Molecular mechanisms of aroma persistence: from noncovalent interactions between aroma compounds and oral mucosa to metabolization of aroma compounds by saliva and oral cells

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Abstract

Aroma persistence plays a major role in the liking and wanting of orally consumed products (food, dental toiletries, tobacco, drugs, etc.). Here, we use an integral approach including *ex vivo* experiments using a novel model of oral mucosa and saliva in well controlled conditions as well as *in vivo* dynamic instrumental and sensory experiments. *Ex vivo* experiments show the ability of the mucosal pellicle, the thin layer of salivary proteins covering the oral mucosa, to interact with aroma compounds, as well as the ability of oral cells and saliva to metabolize carbonyl aroma compounds. *In vivo* evaluation of the exhaled air and perception of individuals after aroma sample consumption confirm *ex vivo* findings in a more real context. Thus, aroma compounds susceptible to be metabolized by saliva and oral cells show a lower aroma persistence than non metabolized compounds, for which other mechanisms such as the adsorption at the surface of the oral mucosa (mucosal pellicle) as a function of their hydrophobicity are involved. Thus, we argue that the physiological aspects occurring during the oral processing, and especially, metabolization of aroma compounds, have to be considered when studying the phenomenon of aroma persistence.

1. Introduction

Everyone keeps in mind a delicious dish or glass of wine with long lasting aromas that prolong the pleasure feels during eating after swallowing. The length of enjoyable final aroma notes has a tremendous impact on the perceived sensory properties and hedonic appreciation of highly enjoyable foods, such as coffee, wine, tea, chocolate or cheeses, but also of other products used on a daily basis, such as dental toiletries (i.e. toothpaste, breath fresheners). At the opposite, unpleasant aroma perceived during a prolonged period is a particularly disagreeable experience. For example, some people evade consuming certain seasoned dishes with raw garlic/onion or even some medicines for the strong long lasting sensations produced after their consumption. This phenomenon, called aroma persistence [1–3], drives consumer behaviour since it is an important criteria for product selection or avoidance. For that reason, food industry, dental products manufacturers or pharmaceutical industry, among others, are increasingly considering the quality, intensity and duration of prolonged aroma perception as a decisive part of the consumer's experience [4].

Aroma perception is a highly dynamic process, resulting from the activation of olfactory receptors by volatile organic compounds (VOCs) released from food within the oral cavity[5]. VOCs (aroma compounds) reach the olfactory receptors, which are in the nasal cavity, via the nasopharynx (Figure 1). In the process of smelling, VOCs perceived as an odor, reach the olfactory receptors via the external nares (orthonasal), avoiding contact with the oral tissues. Aroma persistence, which concerns only aroma perception, involved tissues of the oral cavity and the upper respiratory tracts. However, the molecular mechanisms allowing a continue release of VOCs from the mouth several tens of seconds or minutes after swallowing remain poorly understood [6].

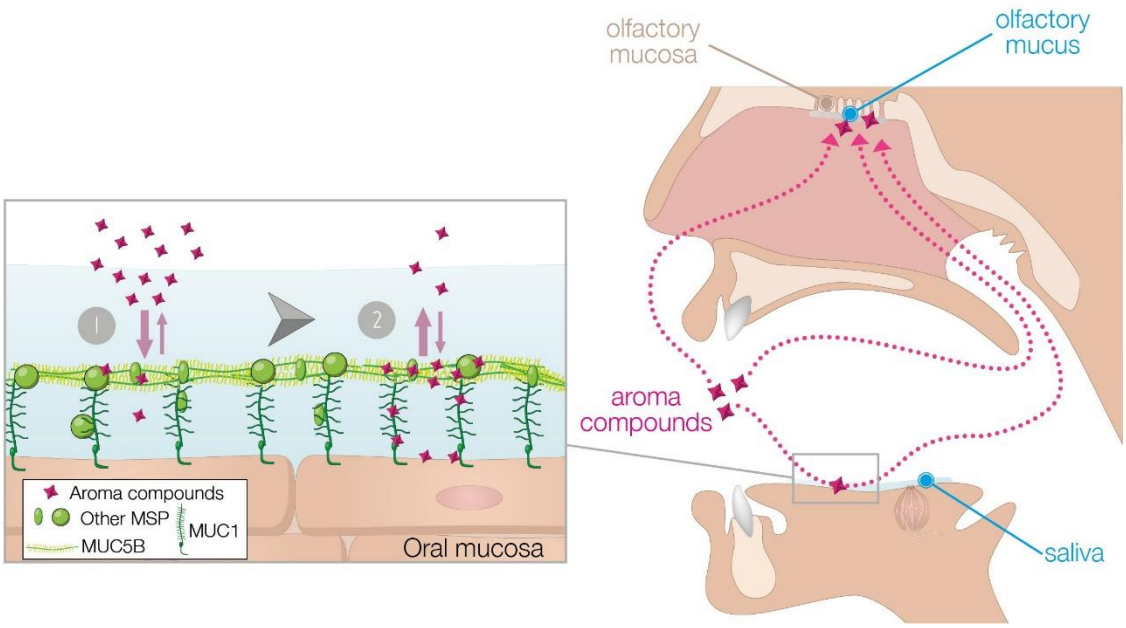

Figure 1. Scheme of aroma perception and hypothesis on the role of the mucosal pellicle on oral aroma persistence.

The firsts experimental works dedicated to study aroma persistence were done at the beginning of the 21st century [3,7–9], thanks to the development of instrumental approaches such as APCI-MS [10] or PTR-MS [11] that allowed to measure *in vivo* aroma release in real time. Results from these studies led to the hypothesis that aroma persistence is mainly driven on the affinity of aroma compounds for the hydrated layer of mucosa, which is mostly linked to their hydrophobicity and volatility in water at physiological temperature. However, this hypothesis is not always fulfilled and several studies have reported that the affinity of aroma compounds for water could not be the only parameter involved in aroma persistence [1,12,13]. For instance, it has been reported that aroma compounds are able to interact with salivary proteins, such as mucins [14]. Salivary mucins can be found free or bound to the mucosal pellicle, which is the thin layer of proteins covering the surface of the oral mucosa (Figure 1). Recently, Ployon and collaborators[15] revealed that constituents of the mucosal pellicle might influence aroma release kinetics (Figure 1). In

the same time, it has been reported that oral epithelial cells are able to metabolize aroma compounds [15,16], as observed for saliva [17–21]. Although these works have been performed in *ex vivo* conditions, there are recent evidences that metabolization of aroma compounds occurs i*n vivo* and that might influence aroma perception [22].

