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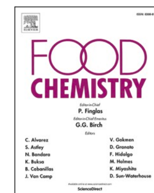

The role of capsaicin stimulation on the physicochemical properties of saliva and aroma release in model aqueous and oil systems

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ABSTRACT

Capsaicin increases saliva production, but the impact of this additional saliva on the food matrix is unknown. This study aimed to explain the impact of capsaicin on saliva properties and in-vivo release of 14 aroma compounds in aqueous [aqu] and oil systems [oil]. To investigate the physicochemical effect from diverse properties of aroma compounds, one healthy subject participated in all the sessions to minimise large variations between individuals. Capsaicin enhanced saliva flow rate (by 172% [aqu] and 85% [oil]) and salivary protein concentration (by 142% [aqu] and 149% [oil]). Furthermore, capsaicin-in-oil stimulated saliva formed a more stable emulsion in the mouth (17% higher zeta-potential and 15% smaller particle size). In-nose release concentrations measured by APCI-MS for certain esters were reduced by capsaicin (e.g., isoamyl acetate was reduced by 65% [aqu] and 76% [oil]), which suggests that capsaicin may induce stronger oral interactions between specific aroma compounds and salivary proteins.

1. Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the main pungent ingredient in “hot” chilli peppers and is an agonist of transient receptor potential vanilloid subtype 1 (TRPV1), which is expressed in a range of secretory epithelia, including salivary glands (Yong-Hwan et al., 2016). Several studies have shown that capsaicin induces various oral physiological responses. These include a significant increase in saliva flow rate and an increase in the concentration of major salivary proteins and salivary citrate (Gardner et al., 2020). Capsaicin also elicits a pleasant warm sensation in the mouth at low concentrations as a result of stimulation of thermoreceptors; it has also been shown to have other physiological impacts, including changing blood flow patterns and localised changes in tongue surface temperature (Lv et al., 2019). However, most studies so far only investigated the effect of capsaicin in a simple model aqueous system, which is not a common spicy food matrix. Capsaicin is a lipophilic compound that is readily soluble in oil, and chilli peppers are usually cooked with oil in many cuisines, so it is essential to understand the impact of capsaicin on saliva properties in both aqueous and oil systems.

Saliva is a naturally secreted oral fluid, which plays a significant role in aroma release and perception. There have been some studies on the influence of capsaicin on flavour perception, and it has been found that the burning sensation in the mouth can affect taste perception (Lawless & Stevens, 1984; Prescott & Stevenson, 1995). A very recent study (Yang et al., 2020a) indicated that capsaicin significantly enhanced aroma perception by 45% during 60 s observation, despite only one aroma compound (3-methylbutanal) being used. In another study, Yang et al. (2020b) investigated three aroma compounds in a model ice-cube system and reported that the presence of capsaicin in the mouth significantly reduced the delivery of volatile aroma compounds to the nasal cavity. It was proposed that a reduction in aroma release could be due to the dilution and dissolution of aroma compounds by capsaicin-enhanced saliva production as the ice started to melt in the mouth. However, there is a lack of study on a wide range of aroma compounds with different physicochemical properties and how capsaicin could affect their release. Additionally, it has been shown that oil has a significant influence on the solubility and the release of food flavour compounds (Ayed et al., 2017; Tamaru et al., 2018), and Linforth et al. (2010) reported a direct impact of lipid content on aroma release, which could be predicted by the hydrophobicity of the aroma compounds. To our knowledge, no study has been conducted to evaluate the impact of capsaicin on aroma release in

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an oil system.

Therefore, the aim of this study was to explore the mechanisms behind the impact of capsaicin on saliva properties and the release of a wide range of aroma compounds in aqueous and oil systems. We proposed the following mechanism related to our hypothesis: capsaicin's impact on aroma release could be not only due to the dilution effect caused by the extra saliva but also the interactions between aroma compounds and the altered saliva's physicochemical properties. Various saliva testing methods (saliva flow rate analysis, saliva total protein analysis, rheological analysis, microscopic analysis) were applied with and without capsaicin stimulation in either model system. Fourteen aroma compounds with different physicochemical properties were selected and their equilibrium partitioning *in vitro* was evaluated by static headspace analysis using atmospheric pressure chemical ionisation with tandem mass spectrometry (APCI-MS/MS). The impact of capsaicin on the *in vivo* release of each aroma compound was measured by breath-by-breath analysis using APCI-MS/MS for the period of before, during and after swallowing.

2. Materials and methods

2.1. Materials

2.1.1. Aroma compounds

All chemicals were purchased from Sigma-Aldrich (Gillingham, Dorset, United Kingdom) and were food grade. Fourteen aroma compounds with different physicochemical properties were used in the experiments (Table 1): pyrazine, methyl acetate, 2,5-dimethylpyrazine, 3-methylbutanal, phenylacetaldehyde, 2,3,5-trimethylpyrazine, (*E*)-2-hexenal, ethyl butyrate, isoamyl acetate, 1-octen-3-ol, ethyl hexanoate, menthone, ethyl heptanoate, and ethyl octanoate. The PubChem CID, CAS number and purity for each compound are listed in Table 1. Their physicochemical properties (including Log P, K_{aw}, Log K_{oa} and vapour pressure) were estimated by *EPI* Suite™ (2016, Microsoft® Windows, v 4.1, United States Environmental Protection Agency, USA). Odour description for each compound was obtained from online resources for the Flavour, Fragrance, Food and Cosmetic Industries: http://www.thegoodscentscompany.com/.

2.1.2. Preparation of aqueous and oil system

Two capsaicin stock solutions were prepared: i) aqueous stock solution and ii) oil stock solution. For the aqueous stock solution, capsaicin was dissolved in 1% ethanol, which was then added into pure water (Purite Ltd, Oxon, UK) to make 5 ppm (mg/kg) for the final aqueous system. This concentration was chosen as it reflects an acceptable level of trigeminal stimulation, and it is also comparable to relevant model systems (Prescott & Stevenson, 1995; Lv et al., 2019; Yang et al., 2020b).

For the oil stock solution, capsaicin was dissolved in pure sunflower oil (Sainsbury's, UK at 1% w/w and then diluted in the same oil to make a series of concentrations (5, 10, 15, 20, 25, 30, 35 and 40 ppm). They were used to identify which concentration of oil-capsaicin (3.5 mL) led to a similar level of saliva enhancement as the 5 ppm aqueous-capsaicin sample (10 mL) (Genovese, Yang, & Linforth, 2018). Prior to consumption, saliva was collected (as detailed below) for 2 min, then the sample was held in the mouth and swallowed after 60 s, and saliva was again collected for 2 min after swallowing. The increased flow rate (g/min) before and after capsaicin stimulation was calculated as follows:

[(weight of saliva produced over 2 min prior to swallowing) – (weight of saliva produced over 2 min after swallowing)] / 2 min.

The increased saliva flow was calculated for the full concentration range of capsaicin in oil (5–40 ppm) with five replicate samples, the mean value was then compared to the results of 5 ppm aqueous-capsaicin ($n = 5$). Increasing levels of capsaicin led to a relatively linear increase in saliva production ($R^2 = 0.9816$) (Supplementary Fig. S1). The 5 ppm capsaicin-aqueous sample led to an increased saliva flow of ~ 0.30 g/min, and the lowest concentration of capsaicin-oil that elicited a similar increase in saliva flow rate was 30 ppm capsaicin-oil. Therefore, 30 ppm capsaicin-oil was chosen to be comparable to 5 ppm capsaicin-aqueous for the purpose of this study. Samples with capsaicin addition or controls without capsaicin were labelled as CAP or CTR respectively, and were placed in screw cap Duran bottles.

