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Understanding the release and metabolism of aroma compounds using micro-volume saliva samples by ex vivo approaches

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ABSTRACT

This study investigated the behaviour of key aroma compounds in the presence of human saliva (200 lL) from different individuals (n = 3) submitted or not to centrifugation (whole vs clarified saliva). HS-GC results showed that human saliva strongly decreased the release of carbonyl compounds (aldehydes and ketones). This effect was dependent on i) the structure of the aroma compounds and ii) the saliva composition. Whole saliva exerted a higher effect than clarified saliva on aroma compounds. Moreover, this effect was individual-dependent and related to the total protein content and the total antioxidant capacity of saliva. HS-SPME and LLE-GC/MS analyses revealed that metabolism of the compounds by salivary enzymes was involved. This observation indicates that some aroma compounds could be metabolized in the oral cavity in an individual manner, which could have implications for aroma perception (e.g., formation of new metabolites with different odor thresholds and qualities) and/or organisms' health status (e.g., compound detoxification).

Keywords: Aroma compounds, Carbonyl compounds, Enzymatic conversion, Saliva composition, Salivary proteins, Total antioxidant capacity

1. *Introduction*

Since aroma perception is one of the most important aspects driving food consumption and it can be modulated during the oral processing of the food, the impact of oral parameters such as saliva on aroma compounds has received great attention in recent years (Ployon, Morzel, & Canon, 2017). In this regard, it has been recently shown that saliva composition is related to in vivo aroma release (Feron et al., 2014) and aroma perception (Guichard, Repoux, Qannari, Laboure, & Feron, 2017) during the consumption of model cheeses. However, in vivo experiments are subjected to the influence of other physiological factors that could affect the transfer of the aroma compounds to the olfactory receptors. Therefore, the effect of saliva in such an approach is difficult to unravel. Consequently, most of the studies performed to elucidate the effects of saliva on aroma compounds have been performed under wellcontrolled in vitro or ex vivo conditions. These studies have highlighted effects of different nature of saliva on aroma compounds. The retention of aroma compounds by salivary proteins in the presence of artificial (Friel & Taylor, 2001; Pages-Helary, Andriot, Guichard, & Canon, 2014; van Ruth, Grossmann, Geary, & Delahunty, 2001) or human salivas (Genovese, Piombino, Gambuti, & Moio, 2009; Munoz-Gonzalez, Feron et al., 2014; Pages-Helary et al., 2014) is well-documented. Moreover, other mechanism such as the metabolism of aroma compounds
(Buettner 2002a, 2002b; Lasekan, 2013) by salivary enzymes, has also been strongly suggested. Interestingly, it has been described that the same compound can be submitted to both effects; e.g., ethyl hexanoate can be retained by mucin solutions (Friel & Taylor, 2001) whilst it is also susceptible to metabolism by salivary enzymes (Buettner, 2002b; Pages-Helary et al., 2014). In addition, an increase in the release of some aroma compounds in the presence of specific salivary constituents (called the salting-out effect) has also been observed (Friel & Taylor, 2001).

However, most of the above-mentioned studies have been carried out with artificial salivas (Friel & Taylor, 2001; Pages-Helary et al., 2014; van Ruth et al., 2001) or pooled salivas submitted or not to a clarification process (Genovese et al., 2009; MunozGonzalez, Feron et al., 2014; Pages-Helary et al., 2014), which could have not completely represented the complexity of human saliva composition as is found in the human mouth (whole saliva). Indeed, human saliva is composed of a wide number of different components, such as electrolytes, proteins and microorganisms, whose profile and proportion is highly individual-dependent (Leake, Pagni, Falquet, Taroni, & Greub, 2016; Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012).

In spite of this well-known inter-individual variability on saliva composition, the possible effects of this variability on aroma compounds (release, metabolism) by ex-vivo approaches have received very little attention and very few studies have tackled this question
(Buettner, 2002a, 2002b; Piombino et al., 2014). This could be related to analytical constraints, such as the collection of suitable volumes of saliva to carry out these studies. Indeed, works on this topic have employed different techniques that require relative high volumes of saliva (6–10 mL/assay) that would be difficult to obtain from one individual. Among them, static headspace methodology has been the preferred approach. This approach is based on the analysis of the headspace (HS) above a solution after an equilibrium time. The headspace can be taken with a syringe (HS) or concentrated in a polymer-coated fiber (headspace solid-phase microextraction; HS-SPME) before being analysed by gas chromatography (GC), gas chromatography-mass spectrometry (GC–MS) or other on-line techniques like proton transfer reaction-mass spectrometry (PTR-MS). Moreover, liquid-liquid extractions (LLE) coupled to GC–MS have also been employed to study the transformations of aroma compounds in the presence of saliva (Buettner, 2002a, 2002b). In fact, due to the complexity of human saliva composition and the wide type of biochemical reactions (e.g., non-covalent interactions, enzymatic conversion) that could occur between salivary and food components, the use of complementary methodologies seems mandatory to elucidate the effects of human saliva on aroma compounds.

With this background, the aim of the present study was to investigate the effects of human saliva composition on aroma compounds. In order to reach this goal, an HS-GC method adapted to low volumes of saliva (200 lL) was firstly developed, validated and then independently applied to 17 key aroma compounds in the presence of saliva from three individuals and submitted to different treatments (whole vs clarified). The selected aroma compounds belonged to three different

chemical families (ketones, aldehydes and alcohols), possessed different structures (carbonyl position, presence of double bonds, linear vs branched) and a range of physicochemical properties (hydrophobicity, volatility). Control samples with water were employed to evaluate the extent of the saliva effect. The relationship between some saliva compositional parameters and HS-GC data was examined. Finally, HS-SPME-GC/ MS and LLE-GC/MS analyses allowed us to investigate the nature of the observed effects.

2. ***Material and methods***

2.1. Aroma compounds

Seventeen compounds were chosen on the basis of their different physicochemical characteristics (chemical family, functional group, carbonyl count, structure, chemical properties), their aroma impact (key food and beverage aroma compounds) and because of their suitability for the analysis technique (sensitivity, solubility). The list of compounds included three main chemical families (ketones, aldehydes and alcohols) and two functional groups (carbonyl vs alcohol) (Table 1). The aroma compounds were of analytical grade (Aldrich, Steinheim, Germany; Fluka, Buchs, Switzerland; Firmenich, Geneva, Switzerland). A gas chromatography-flame ionization detector (GC-FID) analysis confirmed the purity of all aroma compounds (>95%) that was taken into account for calculations. Stock solutions (1%) of the single aroma compounds were prepared in propylene glycol at room temperature under magnetic stirring for 2 h. They were stored at 4 C for a maximum of three months.