Thus, we hypothesised that the intensity and duration of perception of aroma compounds after oral processing is influenced by physiological (i.e. metabolization by the oral mucosa or saliva) and physicochemical mechanisms (i.e. hydrophobic interactions with oral proteins) depending on their chemical structure. To explore this hypothesis, we have executed a multidisciplinary approach coupling analytical chemistry, sensory analysis and using a cutting-edge *in vitro* model of oral mucosa. Indeed, we have recently pioneered the development of a model of oral mucosa considering the mucosal pellicle. Our strategy involved: i) *ex vivo* investigations on the impact of the model of mucosa and saliva on aroma compounds using static experiments, ii) *in vivo* experiments measuring aroma release in dynamic conditions and iii) sensory analysis on aroma persistence, in order to decipher the impact of oral physiology on aroma persistence. Five aroma compounds (linalool, nonan-2-one, pentan-2-one, hexan-2,3-dione and octanal) belonging to different chemical families and presenting different physicochemical and sensory properties were selected for this study. Some of them have been previously reported to be metabolized in the presence of human saliva or oral cells (hexan-2,3-dione, octanal, nonan-2-one), while others not (linalool, pentan-2-one).

2. Experimental section

This study received the approval of the Ethics Committee for Research (CPP Est I. Dijon, #14.06.03, ANSM #2014-A00071-46) and was conducted according to the Declaration

of Helsinki. All participants were selected from the AlimaSSens panel (110 volunteers) based on their good physical and mental status. They provided informed written consent and were financially compensated for their participation.

2.1. Aroma compounds

We selected five food-grade aroma compounds (linalool, pentan-2-one, nonan-2-one, hexan-2,3-dione and octanal) (Sigma-Aldrich, Saint Quentin Fallavier, France) of different chemical families (ketone, aldehyde, terpene-alcohol) and with different physicochemical properties (hydrophobicity, volatility) (Table S1). We prepared independent stock solutions (1 %) of the single aroma compounds in propyleneglycol (Sigma-Aldrich, Saint Quentin Fallavier, France) that were alliquoted and stored at 4 °C for a maximum of one month.

2.2 Oral mucosa model

We used the TR146/MUC1 cell line previously described by Ployon and coworkers[23]. Firstly, we confirmed that the aroma compounds were non-cytotoxic to the TR146/MUC1cells[15] (viability above 90%). Then, we seeded and cultured the cells (density: 4.104 cell/cm^2). For the reconstitution of the mucosal pellicle samples, we deposited whole saliva samples diluted into growth medium (1:1) onto 5-days cells subcultures for 2 h to form the different mucosal pellicles. Afterwards, we washed the samples twice with Phosphate Buffered Saline (PBS) and we carried out the aroma experiments.

2.3 Static headspace analyses

We prepared the aroma solutions at 3 mg/l in PBS from the individual stock solutions (pH=7.4). Then, we performed the static headspace analyses using three different conditions: Condition 1) a vial containing 25 µl of PBS (control); Condition 2) a vial containing the mucosa model (cells); and Condition 3) a vial containing the mucosa model together with the reconstituted mucosal pellicle (MP). To perform the headspace analyses, we added three hundred µl of the aroma solution to the vials prepared under the three different conditions, sealed them with silicone septums (Supelco, Bellefont, PA, USA), and incubated them at 37 °C for 30 min. Then, the headspace above the samples (200 µl of) was automatically sampled (GERSTEL MPS2, Gerstel Inc., Mülheim an der Ruhr, Germany) and analyzed in splitless mode by a gas chromatograph (Agilent 6890 N; Agilent, Santa Clara, CA) coupled to a MS detector (Agilent 5973N) (electron energy = 70 eV). We set the injector temperature at 240 °C, the oven temperature was programmed to increase from 60 (held 1 min) to 150 °C at 5 °C/min and held for 1 min, and the temperatures of the transfer line, quadrupole, and ion source at 250, 150, and 230° C, respectively. We used a DBWAX column (30 m, 0.32 mm i.d., 0.5 µm; Agilent Technologies) with helium as carrier gas at a velocity of 45 cm/s. Each run lasted 20 min. In total, we collected saliva samples from 16 individuals (Table S2) as described previously[24] and used them to form 16 independent models of oral mucosa. Each sample was analysed in triplicate (one injection per sample vial) and the analyses lasted 11 days. We identified the compounds present in the extracts by comparison of their MS spectra with those obtained after the injection of pure compounds and with an internal (INRAMASS) and commercial mass spectra database (NIST 2008, Wiley 138). To validate the methodology, we determined the linearity and repeatability of the procedure in an aqueous solution composed of a mixture of 5 aroma compounds at seven levels of concentration. The methodology presented a good repeatability (relative standard

deviation below 5% for all compounds) and linearity (determination coefficients higher than 99% for all the assayed compounds) (Figure S1).

2.4. Analyses of aroma metabolization by oral components by GC–MS

We incubated vials containing the model mucosa (cells) or 200 µl of whole saliva (saliva) at 37 °C with 300 µl of the aroma solution (3 mg/l in PBS) at different times (0, 5, 30, 60, 120 min) in order to determine whether metabolization of aroma compounds occurs in the presence of oral components. After incubation, we extracted twice the aroma compounds with 1 ml of dichloromethane (Carlo Erba, Val de Reuil, France). We then centrifuged the solutions (15000g, 4 °C, 15 min) to separate the two phases. Prior to the extraction, we spiked the samples with 100 µL of the internal standard (methyl nonanoate at10 mg/l). We finally dried the combined organic extracts over anhydrous Na_2SO_4, and concentrated it under nitrogen to a total volume of 200 µL. We injected one microliter into the GC/MS in splitless mode following the same method described above. As controls, we carried out the same extraction procedure on the aroma solutions without oral components and on the samples with oral components without aroma compounds added. We calculated the relative peak areas (RPAs) by dividing the area of the peak of interest by the area of the internal standard. To validate the methodology, we calculated the linearity and repeatability of the procedure in an aqueous solution composed of a mixture of 5 aroma compounds at six levels of concentration. The methodology presented a good repeatability (relative standard deviation below 7% for all compounds) and linearity (determination coefficients higher than 99% for all the assayed compounds) (Figure S2).

