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In Vitro Measurement of Volatile Release in Model Lipid Emulsions Using Proton Transfer Reaction Mass Spectrometry

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ABSTRACT: The presence of fat in food plays an important role in the way aroma is released during consumption and in the creation of the overall sensory impression. Fat acts as a reservoir for lipophilic volatile compounds and modulates the timing and delivery of aroma compounds in a unique manner. Despite considerable research, reproducible in vitro methods for measuring the effect of fat on volatile release are lacking. An open in vitro cell was used to simulate the open human naso-oropharygeal system and was interfaced with a proton transfer reaction mass spectrometer (PTR-MS) to examine some of the fundamental effects of fat on dynamic volatile release in liquid fat emulsions. Lipid emulsions with various fat contents (0−20%) and droplet sizes (0.25, 0.5, and 5.0 μM) were spiked with flavor volatiles representing a range of lipophilicity ($K_{o/w}$ = 1−1380). Preloaded syringes of spiked emulsion were injected into the cell, and temporal changes in release were measured under dynamic conditions. Significant differences in release curves were measured according to the lipid content of emulsions, the vapor pressure, and $K_{o/w}$ values of the volatile compounds. With increasing addition of fat, the critical volatile release parameters, maximum concentration (I_{max}), time to maximum concentration (T_{max}), and the integrated area under the concentration curve (AUC), were affected. The in vitro curves were reproducible and in agreement with theory and correlated with the preswallow phase of in vivo release data. An exponential model was used to calculate changes in mass transfer rates with increased fat addition.

KEYWORDS: *fat, lipid emulsion, in vitro, volatile release, PTR-MS*

INTRODUCTION

Fat acts as a sink for lipophilic volatiles, slowing the rate of release from food during oral processing. During consumption of lipid emulsions it has been demonstrated that as the fat ratio increases, the maximum volatile concentration (I_{max}) and the total amount (AUC) released decrease for lipophilic compounds ($K_{o/w}$ > 1).[1−4] The presence of fat has also been shown to affect the relative distribution of volatile release before and after swallowing during oral processing;[2] complete removal of fat leads to greater initial preswallow release, relative to postswallow. In vivo measurement techniques have a number of potential drawbacks, however: in vivo release data often lack reproducibility due to variability within and between human subjects, in vivo test materials must meet acceptable standards for human consumption, and initial investment in training of human subjects is required. Use of in vitro systems is attractive, especially to screen new formulations and ingredients in the first instance. Release-modifying prototype technologies, for example, microstructured emulsions, are often manufactured from non-food-grade ingredients and/or under non-food-grade manufacturing processes; hence, there is a need for practical standardized in vitro protocols.

Flavor Release in Lipid Emulsions. The amount, timing, and rate of volatile release from a food matrix are critical to create the characteristic sensory impression. The influence of factors such as food macro- and microstructure, particle size, viscosity, and especially fat content on volatile release has been reported.[1−13] Food aroma is complex, normally composed of a mixture of volatile compounds that vary in their volatility and lipophilicity. Both of these and other factors, such as mass transfer in the liquid and gas phases, as well as changes in surface area over during mastication and swallowing, affect the in-mouth temporal release of volatiles. In multiphase systems the amount of volatile in the headspace is determined by partition equilibria

$$K_{a/p} = \frac{C_a}{C_p} \quad (1)$$

where $K_{a/p}$ is the air/product partition coefficient and C_a and C_p are the concentrations of the volatile compound in the gas and product phases, respectively. In lipid-containing food systems, volatiles with higher lipophilicity tend to partition preferentially into the lipid fraction. The presence of fat in food acts as a solvent (or reservoir) for lipophilic flavors, which are released from the food matrix in a slower and more gradual manner. When the fat content of a given food is reduced, it is well-known that volatiles are released differently, leading to an atypical flavor perception.[5,14,15] $K_{a/p}$ is directly related to the air/water partition coefficient $K_{a/w}$ and inversely related to the octanol/water partition coefficient ($K_{o/w}$), where ϕ_o is the phase volume of oil (eq 2). Equation 2 predicts that as ϕ_o decreases, for compounds with $K_{o/w}$ > 1, the rate of volatile release in the air phase will increase.

$$K_{a/p} = \frac{K_{a/w}}{\phi_o(K_{o/w} - 1) + 1} \quad (2)$$

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Furthermore, $K_{a/w}$ can be related to the vapor pressure[16]

$$K_{a/w} = \frac{C_a}{C_w} = \left[\frac{\gamma P}{P_T}\right] \times \frac{V_w}{V_a} \quad (3)$$

where for a given volatile C_a and C_w are the concentrations of volatile in gas (air) and water (v/v), respectively, γ is the chemical activity coefficient in water, P is the saturated vapor pressure, and P_T is the total pressure. V_w and V_a are the molar volumes of the water and gas phases, respectively. Volatile transfer into the gas phase from an agitated aqueous bulk phase is mainly governed by mass transfer effects.[9,10] Exponential models for volatile release based on convective mass transfer have been widely used[10,17,18]

$$C_{hs}(t) = C_{bp}^{0}\left(1 - \exp\left(-h\frac{A_g}{V_{hs}}t\right)\right) \quad (4)$$

where C_{hs} is the headspace concentration at a given time point t (s), C_{bp}^{0} is the initial concentration of volatile added to the bulk phase (mol/L), V_{hs} is the volume of the model mouth vessel headspace (m^3), h is the mass transfer coefficient, and A_g is the interfacial surface area for gas outflow (m^2). The mass transfer coefficient is related to vapor pressure and in the presence of fat, also related to phase partitioning as well as viscosity, shear, and turbulence considerations.[10]

Measuring Volatile Flavor Release. In vivo methods allow the best indication of food interaction with the human eating apparatus; however, they have a number of disadvantages: "in-nose" measurement requires investment in trained subjects and is known to be affected by intra- and intersubject variation.[19−22] In vivo techniques also involve a significant dilution effect, and there are limitations on the amount of sample that can be tested (∼4−10 g). In the development of novel food prototypes, non-food-grade materials and processes are often used, and the pilot product may not be appropriate for human consumption. Because of these and other considerations, rapid and reproducible in vitro methods are required to test the relative behavior of novel food structures on temporal release. In this study, a simple in vitro system was devised and evaluated to (i) measure volatile release from liquid samples, (ii) examine lipid induced changes on release and the effect of fat droplet size, and (iii) compare in vitro information to in vivo release data obtained in a previous study.[2]

