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

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Title

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Abstract

The present study aims to reveal the molecular mechanisms underlying aroma persistence, as it plays a major role in food appreciation and quality. A multidisciplinary approach including *ex vivo* experiments using a novel model of oral mucosa and saliva as well as *in vivo* dynamic instrumental and sensory experiments was applied. *Ex vivo* results showed a reduction in aroma release between 7 and 86% in the presence of the thin layer of salivary proteins covering the oral mucosa (mucosal pellicle). This reduction was explained by hydrophobic interactions involving the mucosal pellicle and by the ability of oral cells and saliva to metabolize specific aroma compounds. The *in vivo* evaluation of exhaled air and perception confirmed the *ex vivo* findings. In conclusion, this work reveals the need to consider physiological reactions occurring during food oral processing to better understand aroma persistence and open new avenues of research.

Keywords:

mucosal pellicle, aroma release, aroma perception, proton transfer reaction-mass spectrometry (PTR-MS), dynamic sensory evaluation, after-odour

1. Introduction

Everyone retains the memory of a delicious dish or glass of wine with long lasting aromas that prolong pleasure 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 daily, such as dental toiletries (i.e., toothpaste, breath fresheners). In contrast, an 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 some medicines for the strong, long-lasting sensations produced after their consumption. This phenomenon, called aroma persistence (Buettner, 2004; Buettner & Mestres, 2005; Linforth & Taylor, 2000), drives consumer behaviour since it is an essential criterion for product selection or avoidance. For that reason, the food industry, dental product manufacturers and the 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 (Sánchez-López et al., 2016).

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 (Buettner et al., 2008). Sensory active VOCs (aroma compounds) reach the olfactory receptors in the nasal cavity via the nasopharynx (Figure 1). In the process of smelling, aroma compounds reach the olfactory receptors via the external nares (orthonasal), avoiding contact with the oral tissues (Figure 1). Aroma persistence, which only concerns aroma perception, involves tissues of the oral cavity and the upper respiratory tracts (Figure 1). However, the molecular mechanisms allowing the

72 continuous release of aroma compounds from the mouth several tens of seconds or
73 minutes after swallowing remain poorly understood (Canon et al., 2018).
74
75 The first experimental works dedicated to studying aroma persistence were performed at
76 the beginning of the 21st century (Buffo et al., 2005; Hodgson et al., 2004; Linforth &
77 Taylor, 2000; Normand, 2004) thanks to the development of instrumental approaches
78 such as atmospheric pressure chemical ionization-mass spectrometry (APCI-MS)
79 (Linforth & Taylor, 1999) or proton transfer reaction-mass spectrometry (PTR-MS)
80 (Lindinger et al., 1998) that allowed us to measure *in vivo* aroma release in real time. The
81 results of these studies led to the hypothesis that aroma persistence is mainly driven by
82 the affinity of aroma compounds for the hydrated layer of mucosa (Figure 1), which is
83 mainly linked to their hydrophobicity and volatility in water at physiological temperature.
84 However, this hypothesis is not always fulfilled, and several studies have indicated that
85 the affinity of aroma compounds for water cannot be the only parameter involved in
86 aroma persistence (Buettner & Mestres, 2005; Esteban-Fernández et al., 2016; Muñoz-
87 González, Canon, et al., 2019). For instance, it has been reported that aroma compounds
88 can interact with salivary proteins (Figure 1), such as mucins (Pagès-Hélary et al., 2014).
89 Salivary mucins can be found free or bound to the mucosal pellicle, which is the thin layer
90 of proteins covering the surface of the oral mucosa (Figure 1). Recently, Ployon and
91 collaborators (Ployon et al., 2020) revealed that constituents of the mucosal pellicle might
92 influence aroma release kinetics. In addition to these mechanisms, it has been reported
93 that oral epithelial cells are able to metabolize aroma compounds (Perez-Jiménez et al.,
94 2020; Ployon et al., 2020), as observed for saliva (Buettner, 2002a, 2002b; Hussein et al.,
95 1983; Muñoz-González et al., 2018; Muñoz-González, Brulé, et al., 2019). Although
96 these works have been performed under *ex vivo* conditions, there is recent evidence that

metabolization of aroma compounds occurs *in vivo* and might influence aroma perception (Ijichi et al., 2019). Thus, the oral metabolization of aroma compounds could be another unexplored mechanism that explains the phenomenon of aroma persistence.