2.5 *In vivo* aroma persistence by PTR-ToF-MS

In a previous study[24], we selected 54 individuals (Table S2) based on their repeatability to follow a consumption protocol for *in vivo* aroma release analyses. In the present study, these individuals consumed a solution spiked with five aroma compounds (linalool (40 mg/l), nonan-2-one (5 mg/l), pentan-2-one (1 mg/l), hexan-2,3-dione (20 mg/l), octanal (3 mg/l)) following a consumption protocol that consisted in doing mouth-rinsings with the solution during 30 seconds avoiding swallowing, and then, swallow all the liquid in their mouths while breathing normally. Afterwards, and every 30 seconds, we instructed the individuals to swallow the saliva accumulated in their mouths. They evaluated the solution in two different days (once per day). We monitored the individual's nosespace through a Teflon nosespiece, via a helmet connected to a proton transfer reaction-mass spectrometer (PTR-MS) instrument equipped with a Time-of-Flight (ToF) analyser (PTR-ToF 8000, Ionicon Analytik, Innsbruck, Austria). Analytical conditions followed for the PTR-ToF-MS procedure can be found in Muñoz-González et al., 2021[24]. We calculated the release curves of the ions corresponding to the aroma compounds and extracted the area under the curve (AUC) values for each of the selected ions and monitoring time, which corresponded to the swallowing events performed by the individuals (t0-30s, t30-60s, t60-90s, t90-120s, t120-150s). All the release data were analysed from the breath concentration ncps data, using IGOR Pro (WaveMetrics, Inc. Portland, USA).

2.6. *In vivo* aroma persistence by dynamic sensory evaluation

In this section, we selected 26 individuals (Table S2) with a normal sense of smell[25]. The sensory sessions took place in a sensory testing room (21 ± 2 °C) of the ChemoSens platform (Centre des Sciences du Goût et de l'Alimentation, INRAE, Dijon).

For the sensory analyses, we selected two aroma compounds (hexan-2,3-dione and linalool) that showed opposite behaviours in the previous instrumental experiments were studied. A consensus in the assignment of aroma descriptors for each of the two aroma compounds was reached in a first previous session. The final descriptors were "butter" for hexan-2,3-dione and "floral" for linalool. Both aroma compounds were independently evaluated. We prepared the samples immediately prior to the sensory evaluations by diluting the stock solutions in water (Evian, France). The participants were not allowed to smoke, eat or drink starting at least one hour before the different sessions. The participants were trained in the retronasal recognition of the two aroma descriptors and on the discrimination of their aroma intensity by using 10-cm unstructured scales delimited at the ends (0=not very intense, 10=very intense). The evaluation of aroma persistence was done by means of dynamic sensory evaluation. To do so, we firstly familiarized the individuals with the dynamic and discontinuous time–intensity methodology. This methodology consisted in that subjects rated fixed attributes on a scale at predetermined time points. This technique reduces the cognitive load and needs less training than the continuous Time-Intensity technique[26]. For the evaluation session, the aroma compounds were evaluated at a concentration of 9 mg/l for both compounds. This concentration was chosen for providing an accurate stimulus (detection and repeatability) at the retronasal level for all the members of the panel. The samples (10 ml) with random three-digit codes were presented in plastic cups (50 ml) covered with lids. The participants evaluated the samples at room temperature in individual booths illuminated with red light. They were instructed to introduce the entire sample (10 ml) into their mouths at one time, avoiding smell it (they were asked to cover their noses with their hands). Once in the oral cavity, the individuals were instructed to gently rinse their mouths with the solution during five seconds avoiding swallowing. After this time, they

were instructed to swallow all the liquid in their mouths consisting on a mixture of sample and saliva and to breathe normally. Immediately afterwards and every 5 seconds they were asked to rate the intensity of the aroma descriptor on the scale. The individuals were instructed to keep their lips closed during all the evaluation that lasted 90 seconds. They had to wait at least 2 min to evaluate the following sample. A warm-up sample was presented before starting the evaluation. Bread and water were used as mouth cleansers between tests. In each case, the individuals knew the aroma descriptor to be evaluated. The compound hexan-2,3-dione was evaluated first. From the notation in the different scales, time–intensity curves were reconstituted for each aroma descriptor. For each attribute, average time-intensity curves were determined by averaging the data at each time point across the two compounds and the three replicates. The first point (5sec) was considered as 100% and aroma persistence was calculated respect to this point. The sensory analyses were done using the Fizz® software and all the measurements were done in triplicate.

2.7. Statistical analyses

Retention data of the aroma compounds in the three conditions (control, cells, and MP) analyzed by static headspace analyses and data of the effects of the oral mucosa and saliva on aroma compounds analyzed by liquid phase analyses were submitted to univariate analysis of variance (ANOVA) followed by a Tukey multiple comparison test (significance for $p < 0.005$). For PTR-MS and sensory analyses, persistence of aroma compounds was evaluated by ANOVA followed by a post-hoc Tukey multiple comparison test (significance for $p < 0.05$). The XL-Stat (StatSoft, Inc., 2005, www.statsoft.com) and R (R Core Team, www.r-project.org/) programs were used for data processing.

3. Results and discussion

3.1. *Ex vivo* investigation on the impact of a model of oral mucosa on aroma release using static experiments

3.1.1. Impact of oral mucosa on aroma compounds

Using a model of oral mucosa that our group has previously developed [23], we investigated the effect of the oral mucosa model on the release of aroma compounds. This model is based on the TR146/MUC1 cell line [23] that expresses at its surface the extracellular domain of the mucin (MUC1/Y-LSP) [27]. This extracellular domain is involved in the formation of the mucosal pellicle (MP) [23], which is the hydrated layer of epithelial and salivary proteins [28] that is believe to participate to the phenomenon of aroma persistence by retention of aroma compounds in the mouth [15].