2.2. Saliva analysis

2.2.1. Saliva flow rate analysis

The response of an individual to capsaicin is highly dependent on that individual's past exposure to capsaicin. Therefore, to minimise inter-individual variation, this study selected a single subject to observe the impact of capsaicin in different matrices. The panellist (age 28, female, non-smoker, no prior medical history of allergies) was recruited for all the saliva analysis and aroma release analysis. The panellist is a

Table 1
List of aroma compounds selected for APCI-MS/MS analysis.

No.	Compounds	PubChem CID	CAS number	Purity	Log P #	K_{aw} ##	Log Koa ###	VP (est 25 °C) ####	Functional group	Odour description
1	pyrazine	9261	290–37-9	≥ 99%	–0.06	0.0001	3.663	9.66	pyrazine	pungent, sweet
2	methyl acetate	6584	79–20-9	≥ 98%	0.37	0.0047	2.508	52.70	ester	ethereal
3	2,5-dimethylpyrazine	31252	123–32-0	≥ 98%	1.03	0.0001	4.468	3.18	pyrazine	cocoa, roasted
4	3-methylbutanal	11552	590–86-3	≥ 97%	1.23	0.0166	3.011	51.60	aldehyde	ethereal, aldehydic
5	phenylacetaldehyde	998	122–78-1	≥ 95%	1.54	0.0002	5.430	0.35	aldehyde	green, sweet
6	2,3,5-trimethyl pyrazine	26808	14667–55-1	≥ 99%	1.58	0.0002	4.745	1.45	pyrazine	nutty, potato
7	(*E*)-2-hexenal	5281168	6728–26-3	≥ 95%	1.58	0.0020	4.279	4.72	aldehyde	green, sweet apple
8	ethyl butyrate	7762	105–54-4	≥ 98%	1.85	0.0163	3.637	14.60	ester	fruity, juicy
9	isoamyl acetate	31276	123–92-2	≥ 97%	2.26	0.0240	3.870	5.67	ester	sweet, fruity
10	1-octen-3-ol	18827	3391–86-4	≥ 98%	2.60	0.0009	5.625	0.24	alcohol	mushroom, green
11	ethyl hexanoate	31265	123–66-0	≥ 98%	2.83	0.0296	4.359	1.80	ester	sweet, fruity
12	menthone	26447	89–80-5	≥ 99%	2.87	0.0065	5.237	0.37	ketone	cooling, minty
13	ethyl heptanoate	7797	106–30-9	≥ 98%	3.32	0.0204	5.010	0.69	ester	fruity, pineapple
14	ethyl octanoate	7799	106–32-1	≥ 98%	3.81	0.0320	5.095	0.51	ester	fruity, wine

All the values (Log P, K_{aw}, Log K_{oa} and VP) were obtained by EPISuite version 4.10 from the U.S. Environmental Protection Agency.
Log P is the logarithm of partitioning coefficient of the molecule between octanol and water, which was estimated by the KOWWIN program.
K_{aw} is the partitioning coefficient of the molecule between air and water, which was estimated by the KAWWIN program.
Log K_{oa} is the logarithm of partitioning coefficient between oil and air, which was estimated by the KOAWIN program.
VP is the vapour pressure (mm Hg) of the molecule estimated at 25 °C, which was estimated by the mean VP of Antoine and Grain methods.

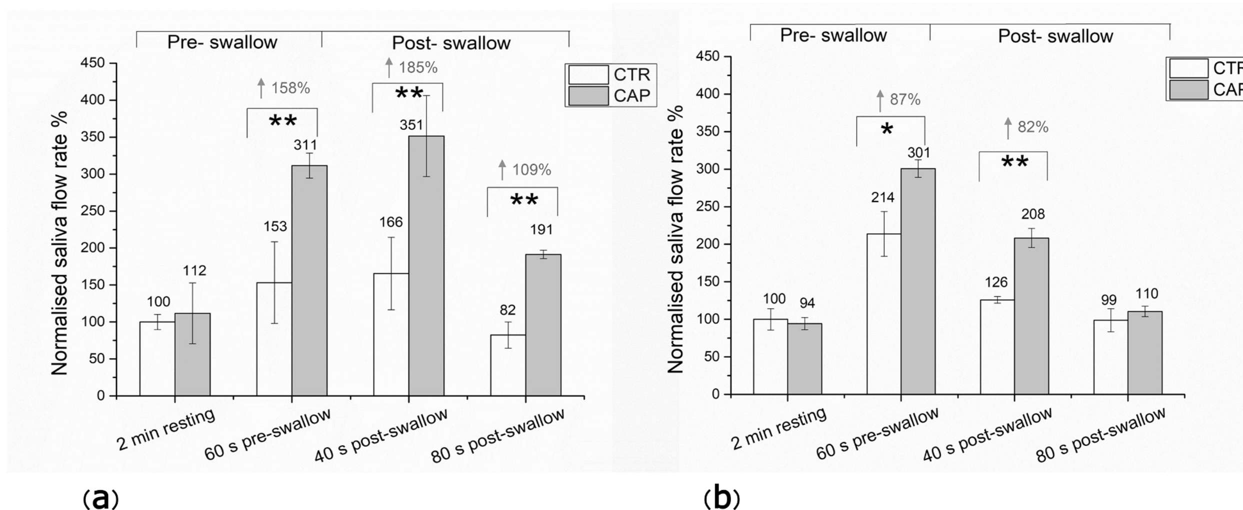

Fig. 1. Normalised salivary flow rate % of the control sample (CTR, white bar) and capsaicin sample (CAP, grey bar) for (a) aqueous system and (b) oil system. The control sample at 2 min resting is set as 100%. The *x*-axis indicated the four collection periods: i) 2 min resting, ii) 60 s pre-swallow, iii) 40 s post-swallow and iv) 80 s post-swallow. Stars within the same comparison group indicate significant differences (* indicated $p < 0.05$; ** indicated $p < 0.01$). The standard deviation is shown as ± error bar based on 5 sample reps ($n = 5$). Arrow symbol ↑ indicated percentage change between CAP and CTR.

regular chilli consumer who eats chilli-containing food at least three times a week.

Prior to saliva collection the panellist was asked to refrain from eating for 1 h, and to only drink a small amount of unflavoured room temperature water if required during this period. The panellist was initially instructed to wash their mouth three times with distilled water for 10 s followed by a short 60-s break. The panellist was then asked to tilt their head forward and open their mouth to allow the saliva to drip passively from the lower lip into the sterile container (Muñoz-González et al., 2018). Saliva was collected at multiple time points before and after placing the sample in the mouth, at each time point five replicated samples were collected ($n = 5$).

1) Saliva was collected continuously for 120 s prior to consumption, the container was weighed and the saliva flow rate (g/s) was calculated based on saliva weight difference divided by 120 s (i.e., 1st set of data for "2 min resting").
2) The sample was then held in the mouth for 60 s, and both saliva and solution were expectorated and weighed at the end of the 60 s period, the weight of the sample was deducted to calculate saliva flow rate over 60 s (i.e., 2nd set of data for "60 s pre-swallow"), which followed a similar timeline for pre-swallow *in-vivo* release by APCI-MS/MS analysis (*Section 2.3.2*).
3) After swallowing at 60 s, saliva naturally generated over the next 40 s was collected and weighed (i.e., 3rd set of data for "40 s post-swallow"), which was comparable with APCI-MS/MS post-swallow procedure (*Section 2.3.2*).
4) Final set of saliva was sampled continuously for another 80 s after the previous collection (i.e., 4th set of data for "80 s post-swallow"). This extended collection was designed to evaluate the long-lasting pungent stimulation of capsaicin on saliva flow rate.