MATERIALS AND METHODS

Volatile Chemicals. Five methyl ketone and two ethyl ester compounds, common in foods and beverages, were sourced from Firmenich (Balgowlah, Australia). Methyl ketones are often formed in fatty foods from β-oxidation reactions and are important aroma components in some lipid-rich foods such as hard (e.g., parmesan, pecorino, mature cheddar) and mold-ripened cheeses.[23] Ethyl butanoate and ethyl hexanoate are ubiquitous flavor volatiles that are present in many manufactured and natural foods. The volatile flavor compounds were selected to span a range of physicochemical parameters (Table 1): octanol/water partition coefficient ($K_{o/w}$) and vapor pressure. The relatively high olfactory thresholds of the methyl ketones were considered to be advantageous, in a related in vivo study[2] where, because of significant in mouth dilution effects, a high volatile concentration was required for proton transfer reaction mass spectrometry (PTR-MS) sensitivity. The flavor volatiles were diluted in food-grade ethanol such that a 5 μL aliquot could be added to bulk phase samples to reach a final concentration of 2.5 mg/L of each, respectively. The final headspace concentration of ethanol (∼50 ppm_v) was below the concentration at which H_3O^+ primary ion depletion occurs.[24] The final molar concentration differed for each compound, which influenced the relative response from the PTR-MS (Table 1).

Table 1. Physicochemical Properties of the Seven Volatile Compounds Used in in Vitro Experiments[a]

	C	molar concn	$K_{o/w}$	VP (Pa at 25 °C)	m/z $[M + H]^+$
2-butanone	4	2.8×10^{-5}	1	11875[b]	73
2-pentanone	5	2.35×10^{-5}	6	4718[c]	87
2-heptanone	7	1.80×10^{-5}	73	514[c]	115
2-octanone	8	1.60×10^{-5}	234	187[b]	129
2-nonanone	9	1.45×10^{-5}	1380	27[d]	143
ethyl butanoate	6	1.89×10^{-5}	80	1510[b]	117
ethyl hexanoate	8	1.50×10^{-5}	641	215[b]	145

[a]Carbon chain number (C), molar concentration based on 2.5 mg/L added to water or emulsion (mol/L), octanal/water partition coefficient ($K_{o/w}$), vapor pressure (VP), measured ion by PTR-MS (m/z $[M + H]^+$). [b]Corvarrubias-Cervantes et al.[41] [c]Rathbun and Tai.[40] [d]Voilley et al.[42]

Preparation of Emulsions. In the first instance food-grade commercial soy-based emulsion (Ivelip, 20%, Baxter Health, Australia) was used to assess the performance of the in vitro cell. The emulsion droplets were experimentally determined to have a Sauter mean diameter, $D_{3,2}$, of 0.25 μm, and a volume moment mean diameter, $D_{4,3}$, of 0.3 μm (see the following section). Custom emulsions were prepared in the laboratory by mixing 40 vol % oil and 60 vol % surfactant solution using various types of shear devices to achieve the average droplet size desired (0.5 or 5.0 μm) as follows. The 0.5 μm emulsions were prepared by first mixing the oil into the surfactant solution (1.5 wt % polyoxyethylene sorbitan monooleate, Tween 80) using a magnetic stirring mantle at 700 rpm. A pre-emulsion was then created from this mixture using a Silverson mixer (2 min at 6000 rpm, small 2 mm round mesh). This pre-emulsion was then homogenized (2 passes at 200 bar) using a Microfluidizer (110Y, equipped with a 75 μm Y emulsification chamber and a 200 μm Z dispersion chamber). The 5.0 μm emulsion was prepared by first mixing the oil into the surfactant solution (0.12 wt % Tween 80) using identical conditions. Emulsion particle size was assessed by laser light scattering using a Malvern Mastersizer 2000 (Malvern Instruments, Worcestershire, U.K.). Samples were diluted with distilled water to approximately 0.002 wt % in an effort to avoid multiple scattering effects. Information about emulsion particle size was then obtained via a best fit between light scattering theory and the measured light scattering pattern. Emulsion particle sizes are quoted as the volume−surface mean diameter $d_{3,2}$ ($d_{3,2} = \Sigma n_i d_i^3/\Sigma n_i d_i^2$) or the volume−length mean diameter $d_{4,3}$ ($d_{4,3} = \Sigma n_i d_i^4/\Sigma n_i d_i^3$). For the 0.5 μm emulsion, $d_{3,2}$ = 0.45 and $d_{4,3}$ = 0.56, and for the 5 μm sample, $d_{3,2}$ = 4.09 and $d_{4,3}$ = 9.57. Lipid emulsions were diluted with Milli-Q water (containing 0.12 wt % Tween 80) to achieve a series of lipid contents ranging from 0 to 20%.

To prepare samples for PTR-MS analysis, a 5 μL aliquot of the volatile mixture was added to a 10 mL volume of emulsion in a plastic 20 mL syringe (Terumo Corp., Macquarie Park, Australia) to obtain a final concentration of 2.5 mg/L for each volatile. Samples were capped and equilibrated overnight in a refrigerator (4 °C) prior to in vitro experiments. Samples were removed from the refrigerator (at least 90 min before) and left to equilibrate to room temperature (∼22 °C) before use. Initial pilot experiments demonstrated negligible volatile adsorption/absorption onto the plastic syringes in a direct comparison with glass syringes. Syringes were shaken before use, although no visual evidence of separation was evident.

In Vitro Cell. The cell was based on a simple Schott bottle system, which could be easily replicated for multiple experiments (Figure 1). The design was based upon previously described systems developed for similar purposes[5,25−31] and consideration of some critical parameters of the human mouth. The in vitro cell incorporated a 100 mL Schott bottle, close to the combined volume of the human oropharyngeal[32] and the nasal airspace volumes.[33] The average human