Static headspace sampling is considered one of the best methods to study the impact of molecular interactions between aroma compounds and other components in solution on aroma release. It consists in the determination of aroma concentration in the gaseous phase of a closed vial after thermodynamic equilibrium. In an attempt to unravel the mechanisms behind aroma persistence, static headspace analyses were performed after 30 min of incubation at 37 °C in three different conditions: a control condition and a model of oral mucosa consisting of a cellular epithelium TR146/MUC1 (cells) with or without a MP. The amount of aroma compounds recovered in the headspace above the samples was expressed as a percentage of the control condition (Figure 2.A). A percentage lower than 100% indicates that the aroma compounds were present in a lower

amount in the gas phase of the vials containing the oral mucosa with or without a MP than in the control samples.

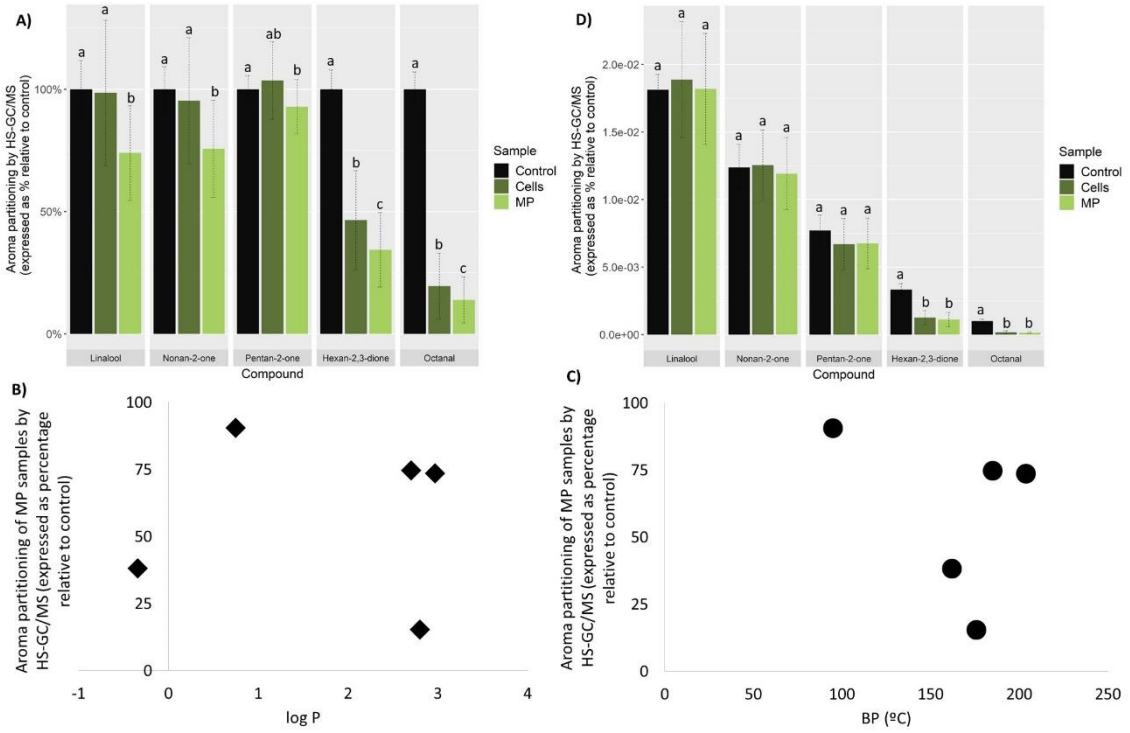

Figure 2. (A) Aroma partitioning in cells or mucosal pellicle (MP) samples by HS-GC/MS expressed as percentage relative to controls. (B) Relationship between hydrophobicity (log P values) of the aroma compounds and their partitioning in MP samples as percentage relative to controls. (C) Relationship between polarity (Boiling Point values) of the aroma compounds and their partitioning in MP samples as percentage relative to controls. (D) Aroma recovered by LLE-GC/MS in the controls, cells and MP samples after incubation with the five aroma compounds. Results from the bar graphs are presented as the mean value ± SD. Different letters indicate significant differences (p-value < 0.005) between the conditions after application of the Tukey test.

Figure 2.A shows that the release of all compounds was significantly affected by the presence of the MP (26% for linalool, 24% for nonan-2-one, 7% for pentan-2-one, 66% for hexan-2,3-dione and 86% for octanal in comparison to the control). Hexan-2,3-dione and octanal were also significantly affected by the oral cells condition and this reduction was even more important in the MP condition for both compounds.

These effects could mainly result from two mechanisms: (i) non-covalent interactions between the surface of the oral cells with or without mucosal pellicle or (ii) metabolization of the compounds. Non-covalent interaction between aroma compounds and salivary proteins, such as mucins, are thought to involved hydrophobic effects[14]. In order to check the first mechanism, we plot the results obtained in the previous HS analyses as a function to the polarity (log P values) of aroma compounds (Figures 2.B). As it can be seen, no correlation, considering the five aroma compounds assayed, is observed. It suggests that the retention by MP was not driven or at least not only driven by hydrophobic effects. However, it is important to note that two compounds (hexan-2,3-dione and octanal) behaved very differently with regard to the other three (linalool, nonan-2-one and pentan-2-one). Interestingly, these two compounds were the only ones significantly affected by the cells condition. No trend was also observed in the plotting of the HS data as a function of aroma compounds volatility (boiling point values) (Figure 2.C).