During each collection point, the saliva flow rate was calculated, and to remove any effect of diurnal variation, the saliva flow rates were normalised to the innate secretion rate at the point of collection (i.e., 1st set of data).

2.2.2. Salivary total protein analysis

Two sets of saliva samples were collected for salivary protein analysis: 1) after holding either oil or aqueous-based samples for 60 s (pre-swallow); 2) 40 s after swallowing the samples (post-swallow). These two sets of points aligned with collection points of aroma *in vivo* release (section 2.3.2). The total protein concentration was determined by the bicinchoninic acid (BCA) method (562 nm) using a total protein assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Fluorescence intensity was measured using a multi-mode microreader (excitation filter set at 562 nm) (SynergyH1; BioTek Instruments, Inc., Winooski, VT).

For the aqueous sample, saliva was centrifuged at 4 °C for 30 min (500 *g*), the supernatant after centrifuging was diluted with 0.9% NaCl ($n = 3$), and the protein concentration was measured. For the oil sample, all samples ($n = 3$) were centrifuged at 4 °C for 20 min (2000 *g*), according to previously established methods (Fan et al., 2017). The appearance of the saliva-oil mixture after centrifugation is shown in Supplementary Fig. S2. The oil phase (a) was the top layer, followed by the cream layer (b), then the aqueous phase (c), and some heavier molecular weight constituents (d) remained at the bottom. The total protein concentration for the oil-saliva system was calculated from its level in the cream layer (b) plus the concentration in the aqueous phase (c). To measure the protein concentration in the cream layer (b), five extra steps were followed: i) the cream layer was carefully removed by a plastic pipette and then diluted in 20 mM phosphate buffer in a 1:1 ratio [*w/w*]; ii) the dilution was centrifuged again under the same conditions; iii) another cream layer was collected and re-suspended in 1% Tween 20 solution with 20 mM phosphate buffer at a 1:1 ratio [*w/w*]; iv) the mixture was vortexed for 3 min and left 2 h to destabilise; v) the final aqueous phase was taken and its protein concentration was measured to present its level for cream layer phase in the oil-saliva system.

2.2.3. Saliva rheological analysis

Collected saliva samples with or without capsaicin stimulation (60 s pre- and 40 s post- swallow) were pre-treated with centrifugation (2000 *g*, 20 min) at 4 °C. The aqueous phase was taken for surface tension and rheology analysis using the methods developed by Yuan (Yuan et al., 2018). The surface tension of saliva samples was obtained by using a Theta Lite Optical Tensiometer (Biolin Scientific, Gothenburg, Sweden). A pendant drop was monitored for 10 s and the data were fitted into the Laplace–Young equation. The average was obtained from three replications.

A DHR-2 shear rheometer (TA Instruments, New Castle, DE) with tapered plate clamp (40 mm diameter, angle 2.08°) set at 28 °C was used for shear viscosity measurement, where shear viscosity was defined as the ratio of shear stress (τ) to shear rate (Shear rate, γ), shown in Eqn 1:

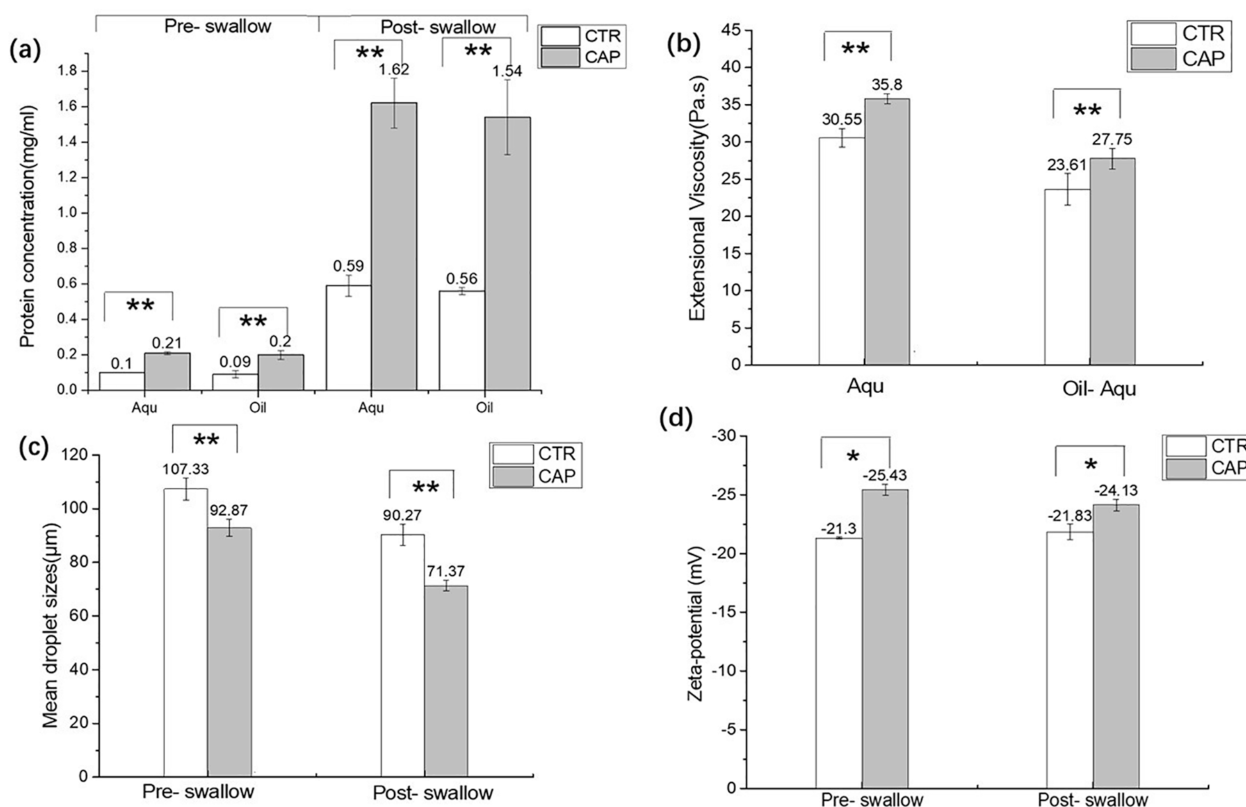

Fig. 2. Effect of capsaicin on saliva properties between CTR (white bar) and CAP (grey bar): (a) total protein concentrations from the aqueous (aqu) and oil-based system; (b) apparent extensional viscosity in the aqu and oil -aqu phase; (c) mean droplet size and (d) zeta-potential of the mixture of oil-saliva (CTR) and capsaicin oil-saliva (CAP). Stars within the same comparison group indicate significant differences (* indicated $p < 0.05$; ** indicated $p < 0.01$). The standard deviation is shown as ± error bar based on triplicate samples. Symbol ↑ or ↓ indicated percentage changed calculated as ((CAP – CTR)/CTR)*100%.

$$\eta_s = \frac{\tau}{\gamma} \quad (1)$$

CaBER-1 extensional rheometer (ThermoHaake, GmbH, Karlsruhe, Germany) was used to measure extensional viscosity with the assistance of a high-speed camera capturing the stretching process of saliva samples (Hadde et al., 2019). A saliva sample (25 μL) was used and each sample was measured 5 to 10 times. The extensional viscosity, η_E, was calculated based on Eqn 2 (McKinley & Tripathi 2000; Tuladhar & Mackley, 2008).

$$\eta_E = (2X - 1)\frac{\sigma}{\frac{-dD_{mid}(t)}{dt}} \quad (2)$$

where X is the geometric constant of the instrument (X = 0.7127), mainly considering the influence of gravity and inertial force; σ is the surface tension of saliva; $D_{mid}(t)$ is the sample's intermediate diameter at time t.

