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Retention effect of human saliva on aroma release and respective contribution of salivary mucin and α-amylase

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ABSTRACT

As great differences were observed in the amount of α-amylase in human saliva, there is a need to better understand the effect of this protein alone or in mixture with mucin on aroma compound partitioning. We report the respective role of mucin and α-amylase on the air/liquid partition coefficients of two series of 5 methyl-ketones and 5 ethyl-esters. We confirm that mucin affects the release of aroma compounds and, for the first time, we demonstrate the ability of α-amylase to decrease the release of aroma compounds. For both proteins, we report the involvement of hydrophobic effects. Interestingly, no cumulative effect was observed when both proteins were mixed together in solution. We hypothesize that protein–protein interactions occur between the two proteins and decrease the total number of available binding sites for aroma compounds. The effect of human saliva is also investigated and compared to that of artificial salivas. In the presence of human saliva the release of ketones is lower than in water and slightly higher than in the presence of artificial saliva composed of α-amylase and/or mucin. Esters are more affected by the presence of human saliva than ketones. This observation is due to the presence of an esterase activity in saliva, which activity increases with the hydrophobicity of esters. The difference observed in aroma release between artificial and human salivas could be explained by the presence of other salivary proteins in human saliva.

1. Introduction

Flavour is one of the most important organoleptic properties of food regarding consumer acceptability. Therefore, the mechanisms involved in the release of flavour compounds during food consumption are a particular subject of interest. Regarding the release of aroma compounds, the global mechanism could be divided into three steps. During the first step, aroma compounds are released from food to saliva during food breakdown in the mouth. Then, they are partitioned between saliva and the air phase in the oral cavity. The intermittent aperture of the velum allows the transfer of aromatized air from the oral to the nasal cavity (Buettner, Beer, Hannig, & Settles, 2001), mostly during masticatory activity and swallowing events (Repoux, Labouré, et al., 2012; Repoux, Semon, Feron, Guichard, & Laboure, 2012). Finally, they reach the olfactory mucosa, where they bind to the olfactory receptors (Gaillard, Rouquier, & Giorgi, 2004).

Several studies have reported a difference between retronasal and orthonasal perception of the same odorant molecule, indicating an influence of the oral processing and physiology on aroma perception (Burdach, Kroeze, & Koster, 1984; Kuo, Pangborn, & Noble, 1993; Linforth, Martin, Carey, Davidson, & Taylor, 2002; Voirol & Daget, 1986). Among the different factors influencing the release of volatile compounds, the impacts of saliva (Genovese, Piombino, Gambuti, & Moio, 2009) and of oral volume (Mishellany-Dutour et al., 2012) were evidenced. Saliva, which results from blood filtration, not only is composed of 99% of water, but it also contains numerous inorganic salts and organic compounds such as proteins (Humphrey & Williamson, 2001). The total protein concentration ranged between 1 and 3.5 $mg \cdot mL^{-1}$ (Bennick, 1982; Neyraud, Heinzerling, Bult, Mesmin, & Dransfield, 2009), although higher concentrations have been reported (Ferguson, 1999). The proteomic analyses of saliva have identified 1166 proteins (Denny et al., 2008). Some of these proteins are able to interact with food molecules and play a role in flavour. For example, proline rich-proteins are involved in astringency as these proteins can bind and aggregate tannins, which are at the origin of this sensation (Canon, Milosavljević, et al., 2013; Canon, Paté, et al., 2013b). Therefore, different investigations on flavour perception have evoked the variation in salivary protein composition to explain inter-individual variability in flavour perception (Dsamou et al., 2012; Mounayar et al., 2013; Poette et al., 2014). Regarding aroma release from food matrices, a lot of studies have demonstrated that proteins

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can interact and bind to aroma compounds and that the nature and strength of the interactions depend on both the type of macromolecules and aroma compounds (Guichard, 2006; Lorrain et al., 2013; Mitropoulou, Hatzidimitriou, & Paraskevopoulou, 2011; Rodriguez-Bencomo et al., 2011; Villamor, Evans, Mattinson, & Ross, 2013). The effect of saliva addition to model wine modified the release of aroma compounds in the vapour phase (Mitropoulou et al., 2011). This effect depends on the type of wine and differs between human and artificial salivas suggesting that other salivary proteins than mucin can affect the release of aroma compounds (Genovese et al., 2009). Moreover, there is an important variability in saliva composition between individuals (Quintana et al., 2009). For example, Perry et al. (2007) have reported great differences in the amounts of α-amylase in saliva through the study of different populations. In addition, the composition of the salivary peptidome is modified with the age of the subject and with his diet (Morzel et al., 2012). Therefore, it has been suggested that differences in salivary protein composition could be at the origin of inter-individual variation in aroma release (Piombino et al., 2014; Repoux, Semon, Feron, Guichard, & Laboure, 2012). As different proteins can interact with aroma compounds and as there is an important variability in protein saliva composition, it is of interest to understand if the protein composition of saliva impacts the release of aroma compounds. Surprisingly, previous investigations in simple model systems have reported no significant difference between human saliva and artificial saliva containing only mucin on aroma release (Benjamin, Silcock, Beauchamp, Buettner, & Everett, 2012; van Ruth, Grossmann, Geary, & Delahunty, 2001). These results suggest that the effect of salivary proteins on aroma release can be assimilated to the effect of one protein: mucin. Among the few results on the effect of other salivary proteins on aroma compound partitioning, it has been reported that the addition of α-amylase to an artificial saliva containing mucin does not significantly affect aroma release (van Ruth, Roozen, & Cozijnsen, 1995). To our knowledge, besides this study, the effect of α-amylase on aroma release has never been clearly investigated, despite the fact that α-amylase is one of the most abundant proteins in saliva (Schenkels, Veerman, & Nieuw Amerongen, 1995). Therefore, to address the lack of clear information on the effect of α-amylase, we have investigated the effect of this protein alone or in mixture with mucin on the air/liquid partitioning of aroma compounds. As hydrophobic effects are generally the main non-covalent interactions driving the binding of aroma compounds onto proteins (Damodaran & Kinsella, 1980; Damodaran & Kinsella, 1981; Guichard, 2006; Tavel, Andriot, Moreau, & Guichard, 2008), two series of linear aliphatic volatile compounds, five methyl ketones and five ethyl esters, were chosen in order to determine the role of these aroma–protein interactions on aroma compound partitioning. These molecules cover a wide-range of hydrophobicity, characterized by the log *P* value (octanol–water partition coefficient). Their air/liquid partition coefficients were determined in water, in buffer solution, in human saliva and in three artificial salivas containing mucin, α-amylase or a mixture of mucin and α-amylase.