Thus, it can be hypothesized that the intensity and duration of perception of aroma compounds after oral processing is influenced by physiological factors (i.e., interaction with salivary proteins, metabolization by the oral mucosa or saliva) and physicochemical mechanisms (i.e., hydrophobic interactions with oral proteins). To explore this hypothesis, we executed a multidisciplinary approach coupling analytical chemistry and sensory analysis and used a cutting-edge in vitro model of the oral mucosa. Our strategy involved i) *ex vivo* investigations on the impact of a model of mucosa and saliva on aroma compounds using static experiments, ii) *in vivo* experiments measuring aroma release under dynamic conditions, and iii) sensory analysis on aroma persistence to decipher the impact of oral physiology on aroma persistence. Five aroma compounds (linalool, nonan-2-one, pentan-2-one, hexane-2,3-dione and octanal) belonging to different chemical families and presenting different physicochemical and sensory properties were selected for this study.

2. Materials and methods

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. Since there is no scientific evidence that aroma persistence is affected by age to date, participants were selected from the AlimaSSens panel (>70 y/o) based on their good physical and mental status. They provided informed written consent and were financially compensated for their participation.

2.1. Aroma compounds

Five food-grade aroma compounds (linalool, pentan-2-one, nonan-2-one, hexane-2,3-dione and octanal) (Sigma–Aldrich, Saint Quentin Fallavier, France) were selected for this study (Table A.1). They belong to different chemical families (ketone, aldehyde, terpene-alcohol) and present different physicochemical properties (hydrophobicity, volatility), which are known to affect their oral behaviour. Additionally, some of them have been previously reported to be metabolized in the presence of human saliva or oral cells (hexane-2,3-dione, octanal, nonan-2-one), while others have not (linalool, pentan-2-one). The purity of aroma compounds was higher than 98%. Independent stock solutions (1%) of the single aroma compounds were prepared in propylene glycol (Sigma–Aldrich, Saint Quentin Fallavier, France) before use and stored at 4 °C for a maximum of one month.

2.2 Oral mucosa model

The oral mucosa model was based on those previously developed by Ployon and coworkers (Ployon et al., 2016) using the TR146/MUC1 cell line. Briefly, the cells were seeded and cultured (density: 4.104 cell/cm^2). For the reconstitution of the mucosal pellicle samples, whole saliva samples were diluted into growth medium (1:1) onto 5-day cell subcultures for 2 h to form the different mucosal pellicles. Afterwards, the samples were washed twice with phosphate buffered saline (PBS) (Thermo Fisher Scientific, Gibco®, catalogue number: 12549079, Illkirch, France) at physiological pH, and the experiments were immediately carried out. The viability of TR146/MUC1 cells in the presence of aroma compounds (Ployon et al., 2020) was higher than 90%

2.3 Static headspace analyses

Static headspace analyses were performed 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). A total of 16 independent models of oral mucosa reconstituted with saliva samples from 16 individuals were analysed with their respective controls (cells and controls) (Table A.2). To perform the headspace analyses, 300 µl of the aroma solution prepared at 3 mg/l in PBS (pH=7.4) was added to the vials prepared under the three different conditions, sealed with silicone septa (Supelco, Bellefonte, PA, USA), and incubated at 37 °C for 30 min. Exposure to aroma compounds and subsequent analyses after washing with PBS was performed immediately, manually, and one by one. Each condition was analysed in triplicate with one injection per sample vial. Then, the headspace above the samples (200 µl) was automatically sampled (GERSTEL MPS2, Gerstel Inc., Mülheim an der Ruhr, Germany) and analysed in splitless mode as described previously (Muñoz-González et al., 2021). Briefly, a gas chromatograph (Agilent 6890 N; Agilent, Santa Clara, CA) coupled to an MS detector (Agilent 5973N, Agilent, Santa Clara, CA) (electron energy = 70 eV) was used for these analyses. The injector temperature was 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 were 250, 150, and 230 °C, respectively. A DBWAX column (30 m, 0.32 mm i.d., 0.5 µm; Agilent, Santa Clara, CA) with helium as the carrier gas at a velocity of 45 cm/s was used for compound separation. Each run lasted 20 min. The compounds present in the extracts were identified by comparing 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). The linearity and

repeatability of the procedure in an aqueous solution composed of a mixture of 5 aroma compounds at seven concentrations were measured to validate the methodology (Figure A.1).