2.2. Saliva samples

2.2.1. Saliva collection

Unstimulated saliva samples were freshly collected from three healthy subjects (two men, one woman), aged between 30 and 52 years old. All subjects were non-smokers and had not taken any antibiotics or other medical treatments during the three months previous to sampling. Participants were asked not to consume any food or drink two hours before saliva was collected. They let the saliva naturally be accumulated in the mouth and then spat it directly into a collection tube. Different saliva collections for each individual were organized over several days and pooled together in order to: i) avoid interday variability in saliva composition among individuals (Buettner, 2002a, 2002b; Neyraud et al., 2012), and ii) get a suitable volume of saliva from each individual to perform the whole investigations while avoiding their fatigue.

From the pooled saliva from each individual, half of the crude saliva (whole saliva) was separated and the other half centrifuged at 15,000g for 15 min at 4 C (clarified saliva). Clarification of human saliva is a treatment frequently employed in the literature to remove excessive mucus, cells and facilitate biochemical analysis. Therefore, two saliva types (whole and clarified saliva) from each of the three individuals were employed for this study. Saliva samples were aliquoted and stored at 80 C until use. Previously, it was verified that the storage of saliva under these conditions did not modify its effect on aroma compounds. To do that, the release of aroma compounds in presence of fresh saliva or saliva submitted to storage (frozen at 80 C and thawed) was studied and no significant differences were observed between samples.

The experimental protocol was approved by the French Ethics Committee for Research (CPP Est I, Dijon, #14.06.03, ANSM #2014-A00071-46).

2.2.2. Saliva biochemical analyses

2.2.2.1. Protein concentration. The protein concentration was determined using the Bradford protein assay with bovine serum albumin (BSA) used as the standard for calibration.

2.2.2.2. Total antioxidant capacity (TAC). The total antioxidant capacity was determined using an ORAC Assay kit (Zen-bio, Research Triangle Park, NC). This assay measures the loss of fluorescein fluorescence over time due to peroxyl radical formation resulting from the breakdown of AAPH (2,2₀-azobis-(2-amidinopro pane) dihydrochloride). Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), a water-soluble vitamin E analog, serves as a positive control to inhibit fluorescein decay in a dosedependent manner. The intensity of fluorescence was measured (excitation filter, 485 nm; emission filter, 538 nm) using a microtiter plate fluorometer (Victor 3-V, PerkinElmer, France). The total antioxidant capacity was expressed in micromolar Trolox equivalents.

2.2.2.3. pH. pH was determined using a pH meter (Mettler Toledo, Schwerzenbach, Switzerland).

2.3. HS-GC analyses

To follow the release of aroma compounds in micro-volume samples, aqueous aroma solutions were freshly prepared by dilution of the stock solutions with water to obtain single solutions of each odorant at 10 mg/L. This concentration is far below the solubility threshold of the assayed compounds in water (Table 1). A 300-ĺL aliquot of this aroma solution was added to 200 ĺL of water or saliva. Vials were immediately closed with a PTFE/silicone septum (Supelco, Bellefonte, PA), stirred and incubated at 37 C. The time needed to reach equilibrium was determined for all the compounds in the control samples with water at 5, 15, 30, and 45 min. From the analysis of the kinetic profiles it was found that 30 min of incubation was enough for the equilibration of the selected aroma compounds. After the incubation time, two hundred microliters of headspace were taken automatically using a preheated (45 C)

Table 1
List of the 17 aroma compounds employed in this study together with their molecular weight (MW), hydrophobic constant (log P), boiling point (BP), solubility in water, GC oven temperature, aroma descriptor, CAS number and structure.

Compound	MW (g/mol)[a]	log P[b]	BP (C)[c]	Water solubility (mg/L)[d]	GC oven temperature (C)	Descriptor[e]	CAS number	Type[f]
Ketones								
2-butanone	72.11	0.26	70.36	76100	50	Butterscotch	78-93-3	L
2,3-butanedione	86.09	1.34	117.70	1000000	60 60	Buttery	431-03-8	U
2-pentanone	86.13	0.75	95.03	21200		Sweet, fruit	107-87-9	L
2,3-pentanedione	100.12	0.85	140.60	615900	70	Butter, cream	600-14-6 111-	U
2-octanone	128.22	2.22	163.60	884	100	Fruity	13-7	L
1-octen-3-one	126.2	2.37	161.99	895	120	Mushroom, metal	4312-99-6	U
Alcohols								
1-pentanol	88.15	1.33	136.95	20890	90	Fruit, balsamic	71-41-0	L
1-hexanol	102.17	1.82	159.09	6885	100	Fatty, floral, green	111-27-3	L
(Z)-3-hexen-1-ol	100.00	1.61	165.73	16000	110	Fruity, green, leaves	928-97-2	U
1-octanol	130.23	2.81	200.67	814	130	Green herbaceous	111-87-5	L
1-octen-3-ol	128.22	2.60	180.31	1836	100	Mushroom, earthy, fungal	3391-86-4	U
Linalool	154.25	3.38	204.05	684	130	Aniseed, citrus, floral	78-70-6	C
Menthol	156.27	3.38	218.94	434	140	Carraway, sweet	89-78-1	C
Aldehydes								
Hexanal	100.16	1.80	132.2	3527	70	Grass, fat	66-25-1	L
Octanal	128.22	2.78	132.20	394	100	Fat, lemon, green	124-13-0	L
Octenal	126.20	2.35	117.37	945	115	Green	25447-69-2	U
Benzaldehyde	106.13	1.71/1.48	181.22	6100	130	Almond, burnt sugar	100-52-7	C

[a] Molecular weight.
[b] Hydrophobic constant estimated using molecular modeling software EPI Suite (U.S. EPA 2000–2007).
[c] Boiling point estimated using molecular modeling software EPI Suite (U.S. EPA 2000–2007).
[d] Water solubility estimated at 25 C using molecular modeling software EPI Suite (U.S. EPA 2000–2007).
[e] From Flavornet database (http://www.flavornet.org; accessed October 2009), from NIST web chemistry book (2005) (http://www.webbook.nis.gov/chemistry).
[f] Chemical structure (L: linear, U: unsaturated; C: cyclic).