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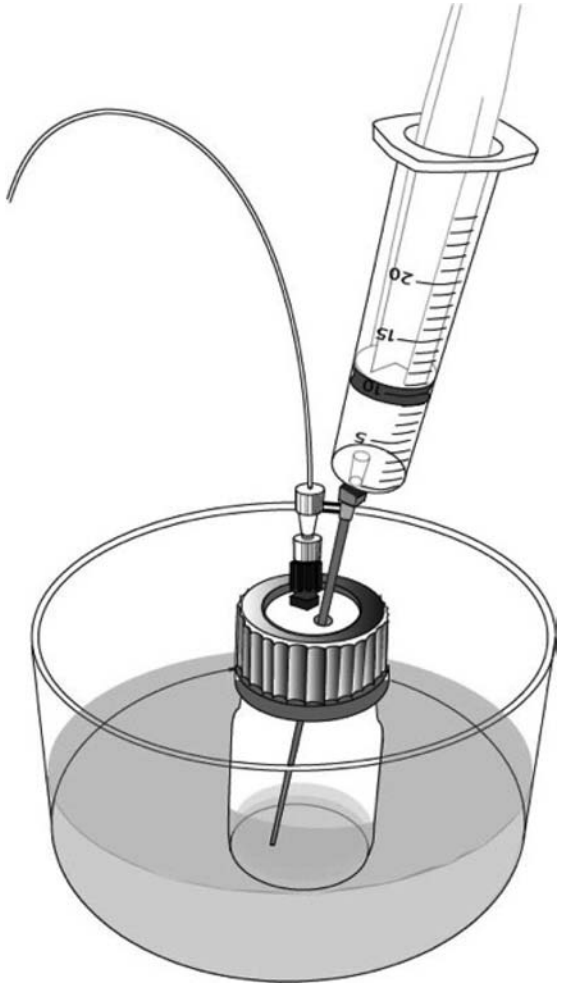

Figure 1. Diagram of the in vitro cell used in the volatile release experiments. The modified Schott bottle was fitted via Luer fittings to the PTR-MS inlet. The liquid bolus was manually injected into 5 mL of water (37 °C).

air exchange is 5000 mL/min, based on a tidal volume of 500 mL and 10 breathing cycles;[34] for practical reasons the flow rate used in our model mouth was 400 mL/min. Distilled water (5 mL) was added to the cell to simulate the dilution by saliva. A (liquid) food bolus (10 mL) spiked with volatiles was introduced via a plastic syringe (20 mL capacity, Terumo Inc.) into the cell, close to the volume of an average mouthful of liquid. Multiple cells were prepared in advance (equilibrated to 37 °C in a separate water bath) for PTR-MS experiments, such that a large number of experiments and replicates could be carried out in one day.

An engineered Teflon insert with a female Luer compatible inlet fitting and an open-hole (4 mm diameter) was held in place by an open plastic screw top and a rubber O-ring onto the Schott bottle (Figure 1). A male Luer fitting was permanently attached to the PTR-MS inlet tubing to facilitate easy gastight connection and disconnection from the cell; the other inlet was left open to allow sample introduction and simulate the open human naso-oropharyngeal system. The whole system was temperature regulated at 37 °C by use of a temperature-controlled water bath. A Teflon stir magnet (100 rpm) was used for stirring experiments.

PTR-MS Conditions. Volatile release was measured using a high-sensitivity quadrupole model PTR-MS (Ionicon Analytik GmbH, Innsbruck, Austria). The design and operational principles of PTR-MS are comprehensively described in the literature.[35,36] Rapid gas phase ionization and instantaneous measurement allow real time monitoring of flavor release on time scales relevant to human consumption. The headspace gas was displaced at a rate of 400 mL/min from the Schott bottle cell through PEEK tubing; 15 mL/min was drawn into the reaction chamber of the PTR-MS instrument. The transfer tubing was held at 60 °C, the reaction chamber temperature was 70 °C, and the drift tube voltage was set at 600 V (2.19 mbar). The acquisition method was started, and after 19 s (19 cycles), the emulsion sample was introduced dynamically via a preloaded syringe fitted with a $^1/_8$ in. i.d. stainless steel cannula, into the bottom of the aqueous layer in the cell. The same operator performed the sample injection (~1 s duration) throughout the in vitro experiments. The headspace volatiles were measured for a total of 130 s postinjection. Preliminary experiments indicated that the emulsions did not contain volatile ions corresponding to the target mass/charge ratios (m/z). Experiments also showed that the methyl ketones used in this study did not undergo significant fragmentation and formed stable $[M + H]^+$ ions. The PTR-MS was used in ion monitoring mode; in addition to the target volatiles listed in Table 1, the protonated water isotope ($H_3{}^{18}O^+$; m/z 21), the water cluster ($H_3O^+{\cdot}H_2O$; m/z 37), and acetone (m/z 59) were measured. Target volatiles were all measured with a dwell time of 100 ms; the full range of target volatiles was measured every second.

Data Processing and Statistical Analysis. Relative flavor volatile concentrations (μg/L) were calculated by the PTR-MS software according to the method of Lindinger et al.[36] The PTR-MS data files were imported into Excel (Microsoft). The curves were first smoothed using a 4-point moving average. After smoothing, MAX and LOOKUP functions were used to find maximum intensity (I_{max}) and time to maximum (T_{max}) values. A rectangular integration function was used to calculate area under the curve (AUC) during the release. Replicate release curve data parameters were analyzed using multivariate analysis of variance (MANOVA) to determine the significance of fat content and volatile compound and their interaction where appropriate (Genstat 13th ed., VSN International, Hemel-Hempstead, U.K.). Least significance difference (LSD) values were calculated by the software. Integration of 4-point smoothed release curve data was performed using the IGOR-Pro software package (version 6.0.5.0, WaveMetrics, Lake Oswego, OR). The cumulative integration curves were fitted with an exponential release function based on eq 4 using the curve fitting option in IGOR-Pro. The volatile parameter data for AUC, I_{max}, and T_{max} did not change in a direct linear fashion with fat addition, so a nonlinear regression curve fitting approach was used. The best fit was obtained by using a linear-by-linear approach to model a rectangular hyperbola (Genstat). Many natural relationships can be described by a decelerating curve or asymptotic regression.[37] The fitted curves were subjected to statistical analysis for goodness of fit and significance of relationship.

RESULTS AND DISCUSSION

Volatile Release Curves and Cell Performance. The most important requirement for the in vitro system was to provide a straightforward, rapid, and reproducible approach to quantify the effects of fat on volatile release in the emulsion system and potentially relate the data obtained by such a system to in vivo data. In the first instance, release curves were obtained in Ivelip emulsions at various fat concentrations (0.5, 1, 1.5, 2, 2.5, 5, 7.5, 10, and 20%) with the in vitro cell under stirred and nonstirred conditions; all samples were measured in triplicate. Typical averaged (n = 3) smoothed release curves corresponding to the nonstirred cell for 2-heptanone ($K_{o/w}$ = 73), 2-octanone ($K_{o/w}$ = 234), and 2-nonanone ($K_{o/w}$ = 1380) are shown in Figure 2. Only selected fat levels are displayed for the purposes of visual clarity. The dynamic release curves were reproducible and displayed distinct release curve parameters I_{max}, AUC, and T_{max}.