2. Materials and methods

2.1. Human saliva

Human saliva (hs) was constituted by a pool of stimulated saliva coming from 15 volunteers (6 females and 9 males, aged 24–60). The subjects were asked to avoid eating or drinking (although water was allowed) for at least 1 h before saliva collection. Stimulated saliva was collected by asking the subjects to chew a piece of Parafilm (American National Can, Chicago, IL) and then to spit out their saliva at regular intervals (Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012). Two saliva collections were organized the same day: one in the morning and one in the afternoon. All saliva samples were immediately stored at 4 °C after each individual collection and then pooled, the same day as the collection, to obtain a total volume of 800 mL. This pool was divided into aliquots of 20 mL stored at −20 °C until use and for a maximal time of two weeks. Before freezing and after defrost of the saliva, the pH was 8.3 ± 0.01 and the viscosity was 6.9 mPa·s at 20.7 °C, 1500 s^{-1}, 30 s. Usually, the pH of human saliva is comprised between 5.56 and 7.41, but pH values up to 8.5 have been reported (Neyraud et al., 2009). Variation in pH can affect the charge of proteins and therefore their conformation (Canon et al., 2011; Giuliani et al., 2012). However, Friel et al. did not find any significant difference on the retention of aroma compounds by mucin for pH values comprised between 6.5 and 8.5. The basicity of the saliva observed here is probably due to the fact that bicarbonate is the main buffer of saliva and could be converted into CO_2 by carbonic anhydrase. As the partial pressure of CO_2 is higher in saliva than in air, the loss of CO_2 leads to a decrease in bicarbonate and therefore an increase in pH (Bardow, Moe, Nyvad, & Nauntofte, 2000). Regarding the effect of the storage on saliva, we verified that the retention of ethyl heptanoate and 2-heptanone by human saliva was not modified after storage at −20 °C.

The protocol for saliva collection was submitted to an Ethics Committee and was approved on 17th April of 2008 by the Comité de Protection des Personnes Est-1 (No. 2008/15) and on 8th August of 2008 by the Direction Générale de la Santé — France (No. DGS2008-0196).

2.2. Preparation of artificial salivas

Water solution (w) was deionized MilliQ water (18.2 $m\Omega \cdot cm^{-1}$, Millipore Corporation Billerica, MA, USA). A buffer solution was made-up using the following components: $NaHCO_3$ (5.208 $g \cdot L^{-1}$), K_2HPO_4, $3H_2O$ (1.369 $g \cdot L^{-1}$), NaCl (0.877 $g \cdot L^{-1}$), KCl (0.477 $g \cdot L^{-1}$), and $CaCl_2$, $2H_2O$ (0.441 $g \cdot L^{-1}$) (van Ruth et al., 2001). Three artificial salivas were constituted by addition in the buffer solution of mucin (2.16 $g \cdot L^{-1}$) for the mucin artificial saliva (m), α-amylase (200,000 $U \cdot L^{-1}$) for the α-amylase artificial saliva (a) and α-amylase (200,000 $U \cdot L^{-1}$) with mucin (2.16 $g.L^{-1}$) for the artificial saliva composed of mucin and α-amylase (ma) in the concentrations used by van Ruth et al. (2001). Mucin from bovine submaxillary salivary glands and human α-amylase were purchased from Sigma Aldrich (Sigma Aldrich, St Quentin Fallavier, France). Before each experiment, fresh artificial salivas were prepared, therefore NaN_3, usually used as a bacterial growth inhibitor, was not added to the solution in order to avoid any effect of this molecule on aroma release (direct interactions or modifications of the protein structure).

The pH of the artificial saliva and buffer solution was adjusted using sodium hydroxide NaOH to 8.3 ± 0.01, which was the pH of the defrosted human saliva. The viscosity of artificial saliva solutions was comprised between 6.6 and 7 mPa·s (20.7 °C, 1500 s^{-1}, 30 s).

2.3. α-Amylase activity

The α-amylase activity was measured in human and artificial salivas containing α-amylase using the amylase CNPG3 assay kit (BIOLABO, Maizy, France), in order to compare the amount of α-amylase in all saliva samples. After the addition of a solution of 2-chloro-p-nitrophenyl-α-D-maltotrioside (CNPG3) at 2.25 · 10^{-3} $mol \cdot L^{-1}$, the tested solution was diluted 4000-fold in NaCl solution at 9 g/L. The degradation of CNPG3 by α-amylase activity is directly proportional to 2-chloro-4-nitrophenol (CNP) concentration, which can be determined by absorbance measurement at 405 nm. A standard curve was established with six different concentrations using a serum containing α-amylase with an activity comprised between 2.25 $U \cdot mL^{-1}$ and 42 $U \cdot mL^{-1}$ (Exatrol-N, BIOLABO, Maizy, France). The α-amylase activity was expressed in International Enzyme Unit Activity (U) per L. One U is defined as the amount of the enzyme that catalyses the conversion of 1 μmol of substrate per minute.

2.4. Aroma compounds

Aroma compounds were purchased from Sigma Aldrich (Sigma Aldrich, St Quentin Fallavier, France): 2-propanone, 2-hexanone, 2-heptanone, 2-octanone, 2-nonanone, ethyl propanoate, ethyl butanoate, ethyl pentanoate, ethyl hexanoate, and ethyl heptanoate (Table 1). A gas chromatography–flame ionization detector (GC–FID) analysis confirmed the purity of all aroma compounds (>99%).

Stock solutions of aroma compounds were prepared in propylene glycol at a concentration of 0.1 mol·L^{-1} and stored at 4 °C.

2.5. Static equilibrium headspace analysis

Stock solutions of aroma compounds were further diluted into water, buffer, artificial saliva solutions and human saliva to obtain a final aroma compound concentration of 1.10^{-4} mol·L^{-1}, which is below the solubility threshold of the tested compounds in water. The final pH was 8.3 ± 0.01 for all solutions. In order to avoid any aroma competition for protein binding sites, we chose to analyse aroma compounds one by one. The final solutions (5 mL) were put into 20.46 mL headspace vials (Supelco, Bellefonte, PA, USA). The headspace vials were immediately sealed with silicone septa in magnetic caps (Supelco). The experiments were performed at a temperature of 30 °C, which is in the range of the temperature measured in the mouth when consuming cold beverages (between 22 and 33 °C) (Engelen et al., 2003). Moreover many air/liquid partition coefficients are available at this temperature. The time needed to reach the static equilibrium was determined in all artificial salivas during preliminary experiments with a mixture of aroma compounds from different chemical classes and log *P* values, including ethyl propanoate, octan-2-one and nonan-2-one. The headspace vials were placed in the incubator of an automatic headspace sampler (GERSTEL MPS 2, Gerstel Inc., Mülheim an der Ruhr, Germany) and equilibrated at 30 °C until a static equilibrium was attained (40 min). A one millilitre sample of headspace was taken automatically using a preheated (35 °C) 2.5 mL gas-tight syringe (Gerstel, manufactured by SGE, Victoria, Australia) and analysed by gas chromatography (TRACE GC Ultra, Thermo Electron, Milan, Italy). The injector temperature was set at 240 °C. A capillary DB-Wax column (30 m, 0.32 mm i.d., 0.5 μm; Agilent J&W Scientific, Folsom, CA, USA) and a flame ionization detector set at 250 °C were used. The carrier gas was hydrogen at a velocity of 0.03 cm^3·s^{-1} in split mode (1/10). For each aroma compound, an oven isothermal temperature was preliminarily determined in order to obtain a retention time comprised between 2 and 5 min and used during the present experiments (Table 1). All experiments were performed in triplicate. In order to compare the effect of saliva composition on aroma release and to avoid day-to-day variations, for each aroma compound, all the studied media were analysed on the same day.