1-mL gas-tight syringe (Gerstel, manufactured by SGE, Victoria, Australia) and analysed by gas chromatography (Agilent 7890B). The injector temperature was set at 240 C and the flame ionization detector at 250 C. A DW-Wax capillary column (30 m 0.32 mm i.d. 0.5 lm; Agilent J&W Scientific, Folsom, CA) was used. The carrier gas was helium at a velocity of 30 cm^3/s in splitless mode. For each aroma compound, an oven isothermal temperature was preliminary determined in order to obtain a retention time between 2 and 5 min (Table 1). In order to avoid any aroma competition for protein binding or catalytic sites in the saliva samples, aroma compounds were analysed one by one. Each sample was analysed in triplicate (one injection per sample vial). A total of 408 injections was carried out.

To validate the methodology, linearity and repeatability of the procedure were determined in an aqueous solution composed of a mixture of 17 aroma compounds at four levels of concentration (1, 5, 10, 25 mg/L). Each sample was analysed in triplicate (one injection per sample vial).

2.4. HS-SPME-GC/MS analyses

HS-SPME-GC/MS analyses were performed to identify the possible formation of new volatile metabolites in the presence of saliva. This technique was chosen since it is a very sensitive technique frequently employed in the literature because of its powerful concentration of the headspace, and therefore suitable to follow metabolites at low concentrations (can reach parts per trillion (ppt) levels for certain compounds). A mixture composed of pooled whole saliva from the three individuals was incubated in the presence of single aroma solutions and the headspace above the samples extracted by HS-SPME-GC/MS. Moreover, control samples with water (water + aroma solution) and blanks of saliva were analysed in parallel and under exactly the same analytical conditions to discard the presence of possible artefacts. The extraction was automatically performed by using a CombiPal system (CTC Analytics, Zwingen, Switzerland) provided with a 50/30 lm DVB/ CAR/PDMS fiber of 2 cm length (Supelco). After the incubation time (25 min), the extraction was performed in the headspace of the vials for 5 min at 37 C. Desorption was performed in the injector of a gas chromatograph (Agilent 6890 N; Agilent, Santa Clara, CA) in splitless mode for 1.5 min at 240 C. After each injection the fiber was cleaned for 30 min at 240 C to avoid any memory effects. The oven temperature was programmed to increase from 40 to 240 C at 4 C/min and held for 1 min. After desorption of the SPME fiber, volatile compounds were separated on a DB-Wax polar capillary column (30 m 0.32 mm i.d. 0.50 lm film thickness) from Agilent (J&W Scientific, Folsom, CA, USA). Helium was the carrier gas at a flow rate of 1 mL/min. The coefficient of variation (CV) of the technique was calculated after analyzing six times an aroma mixture containing the 17 selected compounds under the conditions reported above. The mean CV was 5.7% (with values ranging from 1.6% for (Z)-3-hexen-1-ol to 13.1% for 1-pentanol).

For the MS system (Agilent 5973N), the temperatures of the transfer line, quadrupole, and ion source were 250, 150, and 230 C, respectively. Electron impact mass spectra were recorded at 70 eV ionization voltage and the ionization current was 10 lA. The acquisitions were performed in scan mode (from m/z 35 to 350). The identification of compounds was based on the comparison of retention times and mass spectra from two databases: NIST 2.0 and WILEY 138.

2.5. LLE-GC/MS analyses

In an attempt to confirm the enzymatic metabolism of aroma compounds by saliva, LLE-GC–MS analyses were carried out after submitting the whole saliva samples to different treatments to obtain non enzymatic saliva, sterile saliva and saliva without microorganisms, cells or enzymes. Moreover, the effect of the reducing agent NADH (1 mM; Sigma-Aldrich, Steinheim, Germany) in the presence of whole and non-enzymatic saliva was also evaluated. This technique allows the determination in the liquid phase of a broad range of aroma compounds with very different polarities. Moreover, it does not take into account the possible weak interactions between aroma compounds and salivary components whilst providing a high sensitivity.

After incubation of the samples at 37 C for 30 min, liquid-liquid extractions were carried out. Methodologies based on methods already published by Buettner (2002a), and Esteban-Fernandez and coworkers (Esteban-Fernandez, Rocha-Alcubilla, MunozGonzalez, Moreno-Arribas, & Pozo-Bayon, 2016) were adapted to the present experimental conditions. Briefly, 500 lL of a saturated $CaCl_2$ solution were immediately added to the samples to inhibit possible enzymatic reactions. The solutions were extracted twice with 1 mL of dichloromethane (Carlo Erba, Val de Reuil, France) then centrifuged (5000g, 4 C, 15 min) to separate the two phases. Prior to the extraction, samples were spiked with 100 lL of the internal standard, methyl nonanoate (10 mg/L). The combined organic extracts were dried over anhydrous Na_2SO_4, then concentrated to a total volume of 200 lL. One microlitre was injected into the GC/MS in splitless mode following the same method described above for the HS-SPME/GC–MS analysis. The same LLE procedure was carried out with the salivas and water samples without added aroma compounds, in order to detect any possible compounds coming from the salivas or any artefact formed during the extraction procedure. Relative peak areas (RPAs) were obtained by dividing the area of the peak of interest by the area of the internal standard.

2.6. Statistical analyses

Linear regression analyses were performed on the static HS-GC experiments to establish the linearity of each aroma compound and the lack of fit test was used to judge the adequacy of the linear models. ANOVA analyses were employed to determine significant effects among the studied factors. In addition, for each aroma compound and individual (water, S1, S2 or S3) differences between saliva type (whole vs clarified saliva) were subsequently examined by least significant difference (LSD) test. The significance level was $0.01 < p < 0.1$ throughout the study. The STATISTICA program for Windows version 7.1 and the XLSTAT program were used for data processing (StatSoft, Inc., 2005; www.statsoft.com).

3. ***Results and discussion***

It has been previously demonstrated that human saliva impacts the release of aroma compounds (Genovese et al., 2009; Mitropoulou, Hatzidimitriou, & Paraskevopoulou, 2011; MunozGonzalez, Feron et al., 2014; Pages-Helary et al., 2014; Piombino et al., 2014). However, to study the impact of differences in human saliva composition among individuals on aroma compounds, it is necessary to take into account that the collection of suitable volumes of human saliva for experimental purposes is tedious, unpleasant and sometimes (e.g. hyposalivation) very difficult (Friel & Taylor, 2001). In this work, an HS-GC methodology using small volumes of saliva (200 mL) has been developed, its analytical performance determined and then applied to evaluate the effects of human saliva composition on aroma compounds.