For 2-butanone (curves not shown), a compound with negligible lipophilicity ($K_{o/w}$ = 1), no differences in dynamic release parameters (I_{max}, AUC, or T_{max}) were observed as fat was increased, consistent with the predictions of eq 2, for both

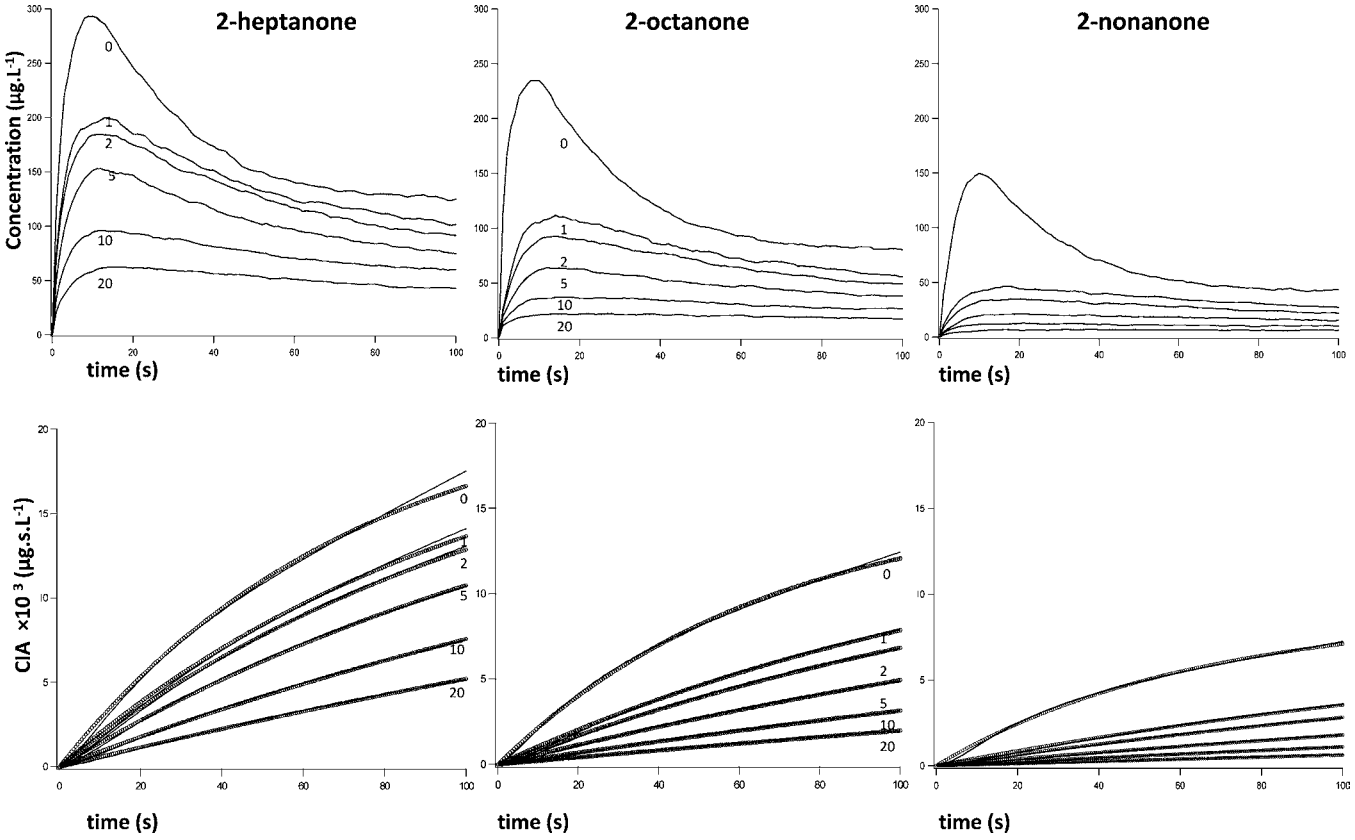

Figure 2. (Top) Typical smoothed in vitro volatile release curves for 2-heptanone ($K_{o/w}$ = 73), 2-octanone ($K_{o/w}$ = 234), and 2-nonanone ($K_{o/w}$ = 1380) obtained in the nonstirred cell in 0, 1, 2, 5, 10, and 20% fat emulsions. (Bottom) Corresponding cumulative integrated area (CIA) curves. The fitted CIA data are shown by the open circle line and the experimental data by the solid line.

Table 2. Statistical Data Corresponding to the Nonstirred Cell (Figure 2) for the Release Parameters I_{max}, AUC, and T_{max}[a]

release parameter	statistical test	2-butanone	2-pentanone	2-heptanone	2-octanone	2-nonanone	ethyl butanoate	ethyl hexanoate
AUC	*P* value fat	ns	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	% fitted curve	ns	58	97	98	99	92	98
	P value fitted curve	ns	0.012	<0.001	<0.001	<0.001	<0.001	<0.001
I_{max}	*P* value fat	ns	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	% fitted curve	ns	54	96	99	99	96	99.6
	P value fitted curve	ns	0.022	<0.001	<0.001	<0.001	<0.001	<0.001
T_{max}	*P* value fat	ns	ns	0.003	<0.001	<0.001	ns	<0.02
	% fitted curve	nc	nc	74	73	85		86
	P value fitted curve	ns	ns	0.002	<0.001	<0.001	<0.001	<0.001
	0→20% s	10→10	8→8	6.5→11.5	6→15.5	6→21	5→6.5	5.5→12

[a] *P* value fat = significance of the effect of fat, % fitted curve = percent of data explained by the rectangular hyperbola curve. *P* value curve = significance of the relationship between fat and release parameter, 0→20%, T_{max} in 0% fat and T_{max} in 20% fat (seconds) for a given volatile. ns = not significant ($p < 0.05$). nc = no change.

the stirred and nonstirred cell. In the case of the release data for the compounds in Figure 1, clear I_{max} values were reached rapidly, within ~20 s of sample introduction, with subsequent gradual decay. As the fat concentration was progressively increased, especially beyond 2%, I_{max} clearly decreased in sequential order. As the fat concentration increased beyond 2%, the time (T_{max}) to reach I_{max}, also visibly increased. In the stirred cell, the release curves had a different shape (data not shown). For the 0 and 0.5% fat systems, the I_{max} for 2-octanone was rapidly reached (~10s), with a slow decrease. For the higher fat samples, I_{max} decreased in sequence with increasing fat; however, the T_{max} was harder to visualize, as the rate of decay in the curves was relatively slow compared to the nonstirred system. Overall, data from the nonstirred and stirred cells were similar. As the data obtained with the former were more reproducible, the remaining experiments were conducted using only the nonstirred cell system.