2.4. Liquid-liquid extraction analyses

Vials containing the model mucosa (cells) or 200 µl of whole saliva (saliva) were incubated at 37 °C with 300 µl of the aroma solution (3 mg/l in PBS) at different times (0, 5, 30, 60, 120 min) to determine whether metabolization of aroma compounds occurs in the presence of oral components. After incubation, the aroma compounds were extracted with dichloromethane (Carlo Erba, Val de Reuil, France) following a previously described methodology (Muñoz-González et al., 2021). Briefly, the samples were spiked with 100 µL of the internal standard (methyl nonanoate (Sigma–Aldrich, Saint Quentin Fallavier, France) at 10 mg/l). Once dichloromethane (1 mL) was added, the samples were centrifuged (15000 g, 4 °C, 15 min). This procedure was repeated twice. Finally, the combined organic extracts were dried over anhydrous Na_2SO_4 (Sigma–Aldrich, Saint Quentin Fallavier, France) and concentrated under nitrogen to a total volume of 200 µL. One microlitre of the extracts was injected into the GC/MS in splitless mode following the abovementioned method. The procedure was also performed on the aroma solutions without oral components and the samples without aroma compounds. Relative areas were calculated by dividing the area of the peak of interest by the area of the internal standard. To validate the methodology, the linearity and repeatability of the procedure were evaluated in an aqueous solution composed of a mixture of 5 aroma compounds at six concentrations (Figure A.2).

2.5 *In vivo* aroma persistence by PTR-MS

In a previous study (Muñoz-González et al., 2021), 54 individuals (Table A.2) were selected 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), hexane-2,3-dione (20 mg/l), octanal (3 mg/l)) following a consumption protocol that consisted of doing mouth rinsing with the solution for 30 seconds to avoid swallowing and then swallowing all the liquid in their mouths while breathing normally. Afterwards, and every 30 seconds, the individuals were instructed to swallow the saliva accumulated in their mouths. Each individual performed the experiment on two different days (once per day). As described previously (Muñoz-González et al., 2021), the individual's nose space was measured through a Teflon nose piece 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-MS procedure can be found in (Muñoz-González et al., 2021). The release curves of the ions corresponding to the aroma compounds were extracted, and the area under the curve (AUC) was calculated for each of the selected ions and monitoring time, and the monitoring times corresponded to the five swallowing events performed by the individuals (t0-30s, t30-60s, t60-90s, t90-120s, t120-150s). The release data were analysed from the breath concentration data using IGOR Pro (WaveMetrics, Inc. Portland, USA).

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

Twenty-six individuals (Table A.2) with a normal sense of smell (Thomas-Danguin et al., 2003) were selected to perform the sensory evaluation. The sensory sessions took place

221 in a sensory testing room (21 ± 2 °C) of the ChemoSens platform (Centre des Sciences
222 du Goût et de l'Alimentation, INRAE, Dijon).
223
224 Two aroma compounds (hexane-2,3-dione and linalool) showed opposite behaviours in
225 the previous instrumental experiments were selected for the sensory analyses. The first
226 previous session reached a consensus in the assignment of aroma descriptors for each of
227 the two aroma compounds. The final descriptors were "butter" for hexane-2,3-dione and
228 "floral" for linalool. Both aroma compounds were independently evaluated. The samples
229 were prepared immediately before the sensory evaluations by diluting the stock solutions
230 in water (Evian, France). The participants were not allowed to smoke, eat or drink starting
231 at least one hour before the different sessions. The participants were trained in the
232 retronasal recognition of the two aroma descriptors and discrimination of their aroma
233 intensity by using 10-cm unstructured scales delimited at the ends (0=not very intense,
234 10=very intense). The evaluation of aroma persistence was performed by means of
235 dynamic sensory evaluation. The individuals were first familiarized with the dynamic and
236 discontinuous time–intensity methodology to do so. This methodology consisted of
237 subjects rating fixed attributes on a scale at predetermined time points. This technique
238 reduces the cognitive load and needs less training than the continuous time-intensity
239 technique (de Lavergne et al., 2016).
240
241 For the evaluation session, the aroma compounds were evaluated at a concentration of 9
242 mg/l for both compounds. This concentration was chosen to provide an accurate stimulus
243 (detection and repeatability) at the retronasal level for all panel members. The samples
244 (10 ml) with random three-digit codes were presented in plastic cups (50 ml) covered
245 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 smelling it (they were asked to cover their noses with their hands). Once the sample was in the oral cavity, the individuals were instructed to gently rinse their mouths with the solution for five seconds to avoid swallowing. After this time, they were instructed to swallow all the liquid in their mouths consisting of 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 evaluations 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 hexane-2,3-dione was evaluated first. From the notation at 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 across the two compounds and the three replicates at each time point. The first point (5 sec) was considered 100%, and aroma persistence was calculated with respect to this point. The sensory analyses were performed using Fizz® software (Biosystemes, Courtenon, France), and all the measurements were performed in triplicate.