Each analysis for each sample was performed with 10 repeats. The extensional viscosity of the saliva in the aqueous system and the aqueous phase in the oil system (Supplementary Fig. S2) was tested in the post-swallowing group. In the pre-swallow samples, the extensional viscosity was too small to be detected after solution mixing and therefore such data were not shown.

2.2.4. Characterisation of saliva-oil emulsions properties

Similar to saliva secretion analysis, the panellist was requested to refrain from eating for at least 1 h before the test. Before consuming the sample, the panellist was instructed to rinse the mouth three times with water for 10 s each time. The panellist was then instructed to hold a 3.5-mL sample in the oral cavity for 1 min without swallowing, then drip oral fluid passively from the lower lip into the sterile container. After collecting the mixture, a new batch of saliva samples was sampled for another 40 s. During the collection period, the head was tilted down and the mouth was open to allow the saliva to drip passively from the lower lip into the sterile container. All the experiments were conducted three times with a 15–20 min break.

The saliva-oil emulsions samples collected at 60 s pre-swallow and 40 s post-swallow were characterised for their droplet size distribution and zeta potential. Droplet size distribution analysis was performed using a Malvern Mastersizer 3000 (Malvern, Great Malvern, UK), according to our established methods (Karthik, Ettelaie, & Chen, 2019). Zeta potential was measured on a Malvern Zetasizer NANO (Malvern, Great Malvern, UK), and followed previous methods (Karthik et al., 2019). The microstructure of saliva-oil mixture during 60 s holding in the mouth (pre-swallow) was observed, an optical microscopy technique fitted with a Leica DM 3000 camera (Meyer, Houston) was used. The standard method established by Glumac et al. (2018) was applied using 10 × magnification.

2.3. APCI-MS/MS analysis

The MS Nose interface (Micromass, Manchester, UK) was fitted to a Quattro Ultima mass spectrometer (Waters, Milford, MA). The selected ion mode was used with a cone voltage of 50 V, the source temperature was 30 °C, and the transfer line temperature was 120 °C. The silica capillary tube used inside the transfer line was 50 m in length and 0.32 mm internal diameter Zebron deactivated tubing (Phenomenex, Torrance, CA). A dwell time of 0.1 s was used for all the acquisition. In order to develop the APCI-MS/MS acquisition method, the static headspace of a standard solution of an individual aroma compound was analysed firstly in the "Full Scan" mode with the mass to charge ratio of *m/z* 20–200. Then based on the major fragment ions, the "Selected Ion Recording (SIR)" mode was used to confirm the precursor ion. Finally,

the "Daughters and Multiple Reaction Monitoring (MRM)" mode was used to identify the respective product ion with the optimised cone voltage and collision energy for the highest signal for each aroma compound (shown in Supplementary Table S1). The concentration for each compound added into either aqueous or oil systems was evaluated in a preliminary *in vivo* study, so their release signal height was at least five times higher than the noise level. All 14 compounds were added at their relevant levels (Supplementary Table S1) for further *in vitro* and *in vivo* analysis.

2.3.1. In vitro release by static headspace analysis

Two sets of *in vitro* tests were conducted by static headspace analysis, and the objectives of the first test were i) to confirm that every compound could be identified and quantified from the mixture of compounds; ii) to evaluate if any *in vitro* release differences existed for each compound between CTR and CAP samples in the aqueous system. Triplicate samples were used for this test. All static headspace analysis was tested after 30 min equilibrium at 20 °C, the headspace for individual sample (100 mL) contained in a 200-mL Duran bottle was evaluated in a randomised order by APCI-MS/MS at 5 mL/ min airflow. MassLynx software (MassLynx v4.1, Micromass, Manchester, UK) was used to integrate the chromatograms and extract the data on the maximum ion intensity (Imax).

The second set of *in vitro* tests was aimed to examine whether the effect of capsaicin on aroma release is solely due to salivary dilution. To mimic the saliva's dilution effect, a similar level of water as the additional amount of saliva stimulated by capsaicin was added to the respective aqueous and oil system. The level of saliva generated by 5 ppm capsaicin was measured when either 10 mL of water or 3.5 mL of oil were held in the mouth for 60 s, which was then spat out to calculate the weight gain with five repetitions. The saliva collected was 0.814 ± 0.075 g (based on 10 mL aqueous) and 0.634 ± 0.037 g (based on 3.5 mL oil), so in the mimic system of 100 mL sample (CTRA), 8.14 g water were added to the aroma-aqueous system, and 18.11 g water were added to the aroma-oil system. The *in vitro* release of each aroma compound from the headspace of the mimic system (CTRA) was measured, which was then compared with its headspace release from the control system (CTR). Triplicate samples were used, and all the samples were analysed in a randomised order by APCI-MS/MS with the operation methods described above. Imax data were used to calculate the release ratio between CTRA and CTR for each aroma compound.

2.3.2. In vivo release pre- and post-swallow

The *in vivo* analysis based on breath-by-breath measurement was carried out following the procedure developed previously (Yang et al., 2011). The test sample was held inside the mouth for a prolonged consumption period (60 s) to allow sufficient time for capsaicin exposure in the oral cavity and adequate saliva stimulation. The panellist was asked to cleanse his/her mouth with water and three exhalations were checked by APCI-MS/MS before sampling for the background check. Then a test sample was carefully transferred into the mouth (10 mL of aqueous or 3.5 mL of oil). The panellist was told to hold the sample in the mouth for 60 s without swallowing or mouth movement, whilst breathing normally into the tube linked to APCI-MS/MS. After 60 s holding the sample and saliva, the panellist swallowed the sample and breathed normally for another 40 s. All the samples were run in a randomised order with triplicates, and a 15–20 min break was given between samples to allow the panellist to swill the mouth with distilled water.

When a panellist consumed samples, the release of aroma compounds to the nasal cavity was monitored in real-time using APCI-MS/MS, following an established protocol (Yang et al., 2020b). The release profile was measured during 60 s pre- and 40 s post-swallow, which was a similar protocol in the saliva tests (*Section 2.2*). For each aroma compound, the data on the area under the curve (AUC) and Imax were extracted from the chromatogram. Then the average Imax and AUC of each compound were calculated for CTR and CAP samples.

2.4. Data analysis

Statistical analysis was conducted using SPSS (IBM® SPSS® Statistics version 25) and XLSTAT Software ©-Pro (2019.3.1; Addinsoft, Inc., New York, NY). Analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) followed with Tukey post hoc test were conducted by SPSS. The level of significance was set at $p < 0.05$. Principal component analysis (PCA) was conducted by XLSTAT to compare *in vivo* aroma release differences based on the AUC data between CTR and CAP pre- and post-swallow of the aqueous and oil samples.