'The decrease in AUC and I_{max} as a consequence of fat content was modeled using a fitted rectangular hyperbola. In most cases, the release data were well described by the fitted curves, especially for compounds with appreciable lipophilicity (Table 2). 'The effect of fat content on the release parameters

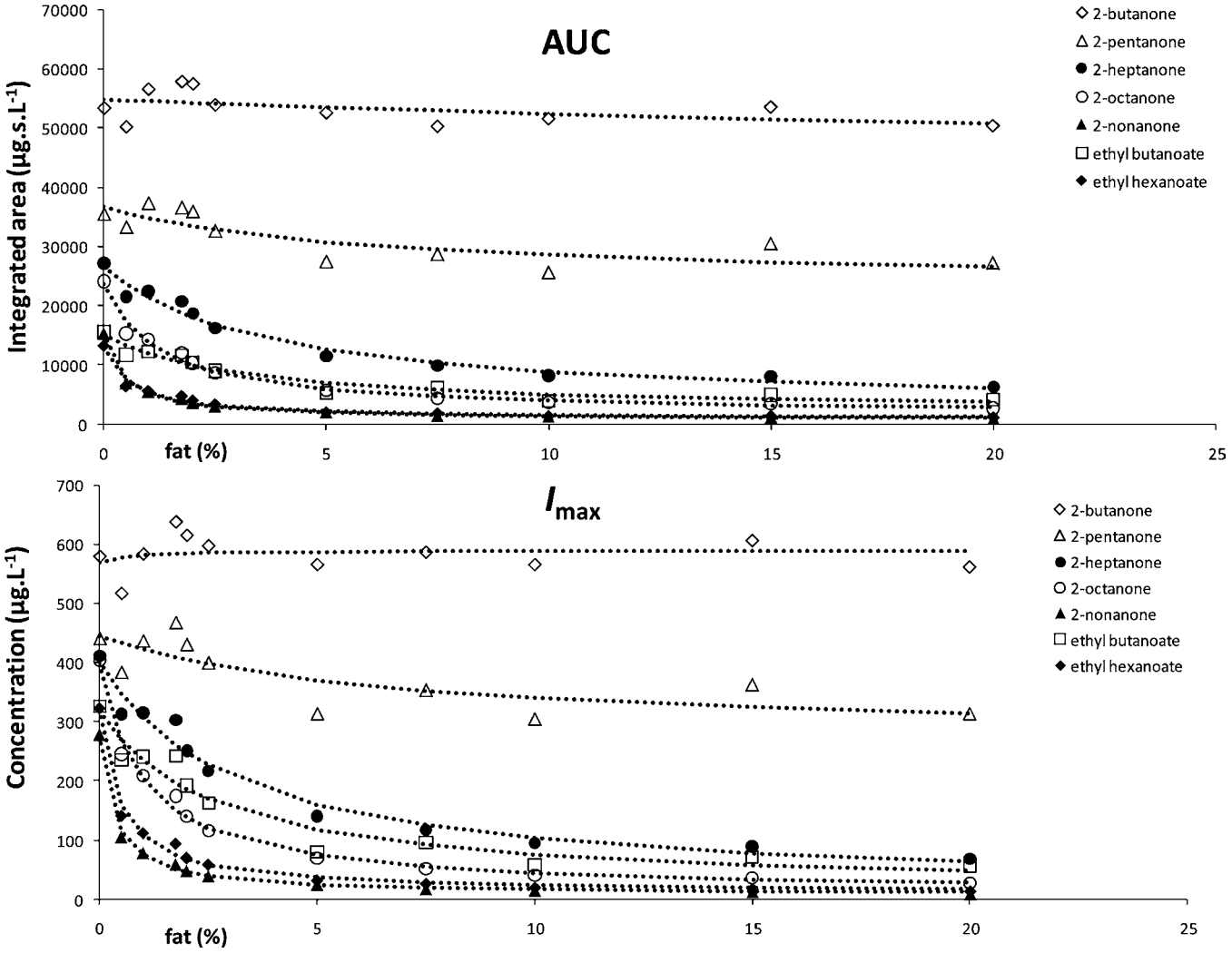

Figure 3. Changes in AUC and I_{max} with increasing fat in the nonstirred cell. Symbols represent mean (n = 3) measured values; the dashed line is the fitted curve.

Table 3. Calculated Values of h Using Equation 4 and Initial Values of C_0[a]

	2-butanone	2-pentanone	2-heptanone	2-octanone	2-nonanone	ethyl butanoate	ethyl hexanoate
C_0	44410	30606	22332	15592	8872	10760	6875
	$h \times 10^{-4}$	$h \times 10^{-4}$	$h \times 10^{-4}$	$h \times 10^{-4}$	$h \times 10^{-4}$	$h \times 10^{-4}$	$h \times 10^{-4}$
0%	5.3	5.3	6.1	6.6	7.1	7.3	8.1
1%	5.1	5.1	4.2	3.1	2.2	5.0	3.8
2%	5.4	5.3	3.8	2.6	1.7	4.5	2.9
5%	5.2	5.4	2.9	1.7	0.9	3.5	1.9
10%	4.9	4.3	1.8	1.0	0.5	2.2	1.1
20%	4.6	3.6	1.2	0.6	0.3	1.5	0.6

[a]The release rate increased with lipophilicity in the aqueous solution. With the addition of fat, the release rates decreased significantly for lipophilic compounds. The initial value of C_0 was the total AUC calculated for each volatile in 0% fat.

are shown graphically in Figure 3. MANOVA indicated that the overall effect of "fat", "volatile", and the "fat × volatile" interaction were highly significant ($p < 0.001$) for AUC, I_{max}, and T_{max}, although effects on individual volatiles may not have been significant. AUC and I_{max} did not change significantly with the addition of fat for 2-butanone, although slightly higher release at low fat levels was indicated. Significant decreases were measured for the remaining more lipophilic volatiles with increasing fat addition. The sensitivity of I_{max} and AUC to changes in the fat concentration became more pronounced with increasing $K_{o/w}$. In general, the T_{max} data were the least reproducible release parameter measured in both cell systems. No changes in T_{max} were measured with increasing fat content for 2-butanone, 2-pentanone, or ethyl butanoate in the Ivelip emulsions. Significant differences ($p < 0.001$) for increases in T_{max} were measured with increasing fat for all of the remaining volatiles (Table 2). The T_{max} increased with increasing carbon chain length when fat was present; however, T_{max} decreased in