To deeply explore this mechanism, a liquid/liquid extraction with an organic solvent was performed in the incubated samples, since this extraction breaks the non-covalent interactions between aroma compounds and MP (especially hydrophobic effects), while extracting the aroma compounds that remain in the liquid phase after incubation. Results are presented in Figure 2.D, which shows that linalool, nonan-2-one or pentan-2-one did not show significant differences in the three assayed conditions while hexan-2,3-dione and octanal were significantly less recovered in the presence of oral cells with or without MP. The fact that no significant differences were found in linalool, nonan-2-one or pentan-2-one among the three assayed conditions in contrast to the decrease observed in the previous headspace analyses in the presence of MP, indicates that differences

observed between figures 2.A. et 2.D. can be attributed to the disruption of noncovalent interactions between the aroma compounds and the MP following the addition of the organic solvent (dichloromethane). The MP contains different mucins such as MUC1, MUC5B and MUC7, while interactions between aroma compounds and mucins have been reported by several authors *in vitro* [14,29]. Actually, it has been shown that mucins are able to retain aroma compounds through non-covalent interactions involving hydrophobic effects [14], which could be dissociated by an organic solvent. However, these results contrast with those previously obtained using a similar model of mucosa, where no effect of MP on aroma compounds at equilibrium was observed [15]. It is important to note that in this previous study centrifuged saliva was used to form the MP, while in the present study we used whole saliva that is richer in high molecular weight proteins such as mucins, which could have increased the available binding sites and thus the retention of aroma compounds. Moreover, the composition of the MP depends on the one of the saliva used to reconstitute the MP. In the present study, we formed 16 models of mucosa using saliva samples from 16 subjects and their composition could also have impacted the MP composition.

The other two compounds assayed, hexan-2,3-dione and octanal, showed a significantly lower recovery in the model mucosa samples (cells and MP) compared to the controls. The decrease of the concentration in the liquid phase of these two compounds in the presence of oral cells could result from metabolization of these aroma compounds by oral cells as previously observed with this model of oral mucosa [15].

Thus, the present results indicate that while MP exerted a compound-dependent retention effect on all the aroma compounds assayed, only two of them (hexan-2,3-dione and octanal) were metabolized in the presence of oral cells.

3.1.2. Impact of oral cells and saliva on aroma compounds over time

In order to deeper explore the metabolization of aroma compounds by oral components, the composition of the liquid phase of aroma solutions incubated at different times in presence of oral cells (cells) or saliva (whole saliva) was characterized by GC–MS. These results are presented in Figure 3.

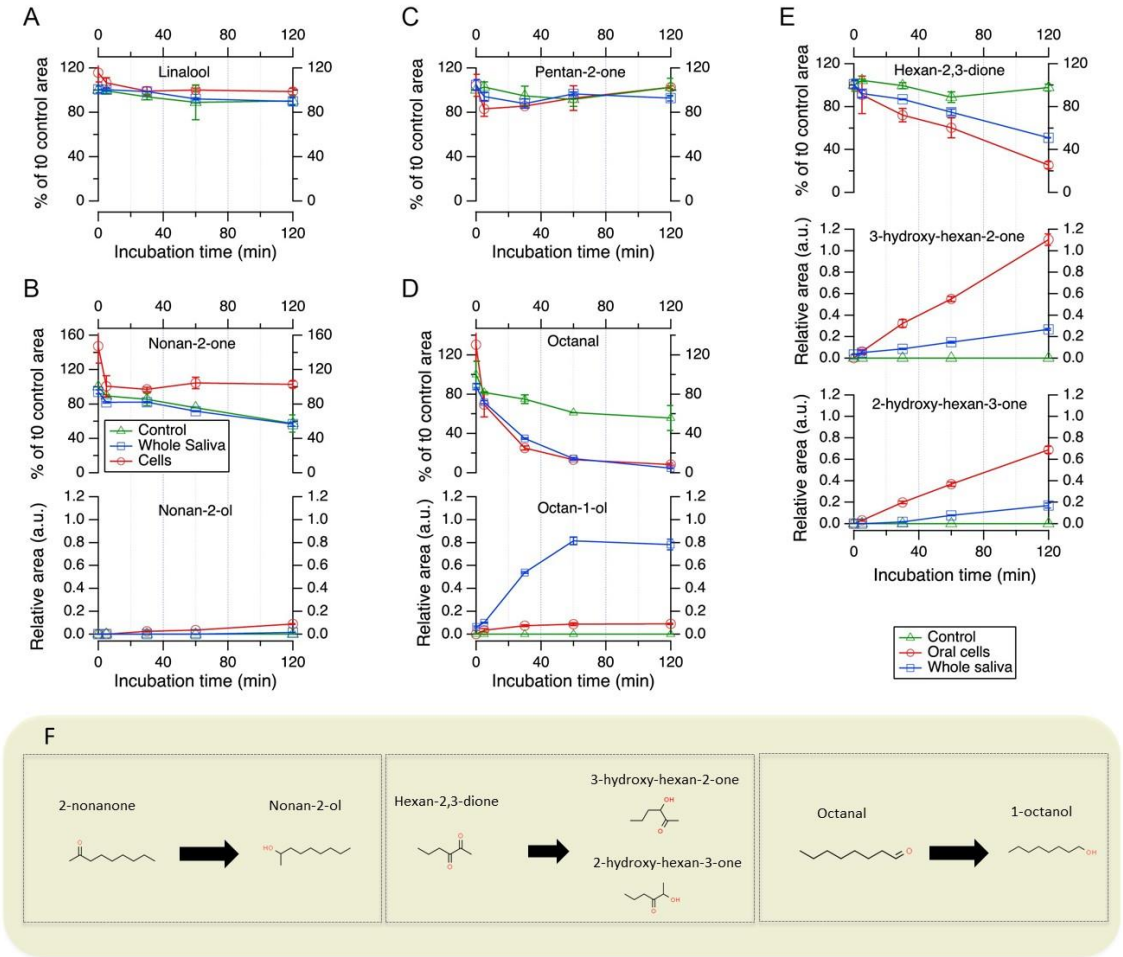

Figure 3. Compounds identified in the controls, cells and whole saliva samples over time for (A) Linalool, (B) Nonan-2-one, (C) Pentan-2-one, (D) Octanal and (E) Hexan-2,3-dione. Data are expressed as percentage relative to the concentration obtained at t0 in the controls for the original compounds and as relative area for their metabolites. All results

are presented as the mean value ± SD. (F) Schema of the reduction of carbonyl compounds observed during *ex vivo* incubations.