3. Results and discussion

3.1. Impact of capsaicin on saliva properties

3.1.1. Saliva flow rate

Capsaicin induced a significantly ($p < 0.05$) higher level of saliva production for both aqueous and oil-based systems when observed during 60 s pre-swallow and 40 s post-swallow periods (Fig. 1). During additional 80 s post-swallow period (end of observation), capsaicin induced a significant increase in the normalised saliva flow rate in the aqueous system ($p < 0.05$), but no significant difference was found for the oil system, where both CAP and CTR samples returned to the innate saliva secretion level. Although 30 ppm capsaicin-oil system was designed to stimulate a similar level of saliva flow rate as 5 ppm capsaicin-aqueous system, this was based on the saliva collection before 2 min and after 2 min of capsaicin exposure (Supplementary Fig. S1). The shorter collection periods (60 s pre-swallow and 40 s post-swallow) showed a greater impact of capsaicin in normalised saliva flow rate (Fig. 1.) in the aqueous system (average increase of 172%) when compared to the oil system (average increase of 85%).

The amount of capsaicin in the 10-mL aqueous system of 5 ppm capsaicin addition was 50 μg, and the 3.5 mL oil system with 30 ppm capsaicin contained 105 μg. So there was a double amount of capsaicin in the oil system than in the aqueous system, but the effect on saliva flow rate was much larger in the aqueous system compared to the oil system. The reason is presumed to be due to the hydrophobic nature of capsaicin, and this tendency to dissolve in the oil droplets would reduce capsaicin's accessibility to the receptor in the tongue and reduce its ability to stimulate saliva secretion.

Compared to the previous study (Yang et al., 2020b), which involved 15 participants with a similar protocol (i.e., measuring saliva flow rate 60 s after consumption of 10 mL aqueous solution with or without 5 ppm capsaicin, labelled as CAP or CTR), the saliva flow ratio (CAP/CTR) was reported with a range of 0.79 and 3.74 with an average of 1.92 ± 0.76. The ratio for the participant in the current study is 2.12 [Fig. 1 a], so the findings reported from this participant might be representative of an average population. However, this is the limitation of using one participant in this study, and further studies are required with a group of panellists to compare the impact of capsaicin on their saliva flow between aqueous and oil systems.

3.1.2. Total salivary proteins

The total concentration of salivary protein after capsaicin stimulation was significantly higher ($p < 0.05$) than that of the control group in both the aqueous and oil-based system during pre- and post-swallow (Fig. 2a). In the aqueous-based system, capsaicin increased the protein concentration by 110% pre-swallow and 175% post-swallow. In the oil system, capsaicin increased the total protein concentration by 122% and 175% for pre- and post-swallow. The results also demonstrated the impact of capsaicin on the total salivary protein was more pronounced post-swallow than pre-swallow, as a 175% higher level was found in both aqueous and oil systems during post-swallow.

The average increase in total protein concentration from pre- and

post-swallow was 142% and 149% in the respective aqueous and oil systems. Capsaicin has been previously identified as a modulator of salivary composition. Gardner et al. (2020) reported an increase in specific saliva proteins (amylase, MUC5B, MUC7, PRP, cystatin and statherin) after exposure to capsaicin in an aqueous system. To the best of the authors' knowledge, our study is the first one that reports on the impact of capsaicin on total salivary proteins in both an oil system as well as an aqueous system. However, this result is based on one participant, and future studies with more participants will be required to validate the findings.

3.1.3. Apparent extensional viscosity

The pre-swallow saliva sample was too thin for extensional measurement and therefore, only apparent extensional viscosity (ASEV) of the saliva secreted after swallowing is shown (Fig. 2 b). After capsaicin stimulation, ASEV was significantly higher in both the aqueous and oil systems when compared with the control samples ($p < 0.05$), ASEV increased by 17% and 18% respectively. Extensional viscosity is a property of saliva that has important implications for adhesion and lubrication. The higher ASEV may reflect the greater polymer load (i.e., salivary protein) and better adhesion properties present in the sample. In this study, the apparent increase in extensional viscosity might be due to the physical entanglement of protein chains and the increased protein concentration by capsaicin stimulation. Gardner et al. (2020) also found that capsaicin increased the apparent extensional viscosity of saliva and concluded that a significant increase in the abundance of major salivary proteins (e.g., amylase and mucin) may contribute to the increase in saliva viscosity.

3.1.4. Saliva-oil emulsion characterisation

During oral processing of oil, it was reported that saliva could also act as an emulsifier to oil both *in vitro* and *in situ* (Glumac, Qin, Chen, Ritzoulis, 2019). In this study, the microstructure result of the saliva-oil mixture during 60 s holding in the mouth (as shown in Fig. 3 a), illustrated the individually stabilised oil droplets by saliva, supported the findings from the previously reported study. Microstructures of the oil-saliva emulsion (CTR) and capsaicin oil- saliva emulsion (CAP) are shown in Fig. 3. Individually stabilised oil droplets were visible, but CAP had many more droplets with a particularly higher number of medium-sized droplets (~100 μm diameter) and smaller droplets (<100 μm diameter), which appeared to have thicker walls compared to droplets in CTR.

Particle size analysis results confirmed that CAP had a significantly ($p < 0.05$) smaller mean droplet size (D_{32}) than that of CTR (Fig. 2 c). The mean droplet size was 13% and 21% smaller in CAP than CTR for pre- and post- swallow samples (average reduction of 17%). The zeta potential (ζ) measurements (Fig. 2 d) indicated a significant increase in CAP for both pre- and post-swallow samples ($p < 0.05$). Zeta potential is a measure of the density of surface charge, representing electric repulsion force and giving protection against aggregation once droplets approach each other. The higher the zeta potential in absolute values, the stronger the repulsive forces and, according to classical stability theory, the more stable the emulsion (Dickinson, 1992). Our results indicated that capsaicin led to an increased ζ by 19% and 10% in the respective pre- and post-swallow samples (average increase of 14.5%), so more stable emulsions could be formed in the capsaicin-saliva-oil matrix.

To summarise, the results for saliva analysis proved that our proposed mechanism is reasonable, at least based on the subject tested in this study. Capsaicin not only stimulates saliva secretion but also alters its physicochemical properties. However, further studies will be conducted with a larger number of participants to validate this finding. Averaging pre- and post-swallow data, oral capsaicin stimulation in aqueous and oil system i) led to 172% and 85% higher saliva flow rate in the respective system; ii) secreted an additional 142% and 149% salivary proteins respectively; iii) resulted in 17% and 18% higher saliva extensional viscosity. The higher level of salivary protein in the capsaicin-oil-saliva system could contribute to a more stable emulsion system in the mouth pre- and post-swallow with 17% smaller droplet size (and 14.5% stronger repulsive forces (ζ). The microscopic picture showed the first evidence that capsaicin-stimulated saliva could form a more stable emulsion in oil-based model foods.

3.2. Impact of capsaicin on aroma release

3.2.1. In vitro release by static headspace analysis

>125 volatile compounds have been identified in fresh and processed Capsicum fruits (Pino et al., 2007). In this study, fourteen common aroma compounds that were found in chilli with different physicochemical properties were selected (Table 1). They vary from very hydrophilic compounds (Log P = –0.06) to very hydrophobic compounds (Log P = 3.81). They also have different values in the respective air–water partition coefficient (K_{aw}), oil-air partitioning coefficient (K_{oa}) and vapour pressure. These 14 compounds represent different functional groups (3 pyrazines, 6 esters, 3 aldehydes, 1 alcohol and 1 ketone) and individual compounds could be identified from the mixture by APCI-MS/MS analysis based on their specific precursor and product ion (Supplementary Table S1).