3.1. Analytical performance of the HS-GC method

The repeatability of this methodology was studied by the calculation of the relative standard deviation (RSD) (n = 6) and results are presented in Supplementary material (Table 1). The RSD average of the method was very low (3.8%) which means a good repeatability across analyses. Among the chemical classes, ketones (mean RSD = 3.2%) and alcohols (mean RSD = 3.3%) presented the lowest RSDs while aldehydes (mean RSD = 5.8%) the highest. This could be due to the higher positive charge of the carbonyl carbon of aldehydes that confers a higher reactivity to these molecules.

For each compound, a linear regression was calculated to determine the linearity of the analytical method (Table 1: Supplementary material). A lack of fit test was also applied to determine whether the calculated model was adequate for the experimental data. As can be seen, a clear linear relationship between the amount of aroma compounds in the headspace and the concentration of aroma compounds added to the vials was obtained. The linear models showed determination coefficients higher than 99% for all the assayed compounds and adequate values of residual standard deviation (s) over the concentration range assayed (Table SM 1). The lack of fit test also showed the adequacy of the proposed regression models (p values > 0.01 for all aroma compounds).

3.2. Effects of human saliva on aroma compounds by HS-GC

Once the validity of the method was proven, the methodology was applied to measure the HS of 17 aroma compounds in the presence of salivas from three individuals (n = 3) submitted or not to a clarification process (whole vs clarified). Results are shown in Table 2 as a function of the aroma chemical family, saliva treatment (whole vs clarified) and assayed sample (w, S1, S2, S3). To facilitate data interpretation, headspace data are expressed relative to water, so that values >100% represent an increase of the compound release in the headspace compared to the water samples, while values <100% denote a decrease. As shown in Table 2, both types of behaviour (decrease or increase of HS concentration compared to water) were observed depending on the molecule.

3.3. Differences on aroma release by compound type

Previous studies have reported that aroma compounds can interact with proteins (Tavel, Moreau, Bouhallab, Li-Chan, and Guichard (2010) and salivary proteins through hydrophobic effects (Pages-Helary et al., 2014). Therefore, physicochemical properties, such as hydrophobicity, have been frequently employed to predict the release of aroma compounds in the presence of other compounds. In an attempt to explain the results observed in this study, the hydrophobicity (log P values) of the compounds and their relative headspace concentrations above the saliva samples (mean of whole saliva values) (Supplementary material Fig. 1) were plotted. As can be observed in the figure, and in spite of the selected molecules covering a wide range of log P values (from 1.34 to +3.38) no clear correlation between the log P and the relative headspace values could be observed ($r^2 = 0.017$). In their study, Friel and Taylor (2001) also reported the absence of a correlation between log P and the relative headspace values of aroma compounds in the presence of artificial saliva solutions containing only mucin. Therefore, non-covalent interactions between salivary proteins and aroma compounds involving hydrophobic effects seem not to be the main or unique mechanism explaining the effects of saliva on aroma compounds.

The impact of human saliva on aroma air–solution partition has been found to be highly chemical family-dependent (Table 2). Aldehydes and ketones, chemical families containing a carbonyl group, were the most affected compounds. Among them, aldehydes were more affected than ketones by the presence of different salivas. This higher effect of saliva on partitioning of aldehydes compared to ketones has already been observed by Friel and Taylor (2001), and van Ruth et al. (2001), in the presence of artificial salivas. Alcohols were less affected than the other chemical families by the presence of saliva (Table 2). Within a chemical family, different effects were observed as a function of the compound structure (e.g. linear vs cyclic/ramified, saturated vs unsaturated).

Table 2

Relative headspace aroma values determined above water and saliva samples by HS-GC. Data represent the comparison of the same aroma compound determined in water or saliva samples and are expressed as percentages (considering the value determined in the water sample as 100% and comparing it with the value of the same compound determined in the saliva sample of each individual). The coloured columns represent the mean values of each saliva type (whole vs clarified saliva).

Compounds	whole saliva					clarified saliva				
	w	S1	S2	S3	mean whole saliva	w	S1	S2	S3	mean clarified saliva
Ketones										
2-butanone	100 b	106 a	104 a	105 a	105	100 a	103 a	101 a	99 a	101
2,3-butanedione	100 a	93 b	90 b	52 c	78	100 a	95 bc	98 ab	94 c	96
2-pentanone	100 a	101 a	99 a	99 a	100	100 b*	105 a*	103 ab*	100 a*	103
2,3-pentanedione	100 a	94 b	92 b	72 c	86	100 a	92 b	91 b	92 b	92
2-octanone	100 a	105 a	101 a	102 a	103	100 a	99 a	97 a	100 a	99
1-octen-3-one	100 a	86 b	85 b	40 c	70	100 a	85 b	85 b	49 c	73
Alcohols										
1-pentanol	100 b*	103 ab*	105 ab*	110 a*	106	100 b	106 a	106 a	103 ab	105
1-hexanol	100 a	103 a	101 a	101 a	102	100 a	103 a	103 a	103 a	103
(Z)-3-hexen-1-ol	100 a	103 a	101 a	101 a	102	100 a	105 a	105 a	105 a	105
1-octanol	100 c	104 b	109 a	107 ab	107	100 b*	103 ab*	105 a*	105 a*	104
1-octen3-ol	100 a*	100 a*	100 a*	96 b*	99	100 a	99 a	102 a	98 a	100
linalool	100 a	102 a	99 a	102 a	101	100 a	103 a	105 a	106 a	105
menthol	100 b*	104 ab*	105 a*	99 b*	103	100 a	102 a	99 a	100 a	100
Aldehydes										
Hexanal	100 a	96 a	88 b	39 c	74	100 a	100 a	98 a	90 b	96
Octanal	100 a	89 b	77 c	23 d	63	100 a	100 a	102 a	94 b	99
Octenal	100 a	105 a	100 a	71 b	92	100 a	101 a	99 a	89 b	96
Benzaldehyde	100 ab	96 b	103 a	73 c	91	100 a	99 a	100 a	103 a	101

Different letters across the different assessors denotes statistical differences ($p < 0.05$ or * $0.05 < p < 0.1$) after application of the LSD test.