Table 1
List of aroma compounds with their molecular weight in g·mol^{-1} (Mw), hydrophobic constant (log *P*), solubility in water at 25 °C in mol·L^{-1} (Sol), enthalpy of vaporisation ($\Delta_{vap}H°$) in kJ·mol^{-1}, GC oven temperature.

Aroma compound	Mw[a]	log *P*[a]	Sol[a]	$\Delta_{vap}H°$[b]	GC oven temperature
Methyl ketones					
2-Propanone	58	−0.24	3.79E+00	31	45 °C
2-Hexanone	100	1.24	7.75E−02	43	80 °C
2-Heptanone	114	1.73	1.88E−02	47.2	95 °C
2-Octanone	128	2.22	6.91E−03	39.8	100 °C
2-Nonanone	142	2.71	1.20E−03	56.4	125 °C
Ethyl esters					
Ethyl propanoate	102	1.36	1.05E−01	39.1	60 °C
Ethyl butanoate	116	1.85	2.37E−02	42	70 °C
Ethyl pentanoate	130	2.34	7.12E−03	46.1	90 °C
Ethyl hexanoate	144	2.83	2.14E−03	51.7	95 °C
Ethyl heptanoate	158	3.32	6.45E−04	55.8	110 °C

[a] Values were calculated by EPISUITE 4.00 software (US Environmental Protection Agency).
[b] http://webbook.nist.gov/chemistry.

2.6. Calculation of air/liquid partition coefficient

For each aroma compound, a calibration curve was established by GC/FID in the same analytical conditions reported above and using a liquid injection (1 μL) of a solution of aroma compounds in CH_2Cl_2 (10^{-5} to 10^{-2} mol·L^{-1}). The calibration curves were used to determine the concentration of each aroma compound in the gas phase (Chana, Tromelin, Andriot, & Guichard, 2006). Then, the partition coefficient of compound i in medium m, $K_{im} = C_{ivap}/C_{im}$, where C_{ivap} is the molar concentration of compound i in the gas phase and C_{im} is the molar concentration of compound i in the medium, was calculated. As $C_{ivap} \ll C_{im}$, C_{im} can be approximated by the initial concentration in the medium.

2.7. Analysis of ester degradation in human saliva

To test the hypothesis that esters are transformed into acid due to the presence of an esterase activity in the human saliva, the concentration of esters was measured in the headspace at 30 °C at different times (20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 min).

Simultaneously, the production of acid from the degradation of esters was quantified in the liquid phase after dichloromethane extraction. Human saliva samples containing ester were thawed and extracted three times with 1 mL CH_2Cl_2 under stirring for 15 min. After extraction, the 3 mL of CH_2Cl_2 was pooled and concentrated with a 5 mL Kuderna Danish apparatus to obtain a final volume of 300 μL. Extracts were then analysed by GC/EI–MS (Agilent Technologies, 78 90A GC system, Les Ulis, France). The injector temperature was set at 240 °C and a capillary FFAP column (30 m, 0.32 mm, 0.5 μm; Agilent J&W Scientific, Folsom, CA, USA) was used. The carrier gas was helium at a flow rate of 0.03 cm^{-3}·s^{-1} in splitless mode. The GC oven temperature started at 40 °C and was increased to 120 °C at a rate of 6 °C·min^{-1} then to 140 °C at a rate of 1 °C·min^{-1}, maintained for 10 min at 140 °C, then increased to 160 °C at a rate of 4 °C·min^{-1}, then to 240 °C at a rate of 6 °C·min^{-1}.

A calibration curve of heptanoic acid ($1.58 \cdot 10^{-10}$ to $7.00 \cdot 10^{-9}$ mol acid in 1 μL CH_2Cl_2, $r^2 = 0.993$) was used to calculate the amount of acid produced by the ethyl heptanoate degradation.

2.8. Data analyses

Air/liquid partition coefficient calculated in the various media that were studied was submitted to univariate analysis of variance (ANOVA), followed by a mean comparison test (Neuman-Keuls test, 5%), by using R® Software (Version 2.14.1. Ink, Foundation for Statistical Computing, Vienna, Austria).

The ratios of the partition coefficient saliva/water were calculated and then plotted against the log *P* value of aroma compounds. The resulting curves were then fitted using the line curve fitting function in Igor Pro (Version 6.22A, WaveMetrics, Portland, USA).

3. Results and discussion

3.1. Air/liquid partition coefficients of ketones and esters in artificial and human saliva

Air/liquid partition coefficients (K) are reported in Fig. 1A for ketones and Fig. 1B for esters. As expected, no significant difference was observed between the air/water (Kw) and air/buffer (Kb) partition coefficients for all molecules (data not shown), thus only Kw values are reported and water constitutes the reference medium. For ketones, Fig. 1A shows an increase in the air/liquid partition coefficients as a function of the ketone aliphatic chain length for all studied media.

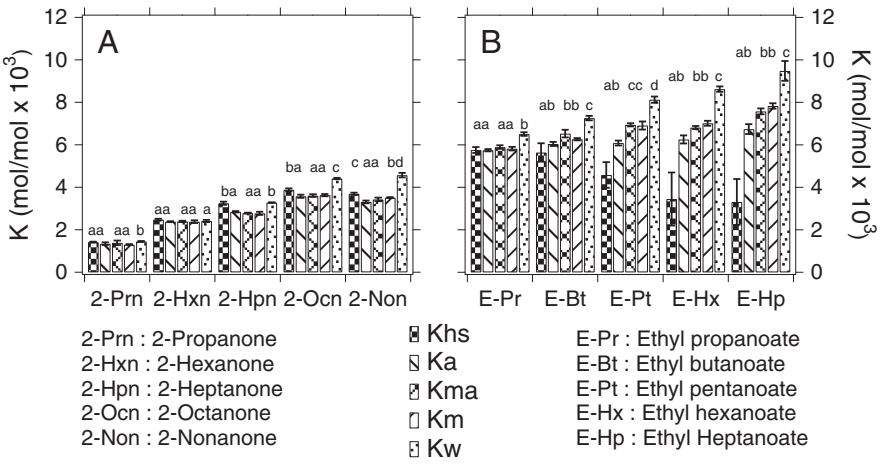

Fig. 1. Air/liquid partition coefficients (K) of aroma compounds for (A) ketones and (B) esters in the presence of water (Kw), mucin in buffer (Km), mucin and α-amylase in buffer (Kma), α-amylase in buffer (Ka), and human saliva (Khs). For each aroma compound, values with different letters are significantly different ($P < 0.05$) after mean comparison (Newman–Keuls-test).