As observed in the precedent experiment (Figure 2), the recovery of linalool (Figure 3.A) and pentan-2-one (Figure 3.C) remain the same through time whatever the tested conditions (control, cells and whole saliva), which indicates that these compounds were not metabolized by oral cells or saliva. A higher recovery of nonan-2-one was observed for the oral cells condition compared to the control and saliva conditions at t0, and from 30 min of incubation onwards. For the control and saliva conditions, the concentration of nonan-2-one decreased through time. Despite the surprising higher recovery of nonan-2-one in the cells condition respect to the control, a reduction of this compound has been previously reported in the presence of oral cells[15]. Thus, we checked the formation of the corresponding nonan-2-ol (Figure 3.B) in the three conditions (control, cells and saliva). In the case of the cells condition, nonan-2-ol started to be detected after 30 min of incubation time and, in the case of saliva, it was only detected at 120 min. However, the decrease of nonan-2-one in the control condition cannot be attributed to a reduction into alcohol since nonan-2-ol was not detected in the controls. Thus, nonan-2-one seemed to be not affected by metabolization. Regarding the compounds octanal and hexan-2,3-dione, their recovering was clearly impacted by oral cells and whole saliva over the time (Figure 3.D, 3.E). The decrease of the concentration of these two compounds suggests that hexan-2,3-dione and octanal are metabolized by saliva and oral cells enzymes. The metabolization of diketones and aldehydes has been previously observed *ex vivo* in the presence of saliva [18,20,21] or cellular enzymes [30–32] and *in vivo* in the oral and nasal cavity [22]. For octanal, the formation of octan-1-ol was observed in both conditions (cells and whole saliva), at different levels (Figure 2.D). The formation of this alcohol was higher in presence of saliva than in oral cells while octanal decrease was similar in both

conditions. For hexan-2,3-dione (Figure 2.E), the formation of 3-hydroxy-hexan-2-one and 2-hydroxy-hexan-3-one was observed in cells and whole saliva conditions, and hexan-2,3-dione metabolization was higher in presence of oral cells than in saliva.

In all cases, the observed reactions corresponded to the reduction of the carbonyl group from aldehydes and ketones (Figure 3.F) giving place to metabolites that present different sensory properties (descriptors or odour thresholds) than the initial compounds. Aldehydes are usually more reactive toward nucleophilic substitutions than ketones because of both steric and electronic effects, while diketones have two carbonyl groups. As a result, hexan-2,3-dione and octanal are reactive compounds, which might be neutralized by the organism via their metabolization. These reactions could be carry out by a range of enzymes named Odorant Metabolizing Enzyme [33] that belong to the xenobiotic metabolism enzymes family. The different metabolization of compounds in the different oral components (oral cells versus saliva) can be due to different factors such as the enzymatic activity of the oral components, which depends on the enzymes and their concentrations, but also on the presence of cofactors [34], among others.

Overall, these experiments confirm that oral cells and whole saliva are able to metabolize aroma compounds *ex vivo* depending on their structure. Thus, we can hypothesize that the oral metabolization of hexan-2,3-dione and octanal could decrease their persistence in the breath compared to the persistence of other compounds that are not metabolized during their oral passage.

3.2. *In vivo* experiments measuring aroma persistence

3.2.1. Instrumental measurements of *in vivo* aroma persistence by PTR-ToF-MS

To validate our hypothesis in an *in vivo* context, we used a real time instrumental approach coupling a PTR-ToF-MS with the nasal cavity of 54 subjects. *In vivo* aroma persistence was monitored after the individuals consumed a model solution flavoured with five aroma compounds following an imposed consumption protocol. Data of the panel (n=54) were averaged considering 30-second intervals after sample swallowing as shown in Figure 4.A. To allow compound comparison, data were expressed considering the AUC values of the first 30 seconds after sample swallowing as 100% and calculating the percentage relative to it for the following 150 seconds.