2.7. Statistical analyses

Retention data of the aroma compounds in the three conditions (control, cells, and MP) were analysed by static headspace analyses, and data of the effects of the oral mucosa and saliva on aroma compounds analysed 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, the 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 (Addinsoft, Paris, France) and R (The R Foundation for Statistical Computing, Vienna, Austria) programs were used for data processing.

3. Results and discussion

3.1. *Ex vivo* investigation of the impact of a model of oral mucosa and saliva on aroma compounds

3.1.1. Impact of oral mucosa on aroma compounds using static headspace and liquid-liquid extraction analyses

The effect of the oral mucosa model on the release of aroma compounds was investigated using a model based on the TR146/MUC1 cell line (Ployon et al., 2016), which expresses the extracellular domain of mucin (MUC1/Y-LSP) on its surface (Zhang et al., 2013). This extracellular domain is involved in the formation of the mucosal pellicle (MP) (Ployon et al., 2016), which is the hydrated layer of epithelial and salivary proteins (Bradway et al., 1989) that is believed to participate in the phenomenon of aroma persistence by retention of aroma compounds in the mouth (Ployon et al., 2020).

In an attempt to unravel the mechanisms behind aroma persistence, static headspace analyses were performed after incubating aroma compounds (30 min, 37 °C) in the presence of the oral mucosa model with or without a mucosal pellicle (MP). Controls without the presence of the oral mucosa model were also evaluated. The number of aroma

296 compounds recovered in the headspace above the samples was expressed as a percentage
297 of the control condition (Figure 2.A). A percentage lower than 100% indicates that the
298 aroma compounds were present in a lower amount in the gas phase of the vials containing
299 the oral mucosa model with or without an MP than in the control samples.
300
301 Figure 2. A shows that the release of all compounds was significantly reduced by the
302 presence of the MP (26% for linalool, 24% for nonan-2-one, 7% for pentan-2-one, 66%
303 for hexane-2,3-dione and 86% for octanal in comparison to the control). Hexane-2,3-
304 dione and octanal were also significantly affected by the oral cell condition, and this
305 reduction was even more critical in the MP condition for both compounds. These effects
306 could mainly result from two mechanisms: (i) noncovalent interactions between the
307 surface of oral cells with or without MP or (ii) metabolization of the compounds.
308 Noncovalent interactions between aroma compounds and salivary proteins, such as
309 mucins, are thought to involve hydrophobic effects (Pagès-Hélary et al., 2014). The
310 results obtained in the previous HS analyses were plotted as a function of the polarity (log
311 P values) of aroma compounds to check the first mechanism (Figure 2.B). No correlation
312 was observed, considering the five aroma compounds assayed. This result suggests that
313 retention by MP was not only driven by hydrophobic effects. Additionally, no trend was
314 observed in the plotting of the HS data as a function of aroma compound volatility
315 (boiling point values) (Figure 2.C). However, it is important to note that two compounds
316 (hexane-2,3-dione and octanal) behaved very differently with regard to the other three
317 (linalool, nonan-2-one and pentan-2-one). Interestingly, these two compounds were the
318 only compounds significantly affected by the cell condition.
319

To deeply explore the impact of oral mucosa on aroma compounds, a liquid/liquid extraction with an organic solvent was performed in the incubated samples since this extraction breaks the noncovalent interactions between aroma compounds and MP (especially hydrophobic effects) while extracting the aroma compounds that remain in the liquid phase after incubation. The 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 hexane-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). MP contains different mucins, such as MUC1, MUC5B and MUC7, while in vitro interactions between aroma compounds and mucins have been reported by several authors (Friel & Taylor, 2001; Pagès-Hélary et al., 2014). It has been shown that mucins can retain aroma compounds through noncovalent interactions involving hydrophobic effects (Pagès-Hélary et al., 2014), which could be dissociated by an organic solvent. However, these results contrast with those previously obtained using a similar model of the mucosa, where no effect of MP on aroma compounds at equilibrium was observed (Ployon et al., 2020). In this previous study, centrifuged saliva was used to form the MP, while in the present study, whole saliva (richer in high molecular weight proteins such as mucins) was used. This differentiation could have increased the available binding sites and thus the retention of aroma compounds. Moreover, the composition of the MP depends on the saliva used to reconstitute the MP. In the present study, 16 models of mucosa

reconstituted with saliva samples from 16 different subjects (>70 y/o) were analysed, which could also have impacted the MP composition.

The other two compounds assayed, hexane-2,3-dione and octanal, showed a significantly lower recovery in the model mucosa samples (cells and MP) compared to the controls. The decrease in the concentration in the liquid phase of these two compounds in the presence of oral cells could result from the metabolization of these aroma compounds by oral cells, as previously observed with this model of the oral mucosa (Ployon et al., 2020).