This increase in air/liquid partition coefficients in the function of log *P* value for homologous series of aroma compounds has already been established at a temperature of 25 °C by Buttery, Ling, and Guadagni (1969) and at 30 °C by Merabtine, Lubbers, Andriot, Tromelin, and Guichard (2010). No significant difference was observed between all media for 2-propanone (P value > 0.06) and 2-hexanone (P value > 0.1). For the other ketones, 2-heptanone, 2-octanone and 2-nonanone, the air/mucin (Km), air/α-amylase (Ka) and air/(mucin + α-amylase) (Kma) partition coefficients are not significantly different from each other, while Km, Ka and Kma are significantly lower than both Kw and air/human saliva partition coefficient (Khs) (P values < 0.001) and Khs is significantly lower than Kw (P value < 0.001). In Fig. 1B in water and artificial salivas, the air/liquid partition coefficients of esters increase also as a function of ester hydrophobicity, whereas the opposite trend is observed in the presence of human saliva. As for ketones, the air/liquid partition coefficients of esters in artificial salivas (Km, Ka and Kma) are not significantly different from each other and significantly lower than the air/water partition coefficient (Kw) (P value < 0.001). But contrary to ketones, the air/human saliva partition coefficients (Khs) of all the esters are significantly lower than the air/liquid partition coefficients in other media (P value < 0.001). Results obtained in artificial salivas suggest that the salivary proteins mucin and α-amylase are both able to retain methyl ketones and ethyl esters and that these interactions are not specific to a family of proteins as previously observed for several proteins in food matrices (Guichard, 2006). The present observation of the retention of aroma compounds in a solution containing only α-amylase seems in contradiction with the previous data reported by van Ruth et al. (1995). Moreover, while previous investigations suggest that artificial saliva containing mucin only and human saliva have similar effects on the behaviour of aroma compounds (van Ruth & Roozen, 2000), our results show significant differences between artificial and human salivas in the function of the protein composition and the nature of aroma compound. Therefore, in the following parts, we will both compare the effect of proteins alone in solution, proteins in mixture and total salivary proteins on aroma partitioning.

3.2. Effect of artificial saliva composition on aroma compound release

In order to gain a deeper understanding on the protein–aroma interactions and the role of hydrophobic effects in such interactions, the relative partition coefficients between saliva media (m, ma, a and hs) and water (w) were calculated for molecules whose the partition coefficients are significantly affected by artificial or human saliva and, then, plotted as a function of the hydrophobicity (log *P* value) of the compounds. The relative partition coefficients give a direct access to the effect of proteins on aroma release.

3.2.1. Effect of isolated mucin and α-amylase on aroma release

The ratio between air/mucin and air/water partition coefficients (Km/Kw) is plotted for esters and ketones as a function of their log *P* value on Fig. 2A. All ratios are lower than 1, which indicates that mucin has a retention effect on all aroma compounds. A linear fit function was used to correlate the relative partition coefficients Km/Kw with the corresponding log *P* value, for aroma compounds which were significantly affected by the presence of salivary protein (i.e. aroma compounds presenting log *P* values above 1.3). The slope of the linear correlation is -0.041 ± 0.017 and its y-intercept is 0.930 ± 0.034. The correlation coefficient (r) is −0.71 and the coefficient of determination (r^2) is 0.50. This negative correlation indicates an increase in the retention of aroma compounds by mucin as a function of their hydrophobicity suggesting that aroma molecules interact with mucin mainly due to hydrophobic effects. Mucins have molecular weights ranging from 0.5 to 20 MDa (Bansil & Turner, 2006). They are composed of a long polypeptidic chain highly glycosylated in the central region. This structure presents hydrophobic domains (Bansil & Turner, 2006), which could constitute binding sites of small molecules such as aroma compounds. Van Ruth et al. also reported a higher retention of the more hydrophobic aroma compounds by salivary proteins at 37 °C (van Ruth et al., 2001). However, it should be noticed that the hydrophobic effect does not explain all the variability of our results because the coefficient of determination r^2 is only of 0.50. Therefore, other non-covalent interactions such as electrostatic interactions could be also involved between aroma compounds and the mucin glycosylations (Tromelin, Merabtine, & Andriot, 2010). Indeed, for a similar log *P* value methyl-ketones exhibit a higher dipole-moment than ethyl-ester, which could be in favour of electrostatic interactions in addition to hydrophobic effects. In their investigation on the effect of mucin on aroma partitioning from solution adjusted at pH 7.0, at 25 °C, Friel and Taylor (2001) did not observe such correlation between the relative concentration of aroma compounds from different chemical classes in air and the corresponding log *P* value. This suggests that this relation is only valid within homologous series and that other types of interactions could be involved.

For similar log *P* value, ketones seem slightly more retained than esters. For example, 2-nonanone is significantly more retained by mucin than ethyl heptanoate (P value < 0.05). Separated analysis of the

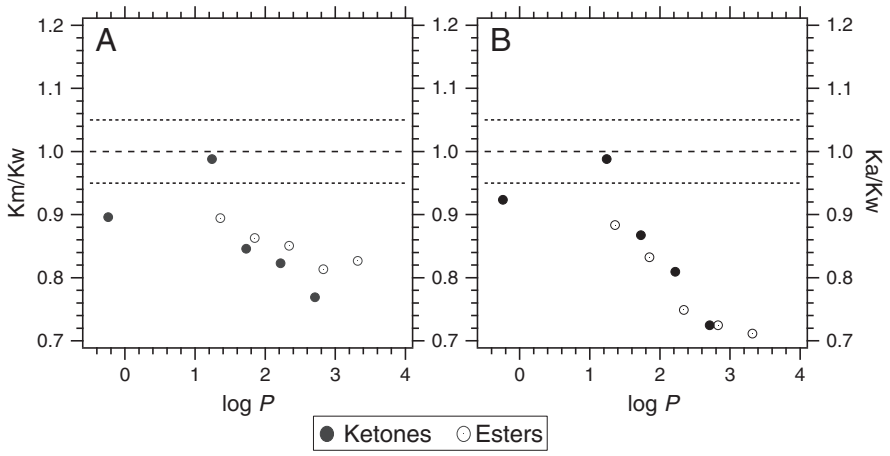

Fig. 2. Relationship between hydrophobicity (log *P* value) of aroma compounds and the ratio of the air/liquid partition coefficient in the presence of (A) mucin (Km/Kw) and (B) α-amylase (Ka/Kw) to the one in water. Dotted horizontal lines represent the ±5% interval around the ratio equal to 1.

correlation between the relative partition coefficients Km/Kw of ester with their log *P* value gives a better negative correlation: $y = -0.037x\ (\pm 0.0091) + 0.0937\ (\pm 0.022)$ ($r = -0.92$ and $r^2 = 0.85$). For ketones, it is too speculative to discuss the negative correlation obtained, as only three molecules are significantly affected and studied in this analysis.

Fig. 2B presents the ratio of K values between α-amylase medium (a) and water (w) as a function of aroma log *P* value. As for mucin, an increase of the aroma retention is observed with the log *P* value for all the esters and for ketones with log *P* values higher than 1.3. Therefore, a linear fit function was used to correlate the relative partition coefficients Ka/Kw of compounds with their corresponding log *P* value (in the range from 1.3 to 3.5). The slope of the curve is -0.101 ± 0.013 and the y-intercept is 1.02 ± 0.032 ($r = -0.95$ and $r^2 = 0.90$). This negative correlation can explain almost all the variability observed, which clearly indicates the involvement of hydrophobic effects in the retention of aroma compounds with a linear hydrophobic chain by α-amylase. Regarding the structure of the proteins, α-amylase is a globular protein with a well-defined three-dimensional structure (Kandra & Gyémánt, 2000; Ramasubbu, Paloth, Luo, Brayer, & Levine, 1996) contrary to mucin. Our results suggest that one or several hydrophobic domain(s) in the α-amylase structure are available for the binding of hydrophobic molecules such as aroma compounds. The correlations observed for mucin and α-amylase with the log *P* value of molecules suggest that aroma compounds with a log *P* value higher than 1.5 are more affected by α-amylase than by mucin. By using a mouth model system under a nitrogen gas flow, van Ruth et al. (1995) did not observe any significant effect of the addition of α-amylase to artificial saliva containing mucin. However these authors observed different trends according to their experimental conditions. It appears thus necessary to analyse the results obtained with artificial saliva containing both mucin and α-amylase.