3.3.1. Ketones

Three classes of ketones were studied: linear saturated (2-butanone, 2-pentanone and 2-octanone), or unsaturated monoketones (1-octen-3-one) and diketones (2,3-butanedione, 2,3-pentanedione). To the authors' knowledge, the only work specifically studying the ex-vivo effect of human saliva on ketones is the one carried out by Pages-Helary et al. (2014). This work focused on the effect of saliva on a series of five linear methylketones (2-propanone, 2-hexanone, 2-heptanone, 2-octanone and 2-nonanone). They showed that the two most hydrophobic ketones assayed in their study (2-octanone and 2-nonanone) presented lower partition coefficients in human saliva than in water solutions. The origin of this observation was attributed to the retention of these compounds by human salivary proteins through noncovalent interactions. In the present study, no significant effects on the assayed linear methyl ketones (2-butanone, 2-pentanone, 2-octanone) have been observed, which could be due to differences in the composition of salivas employed between the two studies. Indeed, in their study Pages-Helary et al. (2014) used pooled human saliva provided by several volunteers. Moreover, they also found different effects of artificial saliva on aroma compounds as a function of its composition. To explain their results, authors proposed that some salivary proteins interact with each other, precluding the access of aroma compounds to the binding sites.

Interestingly, a significant effect of human saliva was found for the diketones (2,3-butanedione and 2,3-pentanedione) and the unsaturated monoketone (1-octen-3-one) assayed in the present study. For example, 2,3-butanedione was reduced on average by 13% in the HS above saliva compared to the control samples. The same trend was observed for 2,3-pentanedione (11% average reduction) and 1-octen-3-one (28% average reduction). The presence of more than one functional group or double bonds in these molecules could have increased their reactivity, making them more susceptible to action by salivary components. To our knowledge this is the first time that an effect of human saliva on these compounds has been described. Only a few works (Friel & Taylor, 2001; van Ruth et al., 2001) have studied the behaviour of one of these molecules (2,3-butanedione) in the presence of artificial saliva, showing contradictory effects. In solutions with mucin, alpha-amylase and salts, van Ruth et al. (2001) did not find any significant effect of saliva on 2,3-butanedione. In contrast, Friel and Taylor (2001) showed that 2,3-butanedione was reduced by up to 50% in the headspace when artificial saliva (mucin and salts) was present. The disparity in these results could be due to the different techniques (HS-GC vs HS-APCI/MS), equilibrium times (6 min vs 5 h), or more probably to the different compositions of the salivas employed in these studies. Indeed, the artificial salivas used in the previous studies contained only mucin with/without alpha-amylase.

3.3.2. Alcohols

In the case of alcohols three different classes were also studied and divided into linear (saturated and unsaturated) and cyclic molecules. However, the effects of human saliva on these compounds were almost negligible and when observed, a salting-out effect was in general the behaviour detected. The limited effect of saliva on alcohols is also in agreement with the results found in different publications (Buettner 2002a; Friel & Taylor, 2001; Genovese, Moio, Sacchi, & Piombino, 2015; Munoz-Gonzalez, Feron et al., 2014; van Ruth et al., 2001) carried out with diverse techniques, matrix (model solutions vs food) and saliva types (human or artificial salivas).

3.3.3. Aldehydes

All the aldehydes assayed in this study were affected by the presence of saliva. The linear saturated molecules (hexanal, octanal) were the most affected molecules, and hydrophobic effects could explain the differences observed between both linear aldehydes: the release of hexanal (log P = 1.80) was reduced to a lesser extent than that of octanal (log P = 2.78) in the presence of saliva. This result was already observed by van Ruth et al. (2001), which stated that a larger molecule size or higher hydrophobicity could result in a higher affinity for salivary proteins. This observation appears to contradict the general trend observed in

this study for which hydrophobicity seems not to be the main or unique determinant explaining HS data. However, it could be possible that all the molecules are not subjected to the same mechanism, and hydrophobicity could be one factor causing the affinity of a serious of analogous compounds for a specific protein via hydrophobic effects as reported previously (Pages-Helary et al., 2014).

The aldehydes less affected were the cyclic benzaldehyde and the unsaturated 2-octenal. In fact, the unsaturated 2-octenal was less affected than its analogue saturated octanal, which was contrary to the effect observed for the ketones. This fact also suggests that different mechanisms could be involved as a function of the structure of the molecule. This could explain the different behaviour observed between aldehydes and ketones in the presence of saliva.

3.4. Differences in aroma release due to saliva type

Table 2 shows the mean headspace data of each aroma compound determined for the whole and clarified saliva samples. Interestingly, the effects of human saliva on the affected compounds (carbonyl compounds) were more marked in the presence of whole than clarified saliva. One compound (benzaldehyde), was affected by whole saliva but not affected by clarified saliva while 1octen-3-one was similarly affected by whole and clarified saliva. During the centrifugation process carried out at 4 C for 15 min at 15,000g, components in suspension and/or with high molecular weight (like aggregates or supramolecular complexes of proteins, mucins, cells, microorganism and food debris or salivary micelles) are separated from saliva solutions. Therefore, some of those components that had been completely or totally removed from clarified saliva could participate in the observed effects. These results pointed out the necessity to apply the minimum treatment on saliva samples, in order to be able to study the effect of human saliva composition on aroma compounds.