Thus, the present results indicate that while MP exerted a compound-dependent retention effect on all the aroma compounds assayed, only two (hexane-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 using liquid-liquid extraction analyses

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

As observed in the preceding experiment (Figure 2), the recovery of linalool (Figure 3. A) and pentan-2-one (Figure 3. C) remained the same over time regardless of the tested conditions (control, cells and whole saliva), which indicates that these compounds were not metabolized by oral cells or saliva. Higher recovery of nonan-2-one was observed for the oral cell condition than 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 over time (Figure 3.B). Despite the surprisingly higher recovery of nonan-2-one in the cell condition than in the control, a reduction in this compound has been previously reported in the presence of oral cells (Ployon et al., 2020). Thus, the formation of the corresponding nonan-2-ol (Figure 3. B) was checked in the three conditions (control, cells and saliva). In the case of the cell 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 in nonan-2-one in the control condition cannot be attributed to a reduction in alcohol since nonan-2-ol was not detected in the controls. Thus, nonan-2-one seemed not to be affected by metabolization in the present experimental conditions. The compounds octanal and hexane-2,3-dione impacted their recovery by oral cells and whole saliva over time (Figure 3. D, 3.E). The decrease in the concentration of these two compounds suggests that hexane-2,3-dione and octanal are metabolized by saliva and oral cell enzymes. The metabolization of diketones and aldehydes has been previously observed *ex vivo* in the presence of saliva (Buettner, 2002b; Muñoz-González et al., 2018; Muñoz-González, Brulé, et al., 2019) or cellular enzymes (Robert-Hazotte et al., 2019; Schoumacker et al., 2016; Zaccone et al., 2015) and *in vivo* in the oral and nasal cavities (Ijichi et al., 2019). 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 saliva than in oral cells, while the octanal decrease was similar under both conditions. For hexane-2,3-dione (Figure 2. E), the formation of 3-hydroxy-hexane-2-one and 2-hydroxy-hexane-3-one was observed in cells and whole saliva conditions, while the disappearance of hexane-2,3-dione was higher in the presence of oral cells than in saliva.

In all cases, the observed reactions corresponded to the reduction of the carbonyl groups from aldehydes and ketones (Figure 3.F), giving rise to metabolites that present different sensory properties (descriptors or odour thresholds) than the initial compounds. Aldehydes are usually more reactive towards nucleophilic substitutions than ketones because of both steric and electronic effects, while diketones have two carbonyl groups. As a result, hexane-2,3-dione and octanal are reactive compounds that organisms might neutralize by their metabolization. These reactions could be carried out by a range of enzymes named odourant metabolizing enzymes (Heydel et al., 2016) that belong to the xenobiotic metabolism enzyme family. The different metabolization of compounds in the presence of 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 (Schwartz et al., 2021), among others.

Overall, these experiments confirm that oral cells and whole saliva can metabolize aroma compounds *ex vivo* depending on their structure. Thus, it can be hypothesized that the oral metabolization of hexane-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-MS

A real-time instrumental approach was used to validate our hypothesis in an *in vivo* context by coupling PTR-MS with the nasal cavities of 54 subjects. *In vivo* aroma persistence was monitored after the individuals consumed a model solution flavoured

420 with five aroma compounds following an imposed consumption protocol. Data from the
421 panel (n=54) averaged considering 30-second intervals after sample swallowing, are
422 shown in Figure 4. Data are expressed considering the AUC values of the first 30 seconds
423 after sample swallowing as 100% to allow compound comparison and calculate the
424 percentage relative to it for the next 150 seconds.
425
426 As expected, once the sample was swallowed, there was a progressive decrease in aroma
427 persistence for all compounds (Figure 4). The extent of the decrease was compound
428 dependent, and the compounds did not disappear at the same rate in the nasal cavity of
429 the individuals. Since the second interval of time ($t_{30\text{-}60\ s}$), significant differences between
430 compounds were observed, with linalool being the most persistent compound, followed
431 by nonan-2-one and pentan-2-one. These three compounds remained in the breath at 61,
432 41 and 35% of their concentrations were recorded during the second interval of
433 monitoring time ($_{t30\text{-}60s}$). As hypothesized above, hexane-2,3-dione and octanal were less
434 persistent in the breath, and their concentrations at this monitoring time represented 19
435 and 12% of the initial concentration ($t_{0\text{-}30s}$), respectively. From the third interval ($t_{60\text{-}90\ s}$),
436 a significant difference was observed between nonan-2-one and pentan-2-one, the former
437 being more persistent than the latter. In the last interval of monitoring time ($t_{120\text{-}150\ s}$),
438 nonan-2-one and linalool concentrations still represented 20-27% of the first interval.
439 Pentan-2-one was found at 13%, while hexane-2,3-dione and octanal almost disappeared
440 in the breath at that time (<4%). Thus, linalool, nonan-2-one and pentan-2-one were
441 significantly more persistent in the breath of the subjects than hexane-2,3-dione and
442 octanal. These results are in accordance with those observed *ex vivo* using oral
443 components (oral mucosa model, MP and saliva). Compounds less susceptible to
444 metabolization (linalool, nonan-2-one and pentan-2-one) displayed lower decay rates in