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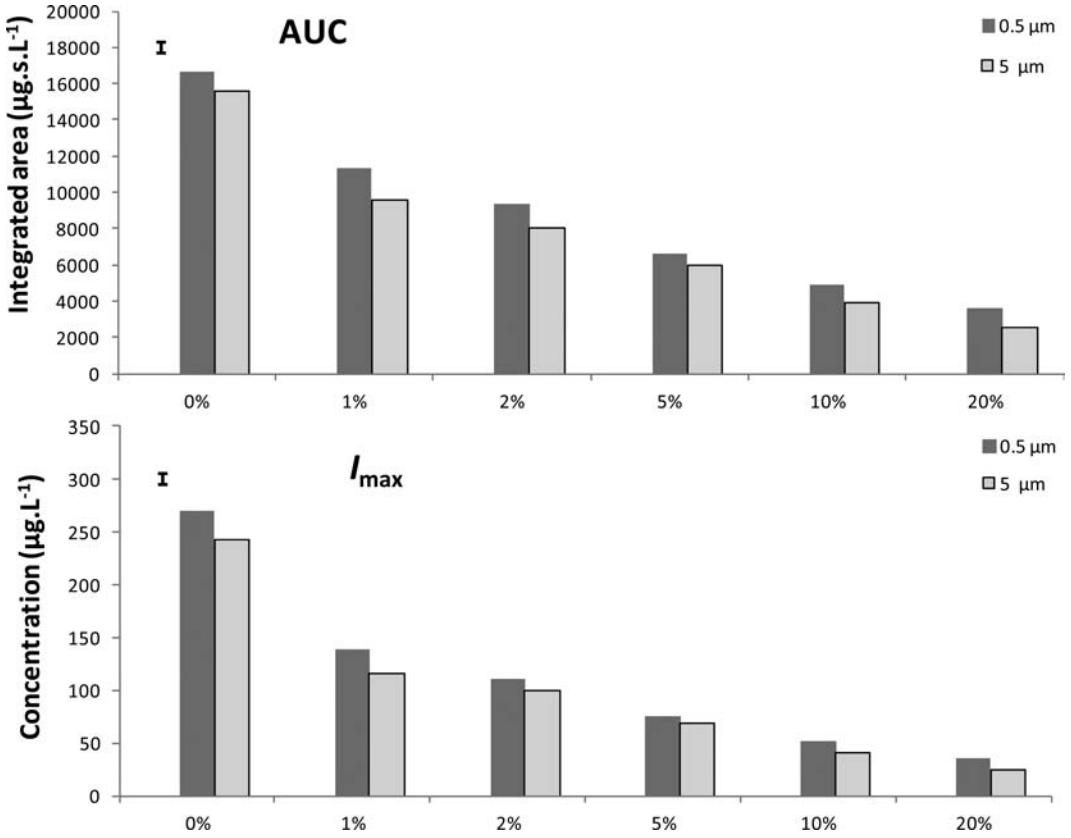

Figure 4. Mean (n = 6) AUC and I_{max} for 2-octanone in the 0.5 and 5.0 μm droplet size emulsions at different fat contents (%). Differences due to fat type were significant ($p < 0.01$). Least significant difference for the effect of fat type was calculated by MANOVA across all fat levels.

zero-fat aqueous solutions with increasing carbon chain length and lipophilicity. These T_{max} data suggested hydrophobic effects dominated in the absence of fat, driving the lipophilic volatiles from solution and more rapidly reaching I_{max}. With the addition of fat the release was attenuated and T_{max} increased.

Release Curve Modeling. Release curve data obtained in the nonstirred cell for the Ivelip emulsion were used to model cumulative release curves according to eq 4. The initial time offset (20 s) was removed from curves before modeling. The cumulative integrated area (CIA) curve was calculated using the rectangular integration function in the IGOR software. The CIA curves were then fitted to eq 4 to determine mass transfer rate coefficients (h). The values for A (interfacial area, 0.0018 m^2) and V_{hs} (volume of the headspace, 8.0×10^{-5} m^3) for the cell were held constant. An initial fixed constant value was given for C_{hs} (the total calculated headspace AUC in aqueous solution for each volatile) and for h (a nonfixed small negative value was given). Individual curves were fitted and h values determined by the software for optimized curves. The average fitted curves for 2-heptanone, 2-octanone, and 2-nonanone across fat levels corresponding to the original PTR-MS release curves are shown in Figure 2; the fitted curves (shown in open circles) closely matched the experimental data (solid black line). Mean h values calculated for release curves at each fat level for 2-butanone, 2-heptanone, 2-octanone, and 2-nonanone are shown in Table 3. The value of h decreased with increasing $K_{o/w}$ and increasing fat. In the absence of fat, h increased with increasing $K_{o/w}$, supporting the T_{max} data for aqueous solutions shown in Figure 5. It can be clearly seen that the mass transfer rates for 2-butanone were greatest within the series and were minimally affected by fat.

Measurement of Release from Other Fat Systems. In vitro release curves for the 0.5 and 5 μm emulsions were obtained in triplicate over all of the fat levels (n = 36) in the nonstirred cell system. The release curves for both emulsions were similar in shape to those shown in Figure 2. Significant overall differences ($p < 0.001$) for all release parameters were measured with increasing fat. MANOVA indicated significantly lower I_{max} and AUC for the 5 μm droplet size compared to the 0.5 μm size (Figure 4; AUC ($p < 0.01$) and I_{max} ($p < 0.002$)). No differences in T_{max} were found between emulsion types. The mean AUC and I_{max} for 2-octanone in the two emulsion systems at different fat levels are shown in Figure 4. Only small differences in release due to droplet size were measured in the current dynamic system. Measurement of such small differences indicates the resolution of the experimental system; however, whether this results in a perceived sensory difference is unlikely. Droplet size effects on release have been reported in static sampling systems; emulsions with smaller droplet size were shown to increase volatile release.[38] In contrast, no size effect was reported in a dynamic system.[29] In the current system an overall small increase in release was found from the smaller droplet size.

Because of the small differences between the two fat systems, the replicate release data from both emulsion systems were combined and subjected to MANOVA. The effect of "fat", "volatile" type, and the "fat × volatile" interaction were all highly significant ($p < 0.001$) for all release parameters. Mean (n = 72) AUC, I_{max}, and T_{max} data for each volatile at the different fat levels are presented in Figure 5.