3.2.2. Effect of mixed mucin and α-amylase on aroma release

We thus investigated the effect of mucin and α-amylase mixed together in solution on the release of aroma compounds. Fig. 3A presents the relative partition coefficient between the medium containing both mucin and α-amylase (ma) and water (w). As for isolated proteins, a decrease in the Kma/Kw ratio as a function of aroma compound hydrophobicity is observed. The application of a linear fit function gives: $y = -0.067x\ (\pm 0.021) + 0.0989\ (\pm 0.050)$ ($r = -0.80$ and $r^2 = 0.63$). The slope obtained with the mixture of the two proteins is intermediate between the ones obtained for isolated mucin or α-amylase. It indicates that the retention of aroma compounds by artificial saliva containing α-amylase and mucin is not the sum of the effects of isolated proteins. Fig. 3B presents the evolution of the relative partition coefficient

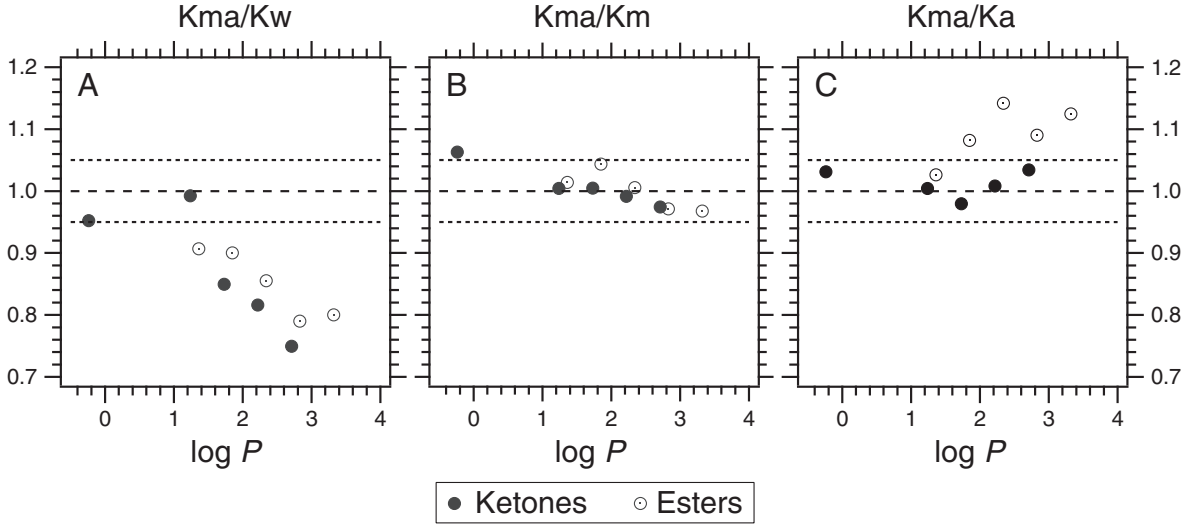

Fig. 3. Relationship between hydrophobicity (log *P* value) of aroma compounds and their relative air/liquid partition coefficient (K) between mucin and α-amylase and (A) water (Kma/Kw), or (B) mucin (Kma/Km) or (C) α-amylase (Kma/Ka). Dotted horizontal lines represent the ±5% interval around the ratio equal to 1.

between the medium containing both mucin and α-amylase and mucin (Kma/Km) as a function of molecule hydrophobicity (log *P* value), which brings information on the effect of the addition of α-amylase to a mucin solution on the release of aroma compounds. Almost all ratio values are comprised between 0.95 and 1.05 indicating that the addition of α-amylase to mucin does not change the retention of aroma compounds. This observation is in agreement with the result obtained by van Ruth et al. (1995). Fig. 3C shows the evolution of the relative partition coefficient between the medium containing both mucin and α-amylase and α-amylase (Kma/Ka) as a function of molecule hydrophobicity (log *P* value) and highlights the effect of the addition of mucin to artificial saliva containing only α-amylase. It appears that the addition of mucin does not affect the retention of ketones but reduces the retention of esters. Mucin has the ability to interact with other proteins and to form supramolecular edifices (Soares et al., 2004). Interestingly, interactions between mucin and α-amylase have been previously reported in saliva (Iontcheva, Oppenheim, & Troxler, 1997). Therefore, protein–protein interactions could be at the origin of our results, as they can modify the protein properties and the ability of each individual protein to interact with other molecules such as aroma compounds. The number of available binding sites of aroma compounds can decrease and consequently affect the retention of aroma compounds by proteins. In the same time, in the presence of mucin in artificial saliva containing α-amylase the retention of esters is decreased while no effect is observed for ketones.

3.3. Effect of human saliva on aroma compound partitioning

In order to compare the effects of artificial salivas and human saliva on aroma retention, the partition coefficient ratios between human saliva and water (Khs/Kw) were also determined. They are reported as a function of the molecule hydrophobicity (log *P* value) in Fig. 4. Ketones and esters seem to be differently affected by human saliva.

For ketones, the effect of human saliva on the air/liquid partition coefficient is only significant (Fig. 1) for 2-octanone and 2-nonanone, which have log *P* values higher than 2. The comparison between the 2 molecules shows that 2-nonanone, which has the highest hydrophobicity, is more retained by human saliva than 2-octanone. The effect of human saliva on ketone release is probably due to retention by salivary proteins. The Khs values of all ketones are higher than their Ka, Km or Kma. Therefore, ketones are less retained by human saliva than artificial saliva. This difference could be due to different amounts of proteins between artificial and human salivas. However, the concentrations in mucin in the artificial salivary media correspond to those typically observed in human saliva. The activity of α-amylase was 180 ± 3 UI·mL^{-1} in human saliva and 144 ± 2 UI·mL^{-1} in artificial saliva. Therefore, the amount of α-amylase is probably higher in human saliva and could not explain the lower retention of ketones in human saliva than that in artificial salivas. As hypothesize in the case of the artificial saliva containing a mixture of mucin and α-amylase, this observation could be explained by protein–protein interactions. Such interactions could decrease the amount of available binding sites for aroma compounds onto proteins. In agreement with this hypothesis, the presence of heterogeneous protein supramolecular edifices in saliva has been previously described (Soares et al., 2004).