445 the *in vivo* experiment and were the most persistent compounds in the breath of the
446 subjects. Accordingly, they showed a significant retention by MP in *ex vivo* experiments
447 (linalool: 26%; nonan-2-one: 24%; pentan-2-one: 7%) under static conditions, which was
448 in line with their hydrophobicity values (linalool: logP 2.97; nonan-2-one: logP 2.70;
449 pentan-2-one: logP 0.75). This behaviour was maintained in the *in vivo* experience, which
450 suggests their retention in the mouth by hydrophobic interactions with MP. Additionally,
451 the two compounds that showed clear metabolization by oral components *ex vivo*
452 presented the lowest persistence *in vivo*. However, these two reactive compounds behave
453 similarly (octanal and hexane-2,3-dione), despite their differences in terms of
454 hydrophobicity (log P 2.80 and -0.35, respectively).
455
456 The m/z corresponding to the metabolites previously reported in the *ex vivo* experiments
457 (99.1 hydroxy-hexane-one and 113.1 octan-1-ol) were extracted from the release curves
458 obtained by PTR-MS to check if metabolization of aroma compounds occurs *in vivo*.
459 They were plotted together with those of hexane-2,3-dione and octanal expressed as the
460 % of aroma released per unit of time (Figure A.3). Since once the solution is swallowed,
461 the formation of metabolites will be dependent on the remaining amount of the original
462 aroma in the mouth, data corresponding to the formation of metabolites were expressed
463 as the ratio between the % of metabolite and that of the original compound released for
464 each time point of analysis (Figure A.4). The ratio of the metabolites showed increases
465 over time, which would support the metabolization of these compounds in *in vivo*
466 conditions.
467
468 Overall, these results highlight that aroma persistence relies not only on the
469 physicochemical properties of aroma compounds, such as hydrophobicity and volatility,

as previously proposed (Buffo et al., 2005; Hodgson et al., 2004; Linforth & Taylor, 2000; Normand, 2004; Sánchez-López et al., 2016) 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* (Ployon et al., 2020) and *in vivo* (Pérez-Jiménez et al., 2021), this is the first experimental work that demonstrates that the metabolism of aroma compounds impacts 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 hexane-2,3-dione. To confirm this hypothesis, a sensory experiment was carried out with two of the compounds that presented contrasting behaviour in the instrumental experiments (linalool and hexane-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 (hexane-2,3-dione) over time was evaluated by 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 every 5 seconds for 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 the monitoring times.

As observed in the instrumental PTR-MS experiment, once the sample was swallowed, there was a progressive decrease in 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, the persistence of the butter descriptor (elicited by hexane-2,3-dione) was significantly lower than that of the floral descriptor (elicited by linalool) ($p<0.05$), confirming previous instrumental findings. However, it is interesting to note that the difference between compounds was higher in the instrumental experiment than in the sensory experiment. Different reasons can explain this fact. First, it is difficult to compare the sensitivity of the human nose with that of PTR-MS. The higher complexity of the sensory study that needs, in addition to in-mouth processing, the integration of the information in the brain, means that interindividual differences among participants are even larger in magnitude.

Moreover, slight differences in the consumption protocol (aroma concentration, rinsing time, duration, etc.) could have influenced the results. Thus, in the instrumental approach, participants were instructed to rinse their mouths for 30 seconds, while in the sensory experiment, the duration of rinsing was only 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 the reduction of hexane-2,3-dione could have contributed to the overall perception by the individuals, as suggested for other aroma compounds by Ijichi and coworkers (Ijichi et al., 2019). They found that the metabolites of aroma compounds produced *in vivo* are perceived as part of the aroma quality of the original aroma. Although the metabolites formed usually present higher odour 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 phenomenon involving the reactivity of the oral mucosa and saliva and is 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, towards aroma compounds. Thus, while the mechanisms of aroma persistence involve the adsorption of aroma compounds at the surface of the oral mucosa as a function of their affinity, aroma compounds that are metabolized by saliva and oral cells are less persistent than unmetabolized compounds.