The first static headspace analysis by APCI-MS/MS was conducted to measure the level of each compound partitioning into the headspace from CTR and CAP aqueous samples. The results of average Imax are summarised (Supplementary Table S2) and the calculated release ratio (CAP/ CTR) for each compound was close to 1. This indicated that *in vitro* release between CTR and CAP was similar and ANOVA showed no

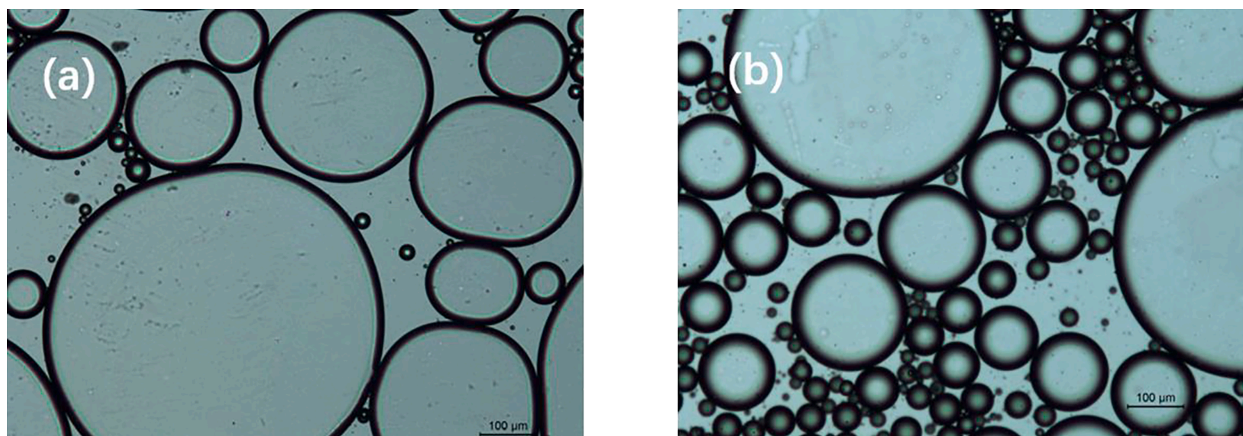

Fig. 3. Microscopic images of a salivary mixture of (a) oil and (b) capsaicin-oil after 60 s holding in mouth.

significant difference between them for each compound ($p > 0.05$). This finding is in agreement with previous observations by Yang et al. (2020a), who reported little impact of 5 ppm on the *in vitro* release of aroma compounds from a simple model aqueous system.

Previous research (Yang et al., 2020a) has shown that trigeminal stimulation by capsaicin can enhance saliva secretion, and hydrophobic aroma compounds are more likely to be retained in the secreted saliva. The second *in vitro* analysis is to evaluate if the impact of capsaicin on aroma release might be solely due to a dilution. An appropriate amount of water was added to the system (CTRA) to mimic the enhanced level of saliva due to capsaicin's oral stimulation. The *in vitro* release ratios (Imax CTRA/Imax CTR) were compared to the *in vivo* release ratios (Imax CAP/Imax CTR) when either aqueous or oil was held in the mouth for 60 s (Fig. 4). The *x*-axis represents the 14 aroma compounds labelled in Table 1, arranged with increasing hydrophobicity (Log P).

In the aqueous system, additional water generally had a dilution effect with a lower level of compounds available at the headspace (as the ratio was mostly ≤ 1.0, Fig. 4 a). However, the release ratio *in vivo* (Fig. 4 b) indicated that some compounds had higher release when CAP solution was consumed, particularly, the more hydrophobic compounds were more likely to be released from CAP samples. Based on Spearman's correlation analysis, there was no significant correlation between *in vitro* and *in vivo* aroma release ratio ($R = 0.442$, $p > 0.05$). In this aqueous system, capsaicin-induced saliva might offer more than just a dilution effect on the *in vivo* aroma release.

For the oil system, extra water in the mimic matrix (CTRA) showed enhanced release of most compounds with much higher Imax (as the ratio > 1.0, Fig. 4 c). Mostly hydrophilic compounds were more likely to move from the oil system to the water phase, which might then lead to a larger amount pushed out to the headspace from the matrix, compared to the pure oil system (CTR). This *in vivo* observation was less apparent than the results *in vitro* (Fig. 4 d), and there is no significant correlation between *in vitro* and *in vivo* results for the oil system by Spearman's correlation analysis ($R = 0.418$, $p > 0.05$). Therefore, our second hypothesis is true, that is, the impact of capsaicin on aroma release *in vivo* might not be simply explained by saliva's dilution effect in both oil and aqueous systems.

Additionally, saliva is made of 98% water, but it also contains more than one thousand proteins, among which are mucins, histatins, statherin, IgA, proline-rich proteins, and enzymes like α-amylase. Many of these proteins are free in saliva, while some are specifically anchored onto the in-mouth surfaces (Ployon et al., 2020). Proteins have been demonstrated to bind and trap aroma compounds, and consequently to modify aroma release and perception (Guichard, 2006). Among the major components of the salivary pellicle that coats the in-mouth surfaces, mucins have been reported to interact with aroma compounds in several studies (Van Ruth et al., 2000; Ployon et al., 2017). Our saliva analysis results in this study indicated that capsaicin caused significant changes to the saliva's physiological and biochemical properties, so other factors, such as the interactions between salivary proteins and aroma compounds might need to be taken into account when exploring the role of capsaicin-induced saliva on aroma release. In future *in vitro* studies, instead of using water in the mimic matrix, additional saliva stimulated by capsaicin can be added into the aroma-aqueous or aroma-oil system. The results will validate the proposed mechanism based on different interactions between aroma compounds and salivary proteins altered by capsaicin.

