whey protein (B and W) solutions than from casein (L) solutions, but the differences observed are not significant at the 5% level; this result is in agreement with that obtained by dynamic headspace analysis (**Table 2**).

The results obtained for the three esters are in agreement with those obtained by static headspace analysis. Retention by proteins is greater for ethyl hexanoate, the most hydrophobic of the three esters, but, even if the optimization of SPME procedure tries to be close to "true" headspace, the percentage of retention is lower by SPME than by equilibrium static headspace. The ranking of the protein mixtures by order of release is the same with the two methods for L, A, and B solutions. The release observed in a mixture of whey proteins (W) is not significantly different from that observed in the A mixture. The rank of the WL mixture is not always between the L and W powders. In fact, this protein solution was made by mixing two powders from two different producers, and thus this WL powder may be not homogeneous.

SPME was the unique methodology that allowed us to detect and quantify hexenol. There is no great difference among the protein solutions concerning the release of this compound. This can be due to its low affinity for the proteins (**Table 1**).

By dynamic headspace, for linalol and mesifuran the differences in release obtained among the different protein solutions were not significant at the 5% level. However, with SPME, differences were significant. Flavor release was less important from B protein solutions than from A protein solutions, as already observed for ethyl hexanoate by static headspace analysis.

γ-Octalactone and methyl dihydrojasmonate could be finally detected and quantified by SPME. The aroma release was lower in the B protein solutions than in other protein solutions.

3-Methylbutanoic acid is detected but not quantified because its amount is at the limit of detection for the mass spectrometer.

SPME-GCMS-SIM allowed the detection of all the flavor compounds and also the differentiation of the protein mixtures. It was thus chosen to assess the effect of the nature of the protein on flavor release from model emulsions.

Application of SPME to the Analysis of Flavor Release from Emulsions (Table 3). Comparison of the results with that of corresponding protein solutions was done to observe if differences existed between protein solutions and emulsions.

For methyl and ethyl hexanoate, the aroma release was more important in protein solutions than in emulsions. In fact, the presence of other constituents (such as fat and emulsifier) in these emulsions could decrease the flavor release (*22*, *23*). No significant difference was observed between the release in the emulsions for ethyl butanoate, but a significant effect of the nature of protein was noticed for methyl and ethyl hexanoate. For ethyl hexanoate, flavor release was more important for L emulsion than for B emulsion, which is in agreement with the effect observed in the L and B protein solutions. Even if the addition of fat induced a greater change in flavor release than the addition of protein in water solution, β-lactoglobulin at the oil/water interface may limit the transfer of hydrophobic compounds from oil to water and thus induce a lower flavor release (*24*).

For hexenol, linalol, and γ-octalactone, the differences in release between the five emulsions were not significant.

For mesifuran, the aroma release in W emulsion was higher than in the other emulsions, which is in agreement with the effect observed in the protein solutions, mainly for B protein solution, due to a greater affinity for β-lactoglobulin.

Conclusions. Concerning the comparison of methods, SPME-GCMS-SIM allowed us to determine flavor release from different mixtures of proteins in aqueous or emulsified media.

Concerning the effect of protein type on flavor release, flavor compounds were found to have different behaviors according to their chemical classes in protein solutions or emulsions, due to their different affinities for the proteins. Even if the other constituents, such as fat and emulsifier, may play a non-negligible role in flavor release, the composition of protein induced significant differences in flavor release for methyl and

ethyl hexanoate, which are known to have a high affinity for β-lactoglobulin.

Further experiments including sensory analysis are in progress to study the retention by proteins in real foods.

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