Credit authorship contribution statement

Conceptualization, C.M-G.; Data curation, C.M-G.; Formal analysis, C.M.-G. and M.B.; Funding acquisition, C.M.-G., G.F. and F.C.; Methodology, C.M.-G., C.M., G.F. and F.C.; Roles/Writing - original draft, C.M.-G.; Writing - review & editing C.M.-G., M.B., C.M., G.F. and F.C. All authors gave final approval for manuscript revision and submission.

Declaration of Competing Interest

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

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693 **Figure Captions.**

694 **Figure 1.** Scheme of aroma perception and hypothesis of the role of oral surfaces in oral
695 aroma persistence. (1) Retention of aroma compounds by the hydrated layer of mucosa,
696 (2) Interaction of aroma compounds with salivary proteins (MSP: mucosa salivary
697 proteins; MUC5B: mucin5B; MUC1: mucin1).

698

699 **Figure 2. (A)** Aroma partitioning in cells or mucosal pellicle (MP) samples by HS-
700 GC/MS expressed as percentages relative to controls. **(B)** Relationship between
701 hydrophobicity (log P values) of the aroma compounds and their partitioning in MP
702 samples as percentages relative to controls. **(C)** Relationship between volatility (boiling
703 point values) of the aroma compounds and their partitioning in MP samples as
704 percentages relative to controls. **(D)** Aroma recovered by LLE-GC/MS in the controls,
705 cells and MP samples after incubation with the five aroma compounds. The results from
706 the bar graphs are presented as the mean value ± SD. Different letters indicate significant
707 differences (p value < 0.005) between the conditions after applying the Tukey test.

708

709 **Figure 3.** Compounds identified in the controls, cells and whole saliva samples over time
710 for **(A)** linalool, **(B)** nonan-2-one, **(C)** pentan-2-one, **(D)** octanal and **(E)** hexane-2,3-
711 dione. Data are expressed as percentages relative to the concentration obtained at t0 in
712 the controls for the original compounds and as relative areas for their metabolites. All
713 results are presented as the mean value ± SD. **(F)** Schematic of the reduction of aroma
714 compounds observed during *ex vivo* incubations.

715

716 **Figure 4.** *In vivo* aroma persistence measured in 54 subjects by PTR-ToF-MS after they
717 consumed a solution flavoured with five aroma compounds. Values are expressed as a

718 percentage considering the AUC of the first 30 sec as 100% and calculating the ratio
719 relative to it for the rest of the monitoring times. A ratio lower than 100% indicates that
720 aroma persistence decreases over time. The results from the bar graphs are presented as
721 the mean value ± SD. Different letters indicate significant differences (p value < 0.001)
722 between the conditions after application of the Tukey test.
723
724 **Figure 5.** *In vivo* aroma persistence was measured in 26 subjects by dynamic sensory
725 analysis after the consumption of two solutions, one flavoured with linalool (floral
726 descriptor) and the other flavoured with hexane-2,3-dione (butter descriptor). Values are
727 expressed as the percentage of persistence considering the first monitoring time (5 sec)
728 as 100% and calculating the ratio relative to it for the rest of monitoring times. A ratio
729 lower than 100% indicates that aroma persistence decreases over time. The results from
730 the bar graphs are presented as the mean value ± SD. Different letters indicate significant
731 differences (p value < 0.05) between the conditions after application of the Tukey test.
732
733