3.2.2. *In vivo* release pre- and post-swallow

The effect of capsaicin on *in vivo* aroma release for 14 compounds

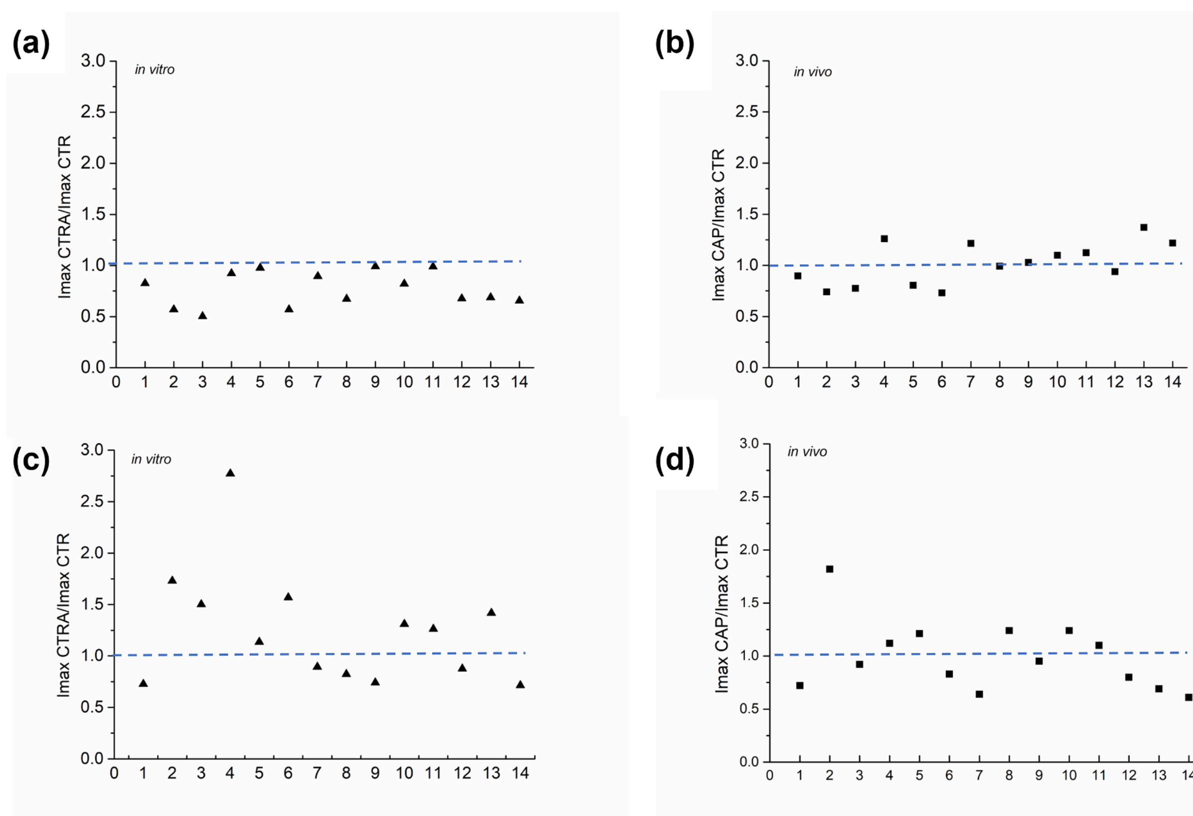

Fig. 4. Average Imax release ratio *in vitro* (triangle points▲) from (a) aqueous system and (c) oil system between water-addition samples (Imax CTRA) and control samples (Imax CTR); average Imax release ratio *in vivo* (square points ■) from (b) aqueous system and (d) oil system between capsaicin samples (Imax CAP) and control samples (Imax CTR). The X-axis is the number of compounds arranged according to their Log P values from low to high (Table 1). The dashed line at the ratio of 1 indicated a similar release level between the two samples.

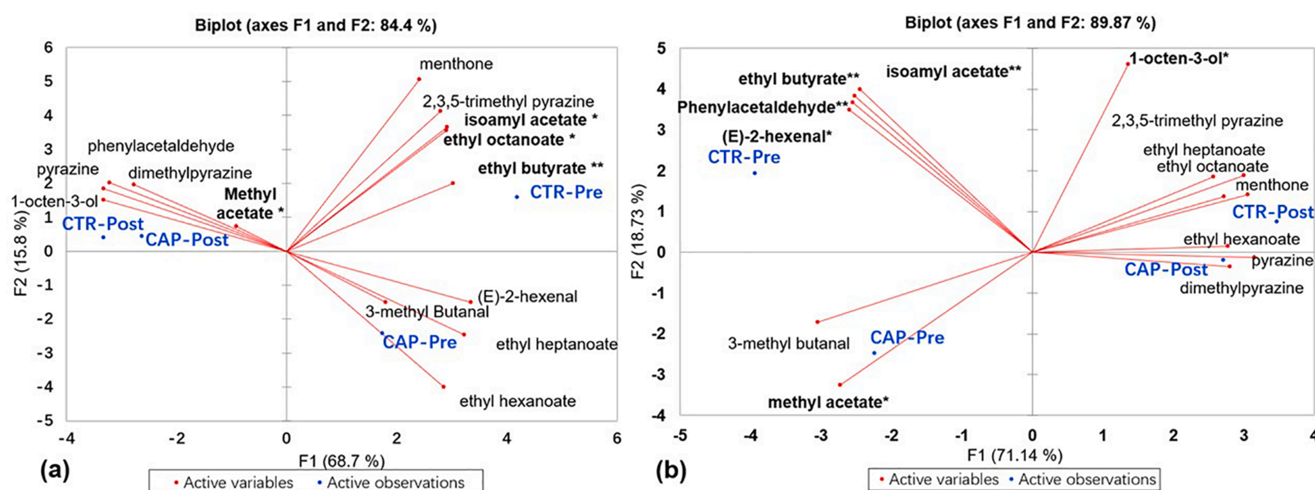

Fig. 5. PCA plot for aroma compounds in-nose release level (AUC data) before and after consumption (pre-/post-swallowing) of the control (CTR) and capsaicin-added (CAP) samples based on (a) aqueous- and (b) oil- systems. Stars represent significantly differences between CTR and CAP with triplicates (* indicated $p < 0.05$; ** indicated $p < 0.01$).

during pre- and post-swallow is summarised in the PCA (Fig. 5). In the aqueous system (Fig. 5 a), the first two principal components (F1 and F2) explained 84.4% of the data (68.7% and 15.8%, respectively). "CTR-Pre" and "CAP-Pre" were projected on the positive side of axis F1, while "CTR-Post" and "CAP-Post" were projected on the negative side. This difference between pre- and post- swallowing data was further confirmed to be significant by ANOVA ($p < 0.05$), which indicated that there might be different mechanisms involved in how capsaicin affects aroma release when holding the solutions for 60 s and 40 s after swallowing. The second axis F2 illustrated the significant difference between "CTR-Pre" data and "CAP-Pre" data ($p < 0.05$). Before swallowing ("Pre" data), there was a significant difference ($p < 0.05$) in the release of isoamyl acetate, ethyl octanoate and ethyl butyrate, that is, a higher level released from CTR compared with CAP.

The compounds that showed significant differences by capsaicin either pre- and post-swallow are summarised (Supplementary Table S3) and the percentage changes between CAP and CTR are listed. Capsaicin significantly reduced the release of isoamyl acetate by 65%, ethyl butyrate by 28% and ethyl octanoate by 42% when the solutions were held in the mouth. Interestingly, they are all fruity esters with relatively high K_{aw} (Table 1), so they might be more affected by additional saliva generated by capsaicin before swallowing.

For post-swallow release from the aqueous system, there were no significant differences between CTR and CAP for most aroma compounds, except for methyl acetate ($p < 0.01$). Methyl acetate is the most hydrophilic ester (Log P = 0.37) and the most volatile compound (VP = 52.70 Pa, estimated at 25 °C). The release of methyl acetate significantly increased by 79% in CAP ($p < 0.01$), which might be due to the capsaicin-induced saliva being more likely to increase the transfer of this compound from the saliva matrix after swallowing. However, further studies are required to explore the mechanisms between the release of methyl acetate and altered physicochemical properties of saliva as the result of capsaicin stimulation post-swallowing.