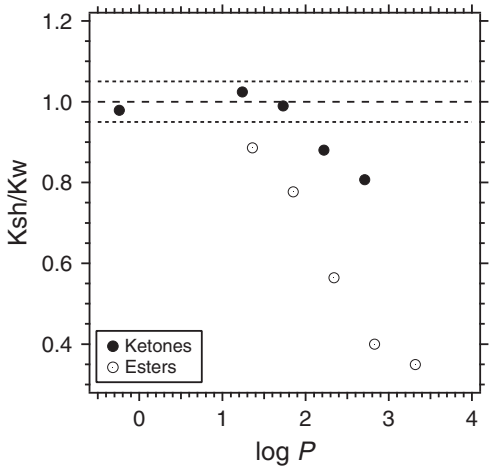

Fig. 4. Relationship between hydrophobicity (log *P* value) of aroma compounds and their relative air/liquid partition coefficient (K) between human saliva and water (Khs/Kw). Dotted horizontal lines represent the ±5% interval around the ratio equal to 1.

For all the studied esters, their air/liquid partition coefficient in the presence of human saliva (Khs) is lower than the one obtained in the presence of water (Kw) (Fig. 1). Fig. 4 shows a decrease of the Ksh/Kw ratio of ester as a function of their log *P* value. A linear relation was found between the Khs/Kw ratio and log *P* values (Fig. 4): $y = -0.296x\ (\pm 0.031) + 1.28\ (\pm 0.075)$ ($r = -0.98$ and $r^2 = 0.97$). This negative correlation indicates that an increase in molecule hydrophobicity induces a decrease in the amount of aroma compounds released in the air phase in the presence of human saliva.

The comparison between esters and ketones indicates that esters exhibit a lower Ksh/Kw value than ketones for a similar log *P* value. The difference of behaviour between esters and ketones in the presence of human saliva could be due to the strongest interactions between esters and salivary proteins, involving other types of non-covalent interaction in addition to hydrophobic effects, or to ester degradation by human saliva. Indeed, Buettner has previously reported an esterase activity of saliva (Buettner, 2002), corresponding to the hydrolysis of ester into the corresponding acid and alcohol. To confirm this hypothesis, we have recorded the concentration of esters in the air phase as a function of time (Fig. 5A). This figure reveals that the ester concentration increases from 5 to 40 min, which is the required time to get the thermodynamic equilibrium. From 40 min, except for ethyl propanoate, the ester concentration decreases as a function of time. It confirms that in the presence of human saliva there is a degradation of esters as a function of time. The composition of the liquid phase was followed through the GC–MS analysis of a dichloromethane extract. This analysis reveals a decrease in ester concentration and the apparition of the corresponding acid (data not shown). The decrease in ester concentrations in the headspace as a function of time and the identification of the corresponding acid in the aqueous phase confirm the hypothesis of an esterase activity in human saliva.

Taking ethyl heptanoate as an example we then calculated the amount of acid produced by the degradation of the ester after 180 min. The initial amount of ethyl heptanoate in the 5 mL human saliva sample was of $5.5 \cdot 10^{-7}$ mol. The production of heptanoic acid was calculated from the calibration curve. A total of $1.85 \cdot 10^{-7}$ mol of heptanoic acid was produced in the 5 mL sample after 180 min, which corresponded to the loss of $3.11 \cdot 10^{-7}$ mol of ethyl heptanoate at the same time, calculated from the GC data. We checked that no acid was produced from the ester solution without the addition of human saliva and that the incubation of human saliva without ethyl heptanoate did not produce the acid. This result confirms the production of heptanoic acid by enzymatic hydrolysis of ethyl heptanoate in the presence of human saliva.

From Fig. 5A, the rate of ester degradation was determined for each ester by determining the slope value of the concentration curve between 40 and 200 min. The rates of ester degradation are reported in Fig. 5B. as a function of the ester log *P* value. It shows a linear correlation between the rate of ester degradation and their log *P* value. The maximal rate of degradation is reached at a log *P* value of three. It should be noticed that the log *P* value is linearly correlated with the carbon number of the ester aliphatic chain. Therefore, the enzymatic activity is also correlated to the length of the aliphatic chain. This observation is in agreement with the study performed by Buettner on the influence of saliva enzymes on esters (Buettner, 2002). As the Khs/Kw ratio of ethyl heptonate is lower than that of ethyl hexanoate, we cannot

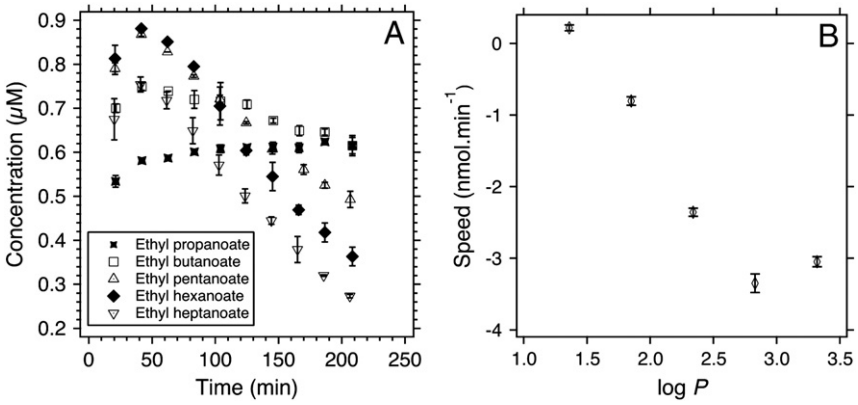

Fig. 5. (A) Concentration of ester in air above the human saliva as a function of time. (B) Relationship between the rate of ester degradation in human saliva and hydrophobicity of aroma compounds (log *P* value).

exclude an additional effect of aroma compound retention by other proteins involving hydrophobic effects. Even if the hydrolysis of esters in our conditions is relatively slow in comparison with real times of consumption, which can vary from a few seconds to a few minutes, this decrease in the amount of esters can impact aroma persistence. During cheese consumption, the analysis of the expired air has revealed a lower persistence of ethyl propanoate than 2-nonanone (Repoux, Labouré, et al., 2012; Repoux, Semon, Feron, Guichard and Laboure, 2012). The persistence of 2-nonanone in the expired air could be due to its retention by the oral, pharyngeal and/or nasal lubricated mucosa, as observed previously (Doyennette et al., 2014) and suggested by Normand, Avison, and Parker (2004) and Buettner and Beauchamp (2010). Until now no experimental results have been obtained on the in vivo persistence of esters. We could suppose that aroma persistence of esters could be also modified due to their degradation by salivary esterases present in the salivary film.

4. Conclusions

This investigation highlights the effect of salivary proteins on aroma release and more particularly on ketone and ester release. For the first time, it is clearly demonstrated that both mucin and α-amylase have the ability to retain aroma molecules. In both cases, hydrophobic effects are involved in these aroma–protein interactions. Regarding the effect of α-amylase on aroma release, the inter-individual variation of α-amylase concentration in saliva (Perry et al., 2007) could be at the origin of variations in aroma release between individuals (Piombino et al., 2014; Repoux, Labouré, et al., 2012; Repoux, Semon, Feron, Guichard and Laboure, 2012). As hydrophobic effects seem mainly at the origin of these protein effects, the differences in the perception of aroma during food consumption by different subjects are more likely to occur for the most hydrophobic molecules. However, the interactions that can occur between mucin and α-amylase could decrease the total number of available binding sites for aroma compounds on proteins. This study shows also that human saliva has the ability to influence the release of volatile molecules and this effect cannot be totally resumed by using artificial saliva containing only mucin and α-amylase. It appears important in the future to have a better understanding of both the protein binding sites involved in the retention of aroma compounds and the protein–protein interactions occurring in saliva. Indeed, the difference observed between artificial and human salivas could be explained not only by the interactions with both mucin and α-amylase, but also by the presence of other salivary proteins, which could impact the release of specific aroma compounds.