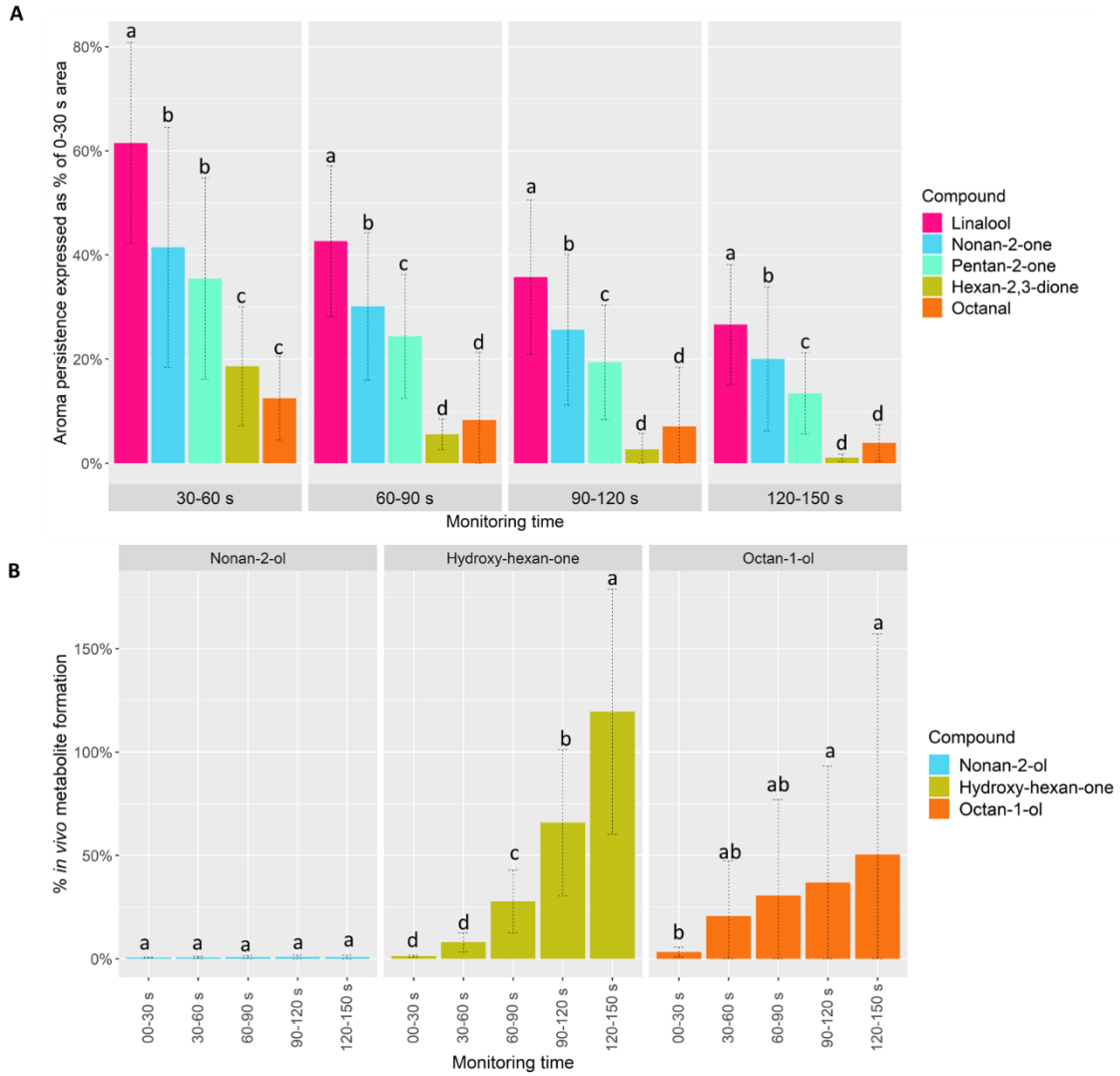

Figure 4. (A) *In vivo* aroma persistence measured in 54 subjects by PTR-ToF-MS after they consumed a solution flavoured with five aroma compounds. Values are expressed as a percentage considering the AUC of the first 30 sec as 100% and calculating the ratio relative to it for the rest of monitoring times. A ratio lower than 100% indicates that aroma

persistence decreases over time. (B) Percentage of metabolites formed during the instrumental *in vivo* aroma analyses. Data are expressed as the ratio of the amount metabolite detected in each monitoring time respect to the amount of the original compound detected in the same monitoring time. Results from the bar graphs are presented as the mean value ± SD. Different letters indicate significant differences (p-value < 0.001) between the conditions after application of the Tukey test.

As expected, once the sample was swallowed there was a progressive decrease of aroma persistence for all compounds (Figure 4.A). The extent of the decrease was compound-dependent and the compounds did not disappear at the same rate in the nasal cavity of the individuals. Since the second interval of time ($t_{30\text{-}60s}$), significant differences between compounds were observed, with linalool being the most persistent compound, followed by nonan-2-one and pentan-2-one. These three compounds remained respectively in the breath at 61, 41 and 35% of their concentration recorded during the second interval of monitoring time ($t_{30\text{-}60s}$). As hypothesized above, hexan-2,3-dione and octanal were less persistent in the breath and their concentration for this monitoring time represented respectively 19 and 12% of the initial concentration ($t_{0\text{-}30s}$). From the third interval ($t_{60\text{-}90s}$), a significant difference was observed between nonan-2-one and pentan-2-one, the former being more persistent than the latter. In the last interval of monitoring time ($t_{120\text{-}150s}$), nonan-2-one and linalool concentrations still represented 20-27 % of the first interval. Pentan-2-one was found at a 13%, while hexane-2,3-dione and octanal have almost disappeared in the breath at that time (<4%). Thus, linalool, nonan-2-one and pentan-2-one were significantly more persistent in the breath of the subjects than hexan-2,3-dione and octanal. These results are in accordance with those above observed *ex vivo* using oral components (oral mucosa model, MP and saliva). Regarding the compounds less susceptible to metabolization (linalool, nonan-2-one and pentan-2-one), they displayed the lower decay rates in the *in vivo* experiment being the most persistent compounds in the breath of the subjects. Accordingly, they showed the higher *ex vivo*

retention by MP (linalool: 26%; nonan-2-one: 24%; pentan-2-one: 7%) in static conditions as a function of their hydrophobicity (linalool: logP 2.97; nonan-2-one: logP 2.70; pentan-2-one: logP 0.75). This behaviour was maintained in the *in vivo* experience, which suggests their retention in the mouth by hydrophobic interactions with MP. Additionally, the two compounds that showed a clear metabolization by oral components *ex vivo*, presented the lowest persistence *in vivo*. However, these two reactive compounds behave in a similar way (octanal and hexan-2,3-dione), despite their differences in terms of hydrophobicity (log P 2.80 and -0.35, respectively).

To check if metabolization of aroma compounds occurs *in vivo*, the m/z corresponding to the metabolites previously reported in the *ex vivo* experiments (127.1 nonan-2-ol; 113.1 octan-1-ol and 99.1 hydroxy-hexan-one) were extracted from the release curves obtained by PTR-MS. Since once the solution is swallowed the formation of metabolites will be dependent on the remaining amount of the original aroma in the mouth, data corresponding to the formation of metabolites are expressed as the ratio between the detected amount of the metabolite and that of the original compound for each time point of analysis (Figure 4.B). As it can be seen, ratio of nonan-2-ol was lower than 1% for all monitoring times, which indicates a very weak metabolization of nonan-2-one. On the contrary, the metabolites corresponding to hexan-2,3-dione and octanal, showed increasing ratios over time, which would support the metabolization of these compounds in *in vivo* conditions. Interestingly, the ratio was higher for hexan-2,3-dione than for octanal.

Overall, these results highlight that aroma persistence relies not only on the physicochemical properties of aroma compounds, such as hydrophobicity and volatility,

as previously proposed [3,4,7–9], but also on the metabolism of aroma compounds. Although the lack of agreement between log P and aroma release has recently been reported *ex vivo* [15] and *in vivo* [35], this is the first experimental work that demonstrates that metabolism of aroma compounds impact on aroma persistence.

3.2.2 Sensory measurements of *in vivo* aroma persistence by dynamic time-intensity evaluation

One can wonder if differences observed instrumentally have an impact on perception. Instrumental results suggest that linalool is more persistent and might contribute in an important way to aroma persistence than compounds, such as octanal or hexan-2,3-dione. To confirm this hypothesis, we carried out a sensory experiment with two of the compounds that presented a contrasted behaviour in the instrumental experiments (linalool and hexan-2,3-dione). Twenty-six individuals were selected and trained in the recognition and rating of intensities of both compounds using a dynamic methodology. The prolonged retronasal perception of floral notes (linalool) and buttery notes (hexan-2,3-dione) over time was evaluated by the 26 volunteers after they consumed the model solutions flavoured independently with these two aroma compounds following specific instructions. Aroma intensity perceived by the subjects was monitored each 5 seconds during 90 seconds after sample consumption. Figure 5 shows the averaged data of the panel. To allow compound comparison, data were expressed considering the data of the first monitoring time (5 seconds) after sample swallowing as 100% and calculating the percentage relative to it for the rest of monitoring times.