In the oil system, the impact of capsaicin on aroma release is shown in Fig. 5 b. The first two principal components (F1 and F2) explained 89.87% of the data (71.14% and 18.73%, respectively). Similar to the PCA for the aqueous system, "CTR-Post" and "CAP-Post" was projected at one side of the F1 axis, while "CTR-Pre" and "CAP-Pre" on the other side. The difference between post- and pre- results was again confirmed to be significant by ANOVA ($p < 0.05$). According to the F2 axis, a significant difference ($p < 0.05$) was observed between "CTR-Pre" and "CAP-Pre". When the samples were held for 60 s ("Pre" data), the release from CAP significantly ($p < 0.05$) reduced for four aroma compounds (Supplementary Table S3): isoamyl acetate and ethyl butyrate both reduced by 76%, phenylacetaldehyde reduced by 67%, and (*E*)-2-hexenal reduced by 60%. These four compounds were in the middle range of Log P (1.54 to 2.26) from all the compounds selected. A possible explanation for reduced aroma release in CAP pre-swallowing could be the formation of a more stable saliva-oil matrix (Fig. 3) and higher saliva protein levels in the capsaicin stimulated saliva (Fig. 2 a), so these aroma compounds were more likely to interact with saliva proteins and be retained in the more stable emulsion. A reduced droplet size formed by capsaicin oil- saliva mixture (Fig. 2 c) could also lead to an increased total surface area of the droplets, which may increase the likelihood of binding/entrapment of the volatiles at the interface (Benjamin et al., 2013).

On the other hand, the in-nose release of the most hydrophilic ester in this study- methyl acetate, increased by 36% in the capsaicin-saliva-oil matrix ($p < 0.05$). This hydrophilic ester was also found to have an enhanced release post-swallowing when capsaicin was added to the aqueous system (Fig. 5 a). Comparing oil and aqueous system, esters were found to be the most likely to be affected by capsaicin than aroma compounds from other functional groups. Esters have previously been reported for their likelihood of interactions with salivary proteins; for example, mucin and amylase have a well-documented capacity to reduce the release of esters (Van Ruth et al., 2000; Ployon et al., 2017). Up to 26% of salivary proteins are mucins, composed of a long polypeptide chain highly glycosylated in the central region (Kupirovi, Elmadfa, & Juillerat, 2017). This structure presents hydrophobic domains (Bansil & Turner, 2006), which could constitute binding sites of small molecules such as aroma compounds. Due to the existence of various types of binding sites of aroma compounds on salivary proteins, their release was affected to a different extent between aroma compounds (Pages-Helary et al., 2014). Additionally, enzymatic degradation activity of esterase in the whole saliva was also reported as an important factor that influences the ester release in oral processing (Pérez-Jiménez et al., 2019, Robert-Hazotte et al.,2019). Munoz-Gonzalez et al. (2017) also reported that human saliva strongly decreased the release of carbonyl compounds and strongly suggested that this was of an enzymatic nature. The most recent *ex vivo* results proved that specific aroma compounds can be metabolised by oral cells and saliva (Muñoz-González et al., 2022). Therefore, capsaicin-enhanced saliva changes are more likely to reduce the *in vivo* release of certain esters in our study.

Compared with pre-swallow data, the effect of capsaicin embedded in oil was less apparent in post-swallow, which was consistent with the findings in aqueous solution. After swallowing ("Post" data), the *in vivo*

release differences between CTR and CAP for most compounds were not significant, except 1-octen-3-ol, which was reduced by 16% in CAP ($p < 0.05$). Among the 14 selected compounds (Table 1), this was the least volatile compound (VP = 0.24 Pa) and relatively hydrophobic (Log P = 2.60). As the swallowing progresses, very few aroma compounds remaining inside the oral cavity, but capsaicin kept stimulating 82% extra saliva (Fig. 1) with a 175% higher total protein concentration (Fig. 2) than the control. Aroma compound 1-octen-3-ol that remained in the mouth after swallowing was likely affected by this additional saliva.

In this study using both aqueous and oil systems, capsaicin-induced saliva enhanced the release of the most hydrophilic ester (methyl acetate), but esters with medium hydrophobicity (ethyl butyrate and isoamyl acetate) were more likely to be metabolised with capsaicin-induced saliva matrices. The results agree with our proposed mechanism that the effect of capsaicin on aroma release was not solely related to the dilution effect by extra saliva generated, but more likely related to the capsaicin-induced changes on saliva properties and salivary proteins, which led to different interactions with aroma compounds of diverse physicochemical properties. In addition, this study chose to use the same subject, focusing on the impact between different matrices and avoiding inter-individual variations of subjects. Further studies could investigate how the capsaicin effect varies with different populations with different oral microenvironments and evaluate subjects with different levels of exposure to chilli and frequency of consumption.

To summarise the effect of capsaicin on the release of 14 aroma compounds with different physicochemical properties: i) there was no significant impact of capsaicin on static *in vitro* aroma partitioning; ii) certain esters were more likely to be affected by capsaicin *in vivo*, in both the aqueous and oil systems. The most hydrophilic ester (methyl acetate) showed a significantly increased release in capsaicin-induced saliva, and esters with medium hydrophobicity (ethyl butyrate and isoamyl acetate) were more likely to be retained, particularly when holding 60 s before swallowing. It will be useful to further investigate the interactions between these aroma compounds and specific capsaicin-stimulated saliva proteins in future studies.

4. Conclusions

In conclusion, the results from both aqueous and oil systems supported the hypotheses we proposed in this study: i) capsaicin not only caused an increase in saliva flow rate but also changed its physicochemical properties; ii) that the capsaicin stimulated saliva directly impacted the release of aroma compounds; this effect is not a simple dilution effect.

Capsaicin not only induced an increase in saliva production (172 % and 85% average increase in the aqueous and oil system respectively), but also increased the concentration of salivary proteins (142% and 149 % increase in the aqueous and oil system) and enhanced extensional viscosity (17% and 18% increase in the aqueous and oil system), and that this effect was matrix dependent. Capsaicin-induced saliva formed a more stable emulsion in the mouth during consumption of the oil-based system. This matrix had more droplets, with a 17% smaller size and 14.5% stronger repulsive force (zeta potential).

The changes in saliva physicochemical properties induced by capsaicin directly impact the release of aroma compounds, which is not a simple dilution effect. The aroma release ratio during consumption (i.e., CAP/CTR *in vivo)* was not proportional to its static equilibrium partition ratio (i.e., CTRA/CTR *in vitro*), despite additional water being added to imitate the diluting effect of additional saliva production. This suggests that the delivery of aroma into the nasal cavity is directly impacted by the capsaicin-induced saliva modifications that were observed.

Overall, this study has revealed the potential mechanism of capsaicin stimulation on saliva properties and aroma release in aqueous and oil systems, although further studies with more participants are required to validate the present finding. The oral exposure of capsaicin in both systems stimulated extra saliva production and more salivary proteins being formed. As a result, the stimulated saliva has a much higher emulsifying property and higher extensional viscosity, which is proposed to be due to the enhanced protein entanglement. Additionally, capsaicin did not only have a significant impact during consumption but also had a long-lasting effect after consumption. Saliva proteins continued to be elevated after 40 s consumption of capsaicin in aqueous and oil systems (both by 175%), and capsaicin's impact is still significant with a 109% increase in the aqueous system during another 80 s after swallowing. This long-lasting impact of capsaicin on food oral processing of more complicated systems could be further investigated.

Ethical review.

Ethics approval was obtained from the Ethics Committee of the Zhejiang Gongshang University, Hangzhou, China (Ref no. 2020040911).

CRediT authorship contribution statement

Xiaoxue Hu: Formal analysis, Investigation, Methodology, Project administration, Data curation, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Charfedinne Ayed:** Conceptualization, Formal analysis, Methodology, Supervision, Software, Writing – original draft, Writing – review & editing. **Jianshe Chen:** Funding acquisition, Conceptualization, Resources, Writing – original draft, Writing – review & editing. **Ian Fisk:** Funding acquisition, Conceptualization, Resources, Writing – original draft, Writing – review & editing. **Ni Yang:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132824.

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