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References

Bansil, R., & Turner, B.S. (2006). Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid & Interface Science*, *11*(2–3), 164–170.

Bardow, A., Moe, D., Nyvad, B., & Nauntofte, B. (2000). The buffer capacity and buffer systems of human whole saliva measured without loss of CO_2. *Archives of Oral Biology*, *45*, 1–12.

Benjamin, O., Silcock, P., Beauchamp, J., Buettner, A., & Everett, D. W. (2012). Tongue pressure and oral conditions affect volatile release from liquid systems in a model mouth. *Journal of Agricultural and Food Chemistry*, *60*(39), 9918–9927.

Bennick, A. (1982). Salivary proline-rich proteins. *Molecular and Cellular Biochemistry*, *45*, 83–99.

Buettner, A. (2002). Influence of human salivary enzymes on odorant concentration changes occurring in vivo. 1. Esters and thiols. *Journal of Agricultural and Food Chemistry*, *50*(11), 3283–3289.

Buettner, A., & Beauchamp, J. (2010). Chemical input–sensory output: Diverse modes of physiology–flavour interaction. *Food Quality and Preference*, *21*(8), 915–924.

Buettner, A., Beer, A., Hannig, C., & Settles, M. (2001). Observation of the swallowing process by application of videofluoroscopy and real-time magnetic resonance imaging-consequences for retronasal aroma stimulation. *Chemical Senses*, *26*(9), 1211–1219.

Burdach, K. J., Kroeze, J. H. A., & Koster, E. P. (1984). Nasal, retronasal, and gustatory perception — An experimental comparison. *Perception & Psychophysics*, *36*(3), 205–208.

Buttery, R. G., Ling, L. C., & Guadagni, D.G. (1969). Volatilities of aldehydes, ketones, and esters in dilute water solution. *Journal of Agricultural and Food Chemistry*, *17*(2), 385–389.

Canon, F., Ballivian, R., Chirot, F., Antoine, R., Sarni-Manchado, P., Lemoine, J. r. m, & Dugourd, P. (2011). Folding of a salivary intrinsically disordered protein upon binding to tannins. *Journal of the American Chemical Society*, *133*(20), 7847–7852.

Canon, F., Milosavljević, A.R., van der Rest, G., Réfrégiers, M., Nahon, L., Sarni-Manchado, P. , Cheynier, V., & Giuliani, A. (2013). Photodissociation and dissociative photoionization mass spectrometry of proteins and noncovalent protein–ligand complexes. *Angewandte Chemie International Edition in English*, *52*(32), 8377–8381.

Canon, F., Paté, F., Cheynier, V., Sarni-Manchado, P., Giuliani, A., Pérez, J., Durand, D., Li, J., & Cabane, B. (2013). Aggregation of the salivary proline-rich protein IB5 in the presence of the tannin EgCG. *Langmuir*, *29*(6), 1926–1937.

Chana, A., Tromelin, A., Andriot, I., & Guichard, E. (2006). Flavor release from ι-carrageenan matrix: A quantitative structure—property relationships approach. *Journal of Agricultural and Food Chemistry*, *54*(10), 3679–3685.

Damodaran, S., & Kinsella, J. E. (1980). Flavor protein interactions. Binding of carbonyls to bovine serum albumin: Thermodynamic and conformational effects. *Journal of Agricultural and Food Chemistry*, *28*(3), 567–571.

Damodaran, S., & Kinsella, J. E. (1981). Interaction of carbonyls with soy protein: Thermodynamic effects. *Journal of Agricultural and Food Chemistry*, *29*(6), 1249–1253.

Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G. S., Boontheung, P., Cociorva, D., Delahunty, C. M., Denny, T., Dunsmore, J., Faull, K. F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S.C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J. A., Loo, R. R. O., Malamud, D., Melvin, J. E.,

Miroshnychenko, O., Navazesh, M., Niles, R., Park, S. K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M.A., Takashima, J., Than, S., Wang, J., Whitelegge, J. P., Witkowska, H. E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D. T., Yates, J. R., III, & Fisher, S. J. (2008). The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *Journal of Proteome Research*, *7*(5), 1994–2006.

Doyennette, M., Deleris, I., Feron, G., Guichard, E., Souchon, I., & Trelea, I. C. (2014). Main individual and product characteristics influencing in-mouth flavour release during eating masticated food products with different textures: Mechanistic modelling and experimental validation. *Journal of Theoretical Biology*, *340*, 209–221.

Dsamou, M., Palicki, O., Septier, C., Chabanet, C., Lucchi, G., Ducoroy, P., Chagnon, M. -C., & Morzel, M. (2012). Salivary protein profiles and sensitivity to the bitter taste of caffeine. *Chemical Senses*, *37*(1), 87–95.

Engelen, L., de Wijk, R. A., Prinz, J. F., Janssen, A.M., Weenen, H., & Bosman, F. (2003). The effect of oral and product temperature on the perception of flavor and texture attributes of semi-solids. *Appetite*, *41*(3), 273–281.

Ferguson, D. B. (1999). The flow rate and composition of human labial gland saliva. *Archives of Oral Biology*, *44*(Suppl. 1), S11–S14.

Friel, E. N., & Taylor, A. J. (2001). Effect of salivary components on volatile partitioning from solutions. *Journal of Agricultural and Food Chemistry*, *49*(8), 3898–3905.

Gaillard, I., Rouquier, S., & Giorgi, D. (2004). Olfactory receptors. *Cellular and Molecular Life Sciences*, *61*(4), 456–469.

Genovese, A., Piombino, P., Gambuti, A., & Moio, L. (2009). Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. *Food Chemistry*, *114*(1), 100–107.

Giuliani, A., Milosavljevic, A.R., Hinsen, K., Canon, F., Nicolas, C., Refregiers, M., & Nahon, L. (2012). Structure and charge-state dependence of the gas-phase ionization energy of proteins. *Angewandte Chemie International Edition*, *51*(38), 9552–9556.

Guichard, E. (2006). Flavour retention and release from protein solutions. *Biotechnology Advances*, *24*(2), 226–229.

Humphrey, S. P., & Williamson, R. T. (2001). A review of saliva: Normal composition, flow, and function. *The Journal of Prosthetic Dentistry*, *85*(2), 162–169.

Iontcheva, I., Oppenheim, F. G., & Troxler, R. F. (1997). Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins supramolecular edificies. *Journal of Dental Research*, *76*(3), 734–743.

Kandra, L., & Gyémánt, G. (2000). Examination of the active sites of human salivary α-amylase (HSA). *Carbohydrate Research*, *329*(3), 579–585.

Kuo, Y. -L., Pangborn, R. M., & Noble, A.C. (1993). Temporal patterns of nasal, oral, and retronasal perception of citral and vanillin and interaction of these odourants with selected tastants. *International Journal of Food Science & Technology*, *28*(2), 127–137.