In order to explore more deeply our results, relative headspace data obtained for each individual and saliva type were independently submitted to one-way ANOVA and LSD tests (Table 2). As can be seen, large variations in the aroma air–solution distribution between individuals have been found. However, these differences were much less important in clarified than in whole saliva, probably due to a reduction in the amount of proteins after centrifugation as mentioned above. This observation suggests that the centrifugation tended to standardize saliva samples across individuals. Interestingly, the compounds affected by the presence of human saliva, presented an aroma distribution pattern quite similar for the affected compounds: S3 showed the highest reduction of aroma compounds in the headspace, while S1 and S2 behaved mostly in the same way and presented values between water and S3. The existence of in vivo inter-individual differences on aroma release patterns during food consumption has been extensively described, both in solid (Feron et al., 2014; Pionnier, Chabanet, Mioche, Le Quere, & Salles, 2004) and liquid food matrices (Deleris et al., 2011; Frank, Appelqvist, Piyasiri, Wooster, & Delahunty, 2011; Munoz-Gonzalez, Martin-Alvarez, MorenoArribas, & Pozo-Bayon, 2014) and have been attributed to anatomical and physiological differences across individuals. Among the factors that can influence in vivo retronasal aroma, respiratory flows (Munoz-Gonzalez, Feron et al., 2014; Pionnier et al., 2004), masticatory parameters (Feron et al., 2014; Pionnier et al., 2004), saliva flow and composition (Feron et al., 2014), oral and pharyngeal mucosa (Buettner, Beer, Hannig, & Settles, 2001; Deleris, Saint-Eve, Saglio, Souchon, & Trelea, 2016; Esteban-Fernandez et al., 2016), and in-mouth oral cavity volume (MishellanyDutour et al., 2012) have been suggested to explain these effects. Therefore, the present results reinforced the idea that interindividual differences in saliva composition could be one factor affecting retronasal aroma, which would explain differences in aroma perception across individuals, as has been recently shown in model cheeses (Guichard et al., 2017).

3.5. Whole saliva composition and its relationship with aroma release among individuals

In an attempt to understand which parameters from whole saliva were more involved on the differences in the HS data observed among individuals, the biochemical characterization of the whole saliva samples (the one showing the more important effects on aroma compounds) was performed and is shown in Table 3. The biochemical characterization was based on three general measurements: total protein content, pH of the medium and total antioxidant capacity. These three parameters were selected for their potential contribution to the observed HS-GC results.

As stated above, aroma compounds can interact with salivary proteins through non-covalent and covalent interactions. Moreover, a wide number of proteins presents in saliva could show potential enzymatic properties on aroma compounds. To investigate the effect of salivary proteins on aroma release, the total salivary content of whole saliva samples (n = 3) was analysed and is shown in Table 3. As can be seen, the three individuals exhibited significant differences in their protein content. S3 showed the highest protein value that was significantly different from S1 and S2. In our study, this individual was the one showing the lowest relative headspace concentrations of carbonyl compounds (aldehydes and some ketones). S1 and S2 presented however a lower effect on aroma compounds and their behaviour was quite similar. Therefore, it seems that a high total protein content was related to a high reduction of carbonyl compounds in the headspace. These results are in agreement with the ones from Piombino et al. (2014), who showed a link between aroma release and protein content in salivas from obese and normal-weight individuals. Therefore, and although proteomic analyses would be needed to confirm the proteins involved in these interactions, the total protein content could be a first parameter to evaluate the potential effect of saliva on aroma release.

The pH of the medium can modify the protein conformation and as a result, the affinity of aroma–protein interactions or the enzymatic activity. In this regard, it has been shown that variations of pH (ranging from 3 to 9) can modify the partition coefficients of some aroma compounds in beta-lactoglobulin solutions (van Ruth & Villeneuve, 2002). Nevertheless, the differences in pH across the saliva samples used in this study were not large (Table 3). S1 presented the highest pH values (7.5), followed by S3 (7.2) and S2 (7.1). Therefore, it is unlikely that the small differences in pH found in the present study could explain the observed results. Moreover, the final pH of all the aroma solutions containing saliva was measured and ranged from 7.2 to 7.5, probably due to the buffering capacity of human saliva (data not shown). Therefore, pH is unlikely the cause of the differences observed in this study. However, it is important to take in mind that in certain consumption situations in which the pH of the product dominates over the oral pH (e.g., consumption of liquid foods, such as wine, characterized by an acidic pH and slight oral processing (high food:saliva ratio, low residence time)), the effects of this variable on aroma release could be different.

Finally, the determination of the total antioxidant capacity (TAC) of the whole saliva samples was carried out, as this parameter has been previously related to the aroma release behaviour in the presence of saliva (Piombino et al., 2014). In that study, authors showed that salivas from obese individuals presented significantly higher TAC values and lower aroma release than salivas from

Table 3 Biochemical composition of the whole saliva samples employed in this study.

	TPC[1] (mg/mL)		pH		TAC[2] (1M Trolox)	
	Mean	SD	Mean	SD	Mean	SD
S1	0.42 b	0.00	7.50 a	0.01	605b	259
S2	0.49 b	0.22	7.10 b	0.00	998 ab	330
S3	1.38 a	0.20	7.20 b	0.01	1277 a	243

Different letters across the different assessors denotes statistical differences ($p < 0.1$). Total Protein Content. Total Antioxidant Capacity.

normal-weight individuals. For example, the headspace concentration of the aldehyde furfural was reduced up to 40% in the saliva from the obese group compared to the saliva from the normalweight group. Authors suggested that these effects were the result of the induction of a systemic antioxidant response in the saliva from obese individuals. This could be especially relevant for compounds prone to be submitted to redox reactions (e.g., lipid oxidation products, such as aldehydes and ketones). In this regard, it has been previously stated that saliva may constitute a first line of defense against oxidative stress (Terao & Nagao, 1991). It has also been speculated that TAC is related to the total amount of reducing agents in saliva (Kohen, Tirosh, & Kopolovich, 1992). Therefore, the higher the TAC is, the higher the reducing power of saliva to control oxidative stress. Our results are in agreement with these findings. As can be seen in Table 3, S3 possessed the highest TAC value, followed by S2 and S1. Therefore, it is likely that, the higher the TAC is, the higher is the ability of saliva to reduce the concentration of carbonyl compounds in the headspace. However, the fact that S3 also showed the highest protein content makes difficult to understand which variable (TPC or TAC) is more related to the aroma release data. Therefore, further studies with a higher number of individuals would be needed to understand the mechanisms at the origin of these effects.

3.6. Metabolism of aroma compounds by whole saliva

Some previous works have shown that human saliva is able to metabolize different groups of aroma compounds, like esters, thiols or aldehydes (Buettner, 2002a, 2002b). Therefore, the possible metabolism of the assayed aroma compounds by saliva was investigated by HS-SPME-GC/MS. These analyses revealed the presence of new metabolites in the HS above the saliva samples that were not present in the controls with water or blanks of saliva samples. Table 4 shows the aroma compounds significantly affected by the presence of human saliva (namely the assayed aldehydes, the diketones and the unsaturated 1-octen-3-one) together with the corresponding new metabolites identified. As can be seen, the main observed effect was the reduction of the carbonyl group to an alcohol group, which is in agreement with the results found by Buettner who observed the reduction of aldehydes to their corresponding alcohols (Buettner, 2002a). However, to the authors' knowledge, this is the first time that the reduction of ketones by human saliva is reported. Moreover for some compounds more than one metabolite was detected. For example, the analysis of the headspace of 1-octen-3-one resulted in the detection of two compounds: 3-octanone and 1-octen-3-ol. This transformation has already been described in mushrooms (Chen & Wu, 1984). Authors suggested the existence of two separated enzymes responsible for the formation of each compound or the existence of only one enzyme whose activity is affected by pH. Other compounds that led to the detection of more than one metabolite were 2,3pentanedione and 2-octenal, although in these cases others hypotheses like isomerization of aroma compounds in the injector cannot be excluded (Le-Quéré, unpublished results).