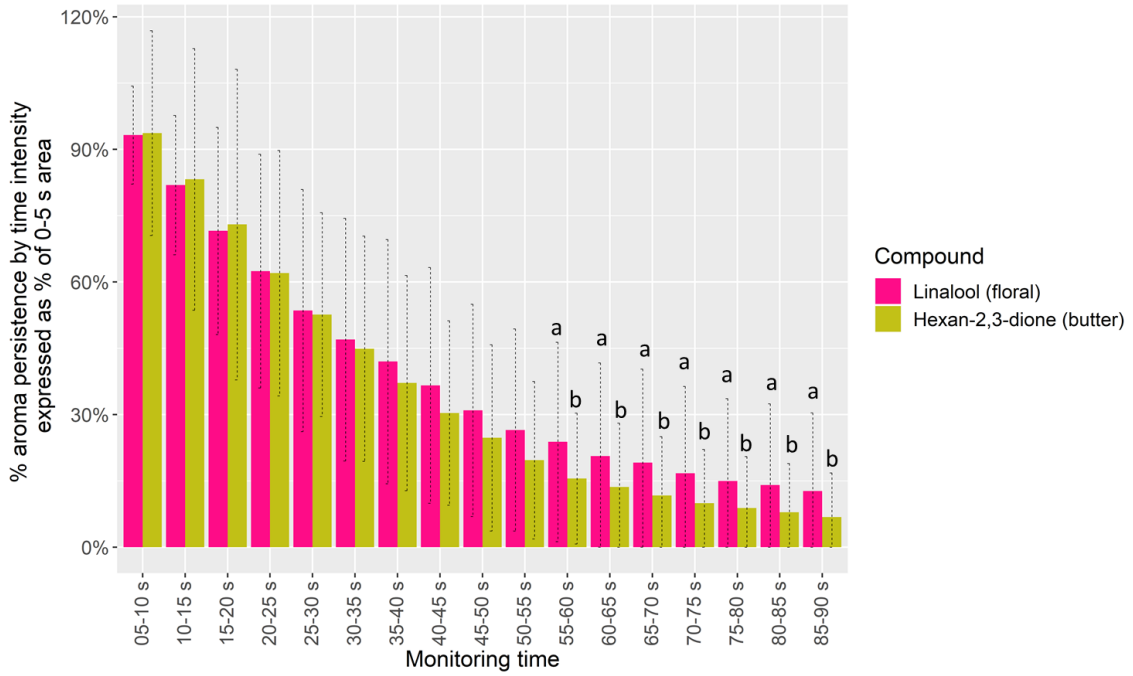

Figure 5. *In vivo* aroma persistence measured in 26 subjects by dynamic sensory analysis after the consumption of two solutions, one flavoured with linalool (floral descriptor) and the other one with hexan-2,3-dione (butter descriptor). Values are expressed as the percentage of persistence considering the first monitoring time (5 sec) as 100% and calculating the ratio relative to it for the rest of monitoring times. A ratio lower than 100% indicates that aroma persistence decreases over time. Results from the bar graphs are presented as the mean value ± SD. Different letters indicate significant differences (p-value < 0.05) between the conditions after application of the Tukey test.

As observed in the instrumental PTR-ToF-MS experiment, once the sample was swallowed there was a progressive decrease of aroma persistence for both compounds (Figure 5), although the extent of the decrease was compound-dependent. Thus, from 60 seconds of monitoring time after sample swallowing onwards, persistence of butter notes (hexan-2,3-dione) were significantly lower than that of floral notes (linalool) ($p<0.05$) confirming previous instrumental findings. However, it is interesting to note that the magnitude of the difference between compounds was higher in the instrumental than in the sensory experiment. Different reasons can explain this fact. Firstly, it is difficult to compare the sensitivity of the human nose with the one of the PTR-MS. The higher complexity of the sensory study that needs additionally to the in-mouth processing, the integration of the information in the brain, makes that interindividual differences among

participants are even of bigger magnitude. Moreover, slight differences in the consumption protocol (aroma concentration, rinsing time, duration, etc) could have influenced results. Thus, in the instrumental approach, participants were instructed to rinse their mouths during 30 seconds while in the sensory experiment only during 5 seconds, which could have affected the metabolization and/or retention of aroma compounds in the mouth. Additionally, it could be possible that the metabolites produced by reduction of hexan-2,3-dione could have contributed to the overall perception by the individuals, as suggested for other aroma compounds by Ijichi and coworkers [22]. Actually, they found that the metabolites of aroma compounds produced *in vivo* are perceived as part of the aroma quality of the original aroma. Although usually the metabolites formed present higher odor thresholds than the original compounds, their formation, could, in some way contribute to aroma persistence.

4. Conclusions

This study showed that aroma persistence is a complex mechanism involving the reactivity of the oral mucosa and saliva and dependent on the structure of aroma compounds. Two different mechanisms are highlighted. The first mechanism involved the mucosal pellicle, the thin layer of proteins covering the oral mucosa, and the affinity of aroma compounds for this biological structure. This affinity seems to depend on the hydrophobicity of aroma compounds suggesting the involvement of hydrophobic effects with salivary mucins. Thus, aroma compounds with a high affinity for the mucosal pellicle can adsorb at the surface of the oral mucosa before being desorbed after changes in the in mouth thermodynamic equilibrium following swallowing. The second mechanism involved the reactivity of oral enzymes, such as xenobiotic metabolizing enzymes, toward aroma compounds. Thus, these two mechanisms have to be considered

in order to understand the phenomenon of aroma persistence observed *in vivo*, opening new avenues of research.

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge at-. Description of the aroma compounds assayed, Characteristics of the participants, Linearity of aroma compounds in static headspace analyses, Linearity of aroma compounds in static liquid phase analyses.

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