The mean data allowed some generalizations to be made regarding the effects of fat in the emulsions systems. The

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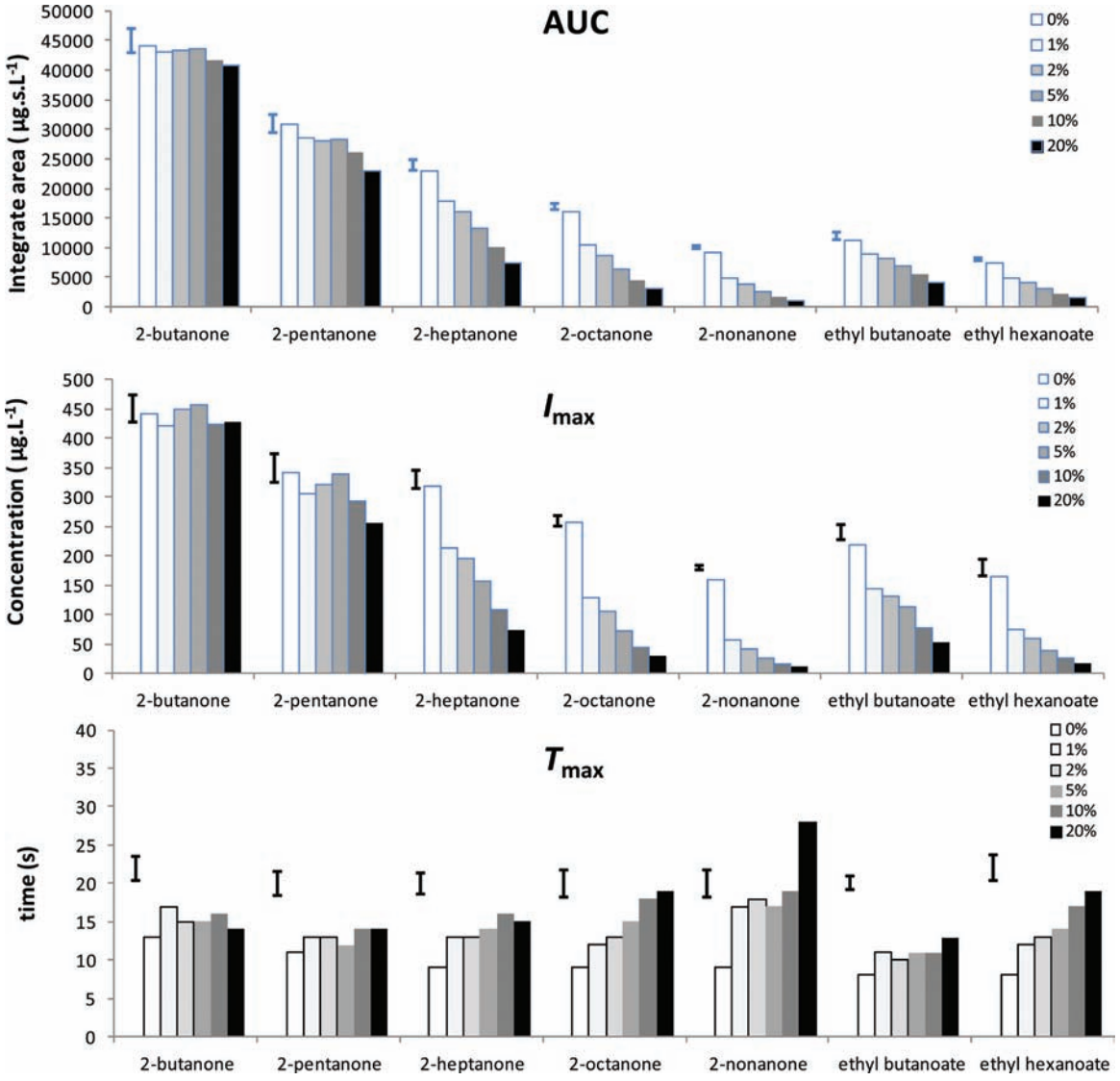

Figure 5. Summary of mean changes (n = 72) in critical volatile release parameters in emulsion systems with increasing fat averaged across 0.5 and 5.0 μm droplet size emulsions, AUC, I_{max}, and T_{max}. LSD values were determined for each volatile separately.

measured headspace concentration (AUC, I_{max}) for 2-butanone was always highest compared to the other volatiles, consistent with the high volatility and negligible interaction of this compound with fat ($K_{o/w} < 1$). For the series of methyl ketones, the headspace concentration in pure water (0% fat) decreased with increasing carbon length and decreasing volatility and molar concentration (Table 1). The measured decrease in headspace concentration with increase in carbon chain is in contrast with increases reported in equilibrium systems[39] but consistent with release data described by Rathbun and Tai[40] using a dynamic two-film model.

Overall, no differences in any of the volatile release parameters were measured for 2-butanone, the most hydrophilic compound. Significant fat effects ($p < 0.001$) were measured for I_{max} and AUC for all of the other volatile compounds. As the $K_{o/w}$ of volatiles increased, the relative decrease in AUC and I_{max} in the transition from 0 to 1% fat became more pronounced, for example, 2-pentanone compared to 2-octanone and ethyl butanoate compared to ethyl hexanoate. The effect of fat on T_{max} was significant ($p < 0.001$) for all individual volatiles, except 2-butanone and 2-pentanone. As the $K_{o/w}$ increased, the influence of fat on T_{max} increased accordingly, with stepwise increases observed. It was of particular interest that T_{max} decreased significantly ($p < 0.001$) in pure water with increasing molecular weight as measured in the Ivelip experiments. Increasing hydrophobicity mirrors chemical activity coefficients[41] and appeared to be a driving force pushing the volatile into the headspace more rapidly under dynamic conditions. At 0% fat, T_{max} was shorter for all volatiles relative to 2-butanone (no fat interaction). As fat was increased to higher concentration (~10% and above), T_{max} values increased relative to 2-butanone, indicating that as the fat phase increased, lipophilic interactions became predominant. Hence, in the case of volatiles with $K_{o/w} > 1$, combinations of both hydrophobic and lipophilic effects were influencing T_{max} and the dynamics of volatile release. These data support the anecdotal and experimental in vivo evidence that volatiles are released in a "burst" in a zero- or low-fat system and the flavor release is modulated or extended after the addition of fat.