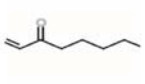
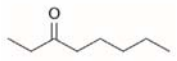
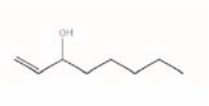
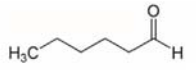
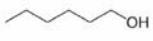
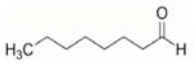
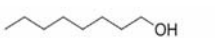
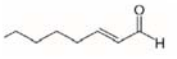
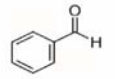
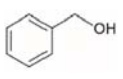
In an attempt to confirm the enzymatic nature of these transformations, four types of saliva and controls with water were incubated at 37 C in the presence of octanal and then extracted and analysed by a previously optimized LLE-GC/MS methodology. Octanal was chosen since it was the compound affected to the highest extent in the previous HS experiments. The four types of saliva assayed were: i) whole saliva, ii) whole saliva submitted to a thermal treatment of 100 C for 10 min (non-enzymatic saliva), iii) whole saliva centrifuged at 2800g for 10 min (4 C), pasteurized (60 C for 60 min) and clarified by a final centrifugation (2800g, 4 C, 10 min) (sterile saliva) and iv) sterile saliva submitted to a thermal treatment of 100 C for 10 min (saliva without microorganisms, cells and enzymes). As can be observed in Fig. 1, the recovery of octanal was only significantly different from water in the whole saliva samples. Moreover, the formation of its metabolite octanol was only observed in this saliva type. Therefore, it appears that the only saliva with metabolizing capacity (enzymatic activity) was the whole saliva (saliva without any treatment). The enzymes responsible for the observed effects could most probably belong to the oxidoreductase superfamily, enzymes NAD(P)H+ dependent. Among them, the aldoketoreductases (AKR), shortchain dehydrogenases (SDR) or alcohol dehydrogenases (ADH) are good candidates to carry out these reactions (Marchitti, Brocker, Stagos, & Vasiliou, 2008), although to the authors' knowledge the secretion of these enzymes by human salivary glands has not been described yet (Denny et al., 2008). Therefore these enzymes could come from different sources (oral tissues or microbiota) and be present in the complex mix of fluids called whole saliva. Indeed, ADH expression and activities have been shown in human gingival and lingual mucosa (Dong, Peng, & Yin, 1996).

To further support the hypothesis that the observed effects were of enzymatic nature, whole and non-enzymatic saliva (100 C – 10 min) were incubated in the presence or absence of NADH. NADH is the reduced form of the coenzyme NAD, working as a reducing agent and found in all living cells to involved in redox reactions. The presence of NADH is essential to the correct efficiency of NAD(P)H-dependent enzymes. As can be seen in Fig. 2a, whole saliva caused a higher degradation of octanal than nonenzymatic saliva. Moreover, the presence of NADH provoked a further reduction of octanal in whole saliva, while in non-enzymatic saliva no significant effects were observed. This strongly suggests the action of an enzymatic mechanism on the metabolism of this compound.

Finally, whole salivas from the three individuals were independently incubated in the presence or absence of NADH (Fig. 2b). As can be seen, salivas incubated in the presence of NADH showed a lower octanal and a higher octanol formation for all the individuals. Interestingly, saliva from S1 (saliva showing the lowest TAC value and possibly the lowest amount of reducing agents) seemed to be most affected by the presence of NADH. It seems for this saliva that the presence of the reducing agent has helped the enzymatic conversion take place at a higher rate. However, for saliva from S3 (saliva showing the highest TAC and possibly the highest amount of reducing agents) no differences on octanol formation

Table 4

Compounds and corresponding metabolites detected by HS-SPME-GC/MS in presence of saliva. Sensory characteristics (attribute and odor thresholds (OT)) are also included in the table.

Compounds significantly reduced by the presence of saliva	Metabolites identified in the HS above saliva samples	
2,3-butanedione (diacetyl)  «Buttery» OT: 2,3-6,5 ppb	3-hydroxy-2-butanone (acetoin)  «Sour milk» OT: 800 ppb	
2,3-pentanedione  «Butter, cream» OT: 78 ppb	3-hydroxypentan-2-one  «Herbaceous, truffle» OT: ?	2-hydroxypentan-2-one
1-octen-3-one  «Mushroom-like» OT: 0,005 ppb	3-octanone  «Fruity, floral» OT: 28 ppb	1-octen-3-ol  «Mushroom» OT : 1 ppb
Hexanal  «Green, grassy» OT: 4,5 ppb	1-hexanol  «Fatty, floral, green» OT: 2500 ppb	
Octanal  «Green grassy» OT: 0,7 ppb	1-octanol  «Green» OT: 110 ppb	
Octenal  «Green» OT: 3 ppb	(E)-2-octen-1-ol  «?» OT: ?	1-octanol, (Z)-3-octen-1-ol, and others
Benzaldehyde  «Almond, burnt sugar» OT: 350 ppb	Benzyl alcohol  «Sweet, flower» OT: 10000 ppb	

Molecular structures from webbook.nist.gov; descriptors from flavornet.com and leffingwell.com; odor thresholds (OT) from leffingwell.com.