Linforth, R., Martin, F., Carey, M., Davidson, J., & Taylor, A. J. (2002). Retronasal transport of aroma compounds. *Journal of Agricultural and Food Chemistry*, *50*(5), 1111–1117.

Lorrain, B., Tempere, S., Iturmendi, N., Moine, V., de Revel, G., & Teissedre, P. -L. (2013). Influence of phenolic compounds on the sensorial perception and volatility of red wine esters in model solution: An insight at the molecular level. *Food Chemistry*, *140*(1–2), 76–82.

Merabtine, Y., Lubbers, S., Andriot, I., Tromelin, A., & Guichard, E. (2010). Retention/release equilibrium of aroma compounds in fat-free dairy gels. *Journal of the Science of Food and Agriculture*, *90*(9), 1403–1409.

Mishellany-Dutour, A., Woda, A., Laboure, H., Bourdiol, P., Lachaze, P., Guichard, E., & Feron, G. (2012). Retro-nasal aroma release is correlated with variations in the in-mouth air cavity volume after empty deglutition. *PLoS ONE*, *7*(7), e41276.

Mitropoulou, A., Hatzidimitriou, E., & Paraskevopoulou, A. (2011). Aroma release of a model wine solution as influenced by the presence of non-volatile components. Effect of commercial tannin extracts, polysaccharides and artificial saliva. *Food Research International*, *44*(5), 1561–1570.

Morzel, M., Jeannin, A., Lucchi, G., Truntzer, C., Pecqueur, D., Nicklaus, S., Chambon, C., & Ducoroy, P. (2012). Human infant saliva peptidome is modified with age and diet transition. *Journal of Proteomics*, *75*(12), 3665–3673.

Mounayar, R., Morzel, M., Brignot, H., Tremblay-Franco, M., Canlet, C., Lucchi, G., Ducoroy, P., Feron, G., & Neyraud, E. (2013). Salivary markers of taste sensitivity to oleic acid: A combined proteomics and metabolomics approach. *Metabolomics*, *10*(4), 688–696.

Neyraud, E., Heinzerling, C. I., Bult, J. H. F., Mesmin, C., & Dransfield, E. (2009). Effects of different tastants on parotid saliva flow and composition. *Chemosensory Perception*, *2*(2), 108–116.

Neyraud, E., Palicki, O., Schwartz, C., Nicklaus, S., & Feron, G. (2012). Variability of human saliva composition: Possible relationships with fat perception and liking. *Archives of Oral Biology*, *57*(5), 555–566.

Normand, V., Avison, S., & Parker, A. (2004). Modeling the kinetics of flavour release during drinking. *Chemical Senses*, *29*(3), 235–245.

Perry, G. H., Dominy, N. J., Claw, K. G., Lee, A. S., Fiegler, H., Redon, R., Werner, J., Villanea, F. A., Mountain, J. L., Misra, R., Carter, N.P., Lee, C., & Stone, A.C. (2007). Diet and the evolution of human amylase gene copy number variation. *Nature Genetics*, *39*(10), 1256–1260.

Piombino, P., Genovese, A., Esposito, S., Moio, L., Cutolo, P. P., Chambery, A., Severino, V., Moneta, E., Smith, D. P., Owens, S. M., Gilbert, J. A., & Ercolini, D. (2014). Saliva from obese individuals suppresses the release of aroma compounds from wine. *PLoS ONE*, *9*(1), e85611.

Poette, J., Mekoué, J., Neyraud, E., Berdeaux, O., Renault, A., Guichard, E., Genot, C., & Feron, G. (2014). Fat sensitivity in humans: Oleic acid detection threshold is linked to saliva composition and oral volume. *Flavour and Fragrance Journal*, *29*(1), 39–49.

Quintana, M., Palicki, O., Lucchi, G., Ducoroy, P., Chambon, C., Salles, C., & Morzel, M. (2009). Inter-individual variability of protein patterns in saliva of healthy adults. *Journal of Proteomics*, *72*(5), 822–830.

Ramasubbu, N., Paloth, V., Luo, Y., Brayer, G. D., & Levine, M. J. (1996). Structure of human salivary [alpha]-amylase at 1.6 A resolution: Implications for its role in the oral cavity. *Acta Crystallographica Section D*, *52*(3), 435–446.

Repoux, M., Labouré, H., Courcoux, P., Andriot, I., Sémon, É., Yven, C., Feron, G., & Guichard, E. (2012). Combined effect of cheese characteristics and food oral processing on in vivo aroma release. *Flavour and Fragrance Journal*, *27*(6), 414–423.

Repoux, M., Semon, E., Feron, G., Guichard, E., & Laboure, H. (2012). Inter-individual variability in aroma release during sweet mint consumption. *Flavour and Fragrance Journal*, *27*(1), 40–46.

Rodriguez-Bencomo, J. J., Munoz-Gonzalez, C., Andujar-Ortiz, I., Martin-Alvarez, P. J., Moreno-Arribas, M. V., & Pozo-Bayon, M.A. (2011). Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis. *Journal of the Science of Food and Agriculture*, *91*(13), 2484–2494.

Schenkels, L. C., Veerman, E. C., & Nieuw Amerongen, A. V. (1995). Biochemical composition of human saliva in relation to other mucosal fluids. *Critical Reviews in Oral Biology and Medicine*, *6*(2), 161–175.

Soares, R. V., Lin, T., Siqueira, C. C., Bruno, L. S., Li, X., Oppenheim, F. G., Offner, G., & Troxler, R. F. (2004). Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycosylated proline-rich protein and lysozyme. *Archives of Oral Biology*, *49*(5), 337–343.

Tavel, L., Andriot, I., Moreau, C. l, & Guichard, E. (2008). Interactions between β-lactoglobulin and aroma compounds: Different binding behaviors as a function of ligand structure. *Journal of Agricultural and Food Chemistry*, *56*(21), 10208–10217.

Tromelin, A., Merabtine, Y., & Andriot, I. (2010). Retention–release equilibrium of aroma compounds in polysaccharide gels: Study by quantitative structure–activity/property relationships approach. *Flavour and Fragrance Journal*, *25*(6), 431–442.

van Ruth, S. M., Grossmann, I., Geary, M., & Delahunty, C. M. (2001). Interactions between artificial saliva and 20 aroma compounds in water and oil model systems. *Journal of Agricultural and Food Chemistry*, *49*(5), 2409–2413.

van Ruth, S. M., & Roozen, J. P. (2000). Influence of mastication and saliva on aroma release in a model mouth system. *Food Chemistry*, *71*(3), 339–345.

van Ruth, S. M., Roozen, J. P., & Cozijnsen, J. L. (1995). Changes in flavour release from rehydrated diced bell peppers (*Capsicum annuum*) by artificial saliva components in three mouth model systems. *Journal of the Science of Food and Agriculture*, *67*(2), 189–196.

Villamor, R. R., Evans, M.A., Mattinson, D. S., & Ross, C. F. (2013). Effects of ethanol, tannin and fructose on the headspace concentration and potential sensory significance of odorants in a model wine. *Food Research International*, *50*(1), 38–45.

Voirol, E., & Daget, N. (1986). Comparative study of nasal and retronasal olfactory perception. *Food Science and Technology - Lebensmittel - Wissenschaft & Technologie*, *19*, 316–319.