Comparison of in Vitro versus in Vitro Data. In vivo flavor release occurs in a complex multichamber geometry composed of the oral and nasal cavities and soft palate, layered

with mucus membranes and subject to changing airflows. Consumption of liquid (emulsions) consists of pre- and post-deglutition release events, which are different, and it is unlikely that these events could be mimicked exactly in an artificial glass system. Oral processing occurs in a dynamic open system into which a bolus of food is introduced, processed, and cleared (after swallowing). On the time scales of eating, it is unlikely that volatile equilibration between the bulk and gas phase occurs. Hence, the release of volatiles is mainly determined by mass transfer effects, which apart from fat partitioning are influenced by vapor pressure and surface area. Most reported in vitro cells are based on volatile depletion rates from an initial headspace equilibrium system over a specified time interval. Although mass transfer rates can be easily measured, the curves obtained by these methods do not provide clear-cut I_{max} or T_{max} data[26] and do not correspond well to in vivo release curves. In the simple system reported here, where the liquid bolus preloaded with volatiles was dynamically introduced, the release curves were visually similar to in vivo data and the classic release curve parameters were easily characterized and quantified in a reproducible manner. The data obtained with the system were amenable to straightforward statistical and curve fitting analyses.

In vitro AUC and I_{max} data obtained for the volatiles 2-heptanone through ethyl hexanoate in the Ivelip were used for comparison to data obtained in the same emulsion in vivo from previously published work.[2] Only compounds with appreciable lipophilicity were considered; that is, data for 2-butanone and 2-pentanone were not included. The data were first normalized, by referencing all AUC and I_{max} values to those obtained for 2-heptanone in water (0% fat), as this was always the highest value within the volatile range. Relative percent decreases in AUC and I_{max} with increasing fat for each of the volatiles for the in vitro and in vivo pre- and postswallow data are shown individually in Table 4. In vitro and in vivo preswallow and postswallow AUC and I_{max} values in 0, 2, 5, 10, and 20% fat (n = 25) were all positively correlated as expected. The in vivo AUC pre- and postswallow data were in most cases similar to each other and the in vitro results. Correlation with preswallow in vivo data was stronger, based on all fat levels and volatiles considered (n = 25): preswallow AUC (r = 0.97, p < 0.001) and postswallow AUC (r = 0.92, p < 0.001). The in vivo preswallow and postswallow I_{max} data were quite different, with smaller postswallow changes between each fat level (Table 4). The preswallow I_{max} data were closest to the values obtained in the in vitro cell: preswallow I_{max} (r = 0.95, p < 0.001) and postswallow I_{max} (r = 0.68, p < 0.001). It is worthy of comment that in general terms the early phase, up to around 20 s, of the in vitro release curves (Figure 2) displayed greater fat-induced differences, compared to later sections. This trend paralleled to a large extent the in vivo data, for which the biggest differences in I_{max} and AUC were measured in the preswallow phase compared to postswallow.[2]

There have been few reported in vivo differences in T_{max} as a result of increasing fat content.[1,5] One reason for this is the much greater variation in this parameter in human release studies, compared to AUC or I_{max}. Few in vitro studies have demonstrated clear changes in T_{max} due to fat. Using the current experimental setup, clear increases in T_{max} as a result of increasing fat content were measured. The effect increased with increasing volatile lipophilicity; however, the T_{max} data were less reproducible than the other release parameters. Samples were manually introduced into the cell, and slight variation in the force of injection may have led to differences in T_{max}. Future improvements to the system will include incorporation of an automated syringe plunger to control the force and rate of sample introduction.

Table 4. Correlations between Normalized Release Ratios for Each Volatile (AUC and I_{max}) at Different Fat Concentrations Obtained in the in Vitro (IV) Cell Compared to Preswallow and Postswallow in Vivo Data Reported in Frank et al.[2a]

	AUC			I_{max}		
	IV	pre	post	IV	pre	post
2-Heptanone						
0%	100	100	100	100	100	100
2%	60	61	79	70	80	92
5%	36	50	54	34	54	92
10%	25	38	23	25	23	66
20%	20	29	20	18	20	56
r		0.99	0.95		0.97	0.82
2-Octanone						
0%	75	69	66	86	66	57
2%	33	36	34	39	34	47
5%	17	27	19	18	19	40
10%	11	16	9	10	9	29
20%	8	19	8	7	8	22
r		0.99	0.99		0.99	0.91
2-Nonanone						
0%	46	36	31	56	31	22
2%	12	19	9	13	9	18
5%	6	13	5	6	5	14
10%	4	9	3	3	3	11
20%	2	8	3	2	3	8
r		0.99	0.99		0.99	0.83
Ethyl Butanoate						
0%	51	51	80	72	80	58
2%	35	29	59	53	64	45
5%	17	21	34	22	25	53
10%	13	18	23	15	24	42
20%	12	13	24	15	22	31
r		0.97	0.99		0.99	0.66
Ethyl Hexanoate						
0%	38	38	62	61	62	29
2%	14	30	19	18	18	22
5%	7	10	15	12	15	20
10%	4	6	5	9	5	16
20%	3	7	4	6	4	10
r		0.91	0.99		0.99	0.87

[a]In general, the in vivo data corresponded more closely with the preswallow in vivo data. Data were normalized to the value obtained for 2-heptanone in water (0% fat). r = Pearson's correlation coefficient

In addition to measuring systematic changes in release due to fat and $K_{o/w}$, the simple cell can potentially be used to predict the release behavior of novel fat replacement systems in liquid form. The mean AUC and I_{max} data for all volatiles except 2-butanone obtained from 1 to 20% fat from 0.5 and 5.0 μm droplet size emulsion experiments were combined and used to construct a predictive model using partial least-squares and cross-validation. The model (not shown) indicated high predictive ability (r = 0.99) with acceptable root-mean-square error values for calibration and validation models; hence, the relative volatile release capacity of novel formulations may be

possible by spiking novel samples and assessing them under the same conditions in the same cell.

Overall, the in vitro method was able to generate useful information regarding the effect of fat on volatile release from emulsions. Depending on the product of interest, the volatiles used should be varied to reflect the actual volatile composition of the target product; the volatile compounds used in this study were limited in scope and may not reflect the behavior of other important flavor volatiles compounds commonly found in food systems. The cell provided new data regarding systematic changes in T_{max} and release rates due to competing hydrophobic and lipophilic interactions, which may correspond to previously reported in vivo effects with respect to relative partitioning between pre- and postswallow phases of eating.

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Notes

The authors declare no competing financial interest.

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