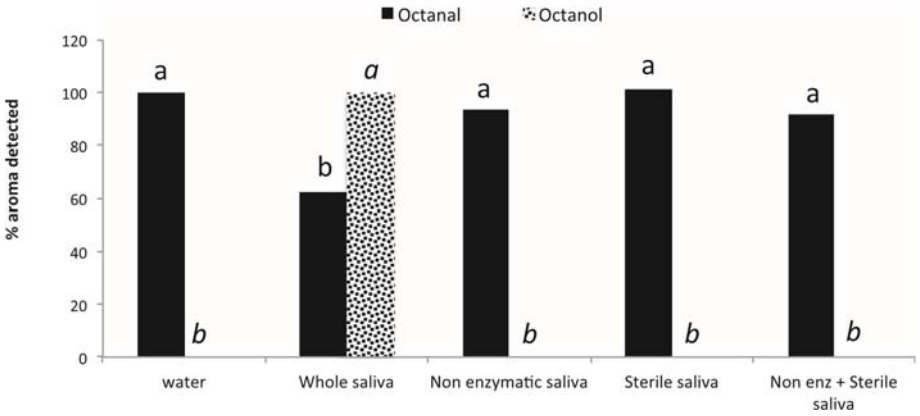


Fig. 1. Average values of octanal and octanol in presence of water and the four saliva types by LLE-GC/MS. Data represent the comparison of the same aroma compound determined in the different saliva/water samples and are expressed as percentages (considering the highest value of the specific compound as 100% and comparing this value with the amounts of the same compound determined in the other samples).

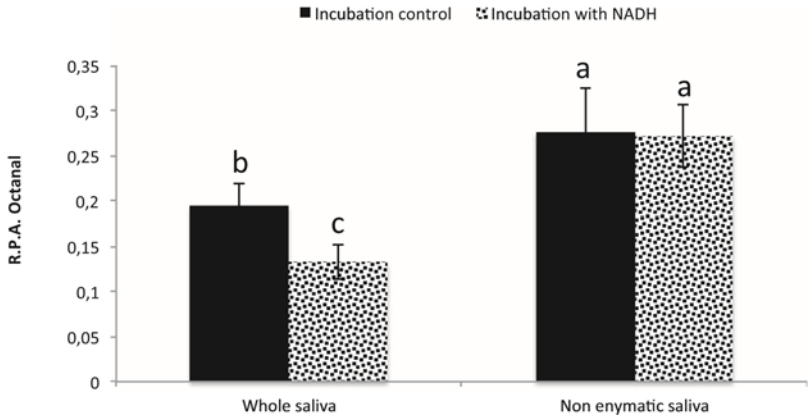


Fig. 2a. Average values of octanal (relative peak areas) in the presence of whole saliva and non-enzymatic saliva with/without NADH by LLE-GC/MS.

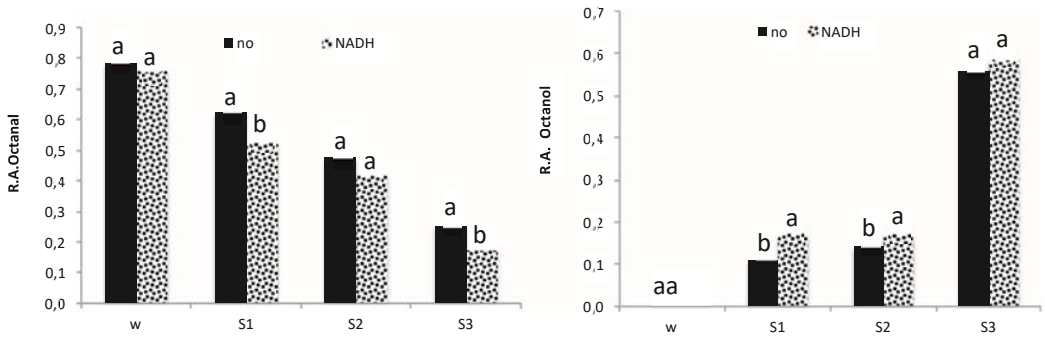


Fig. 2b. Average values of octanal and octanol (relative peak areas) in presence of water or whole salivas (n = 3) with/without NADH by LLE-GC/MS.

in the presence or absence of NADH were observed, possibly because a plateau for the conversion was already reached.

Finally, it is important to underline that although the reduction of the carbonyl group has been proven, it may not be the only mechanism occurring. Therefore, the existence of alternative processes like the retention of aroma compounds by salivary proteins through non-covalent interactions or the formation of conjugates with glutathione or other proteins to form condensed products cannot be discarded from the present investigation. Moreover these experiments have been carried out at pH closer to 7. Therefore the metabolic capacity of human saliva during food consumption in which saliva pH might drop or rise depending on the food should be evaluated in further experiments.

4. ***Conclusions***

In conclusion, the new HS-GC method developed in this study adapted to small sample volumes of saliva has allowed the study of the effects of human saliva composition on 17 aroma compounds. The results showed a decrease of carbonyl compounds (ketones, aldehydes) in the headspace, while alcohols were mostly unaffected by the presence of saliva. These effects were more important for whole than clarified salivas, revealing the necessity to use the most representative saliva

possible to elucidate the effects of human saliva on aroma compounds. Even if the saliva of only three individuals was studied, the present study clearly demonstrates an effect of inter-individual variation of saliva composition on the release of aroma compounds. Salivary biochemical parameters, like the total protein content and the total antioxidant activity determined in whole saliva samples, seemed to be related to the HS data. Finally, HS-SPME-GC/MS and LLE-GC–MS analyses confirmed the reduction of the carbonyl group with the corresponding formation of an alcohol group in the presence of whole saliva and strongly suggested that this was of enzymatic nature. The metabolites formed are in general less odoriferous than the initial compounds and with possibly different sensory attributes. Moreover, by converting carbonyl (xenobiotic) compounds to less reactive products (alcohols), the organism would decrease the overall chemical reactivity of these molecules and prevent their possible toxicity. Therefore, the ability of human saliva to catalyze these reactions could represent one mode of inactivation and detoxification of toxic compounds at the entrance of the organism. Consequently, these results could have implications both in aroma perception and/or an individual's health status.

It should be emphasized that these data were obtained in ex vivo conditions, which could never represent all the complex mechanisms occurring in vivo. For example, incubation times employed cannot be compared to the consumption situation, occurring generally at shorter times. However, under in vivo conditions odorants are continuously exposed to a constant flow of freshly saliva so that the turnover might be considerably higher. Also, the reaction rate in vitro might be decreased by depletion of the reducing agent during incubation. As a result, the effects here shown could have over/under estimated the role of saliva on aroma compounds. Further work, considering different groups of populations (old vs young or hyposalivator vs normal salivator) and matrix effects (non-volatile components) are needed to describe in more detail the observed phenomena, and to determine whether they are significant in vivo.

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