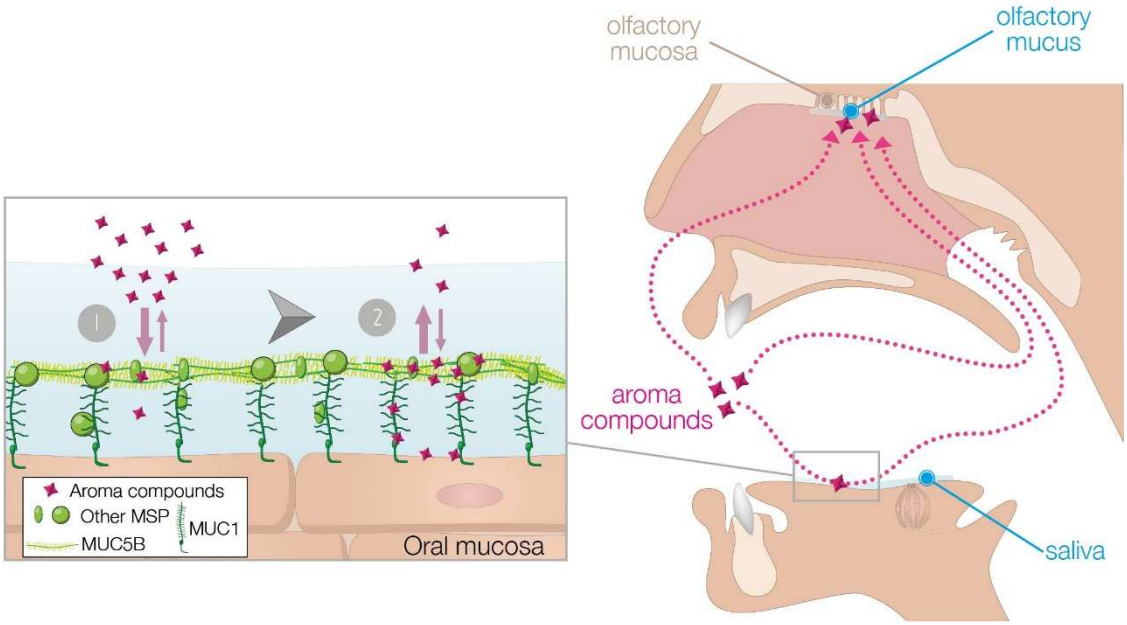
olfactory mucosa
olfactory mucus
aroma compounds
saliva
1
2
Aroma compounds
Other MSP
MUC1
MUC5B
Oral mucosa

Figure 1.

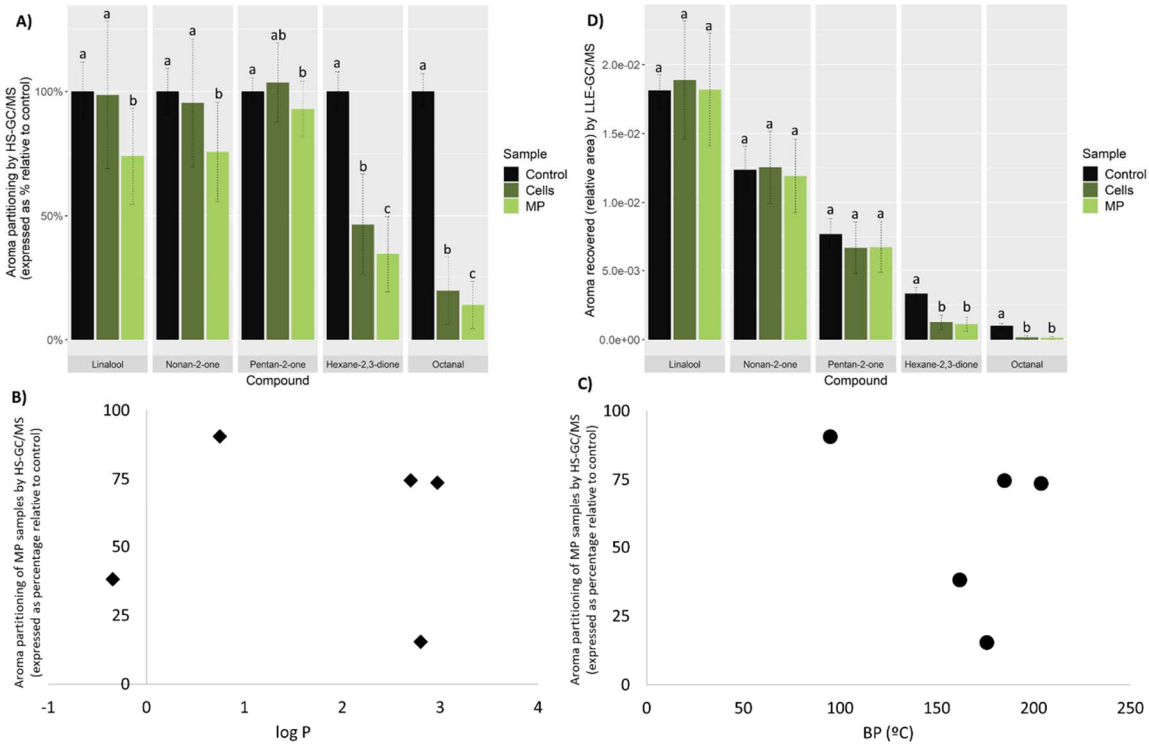
A)
Aroma partitioning by HS-GC/MS (expressed as % relative to control)
100%
50%
0%
Linalool
Nonan-2-one
Pentan-2-one
Hexane-2,3-dione
Octanal
Compound
Sample
Control
Cells
MP
D)
Aroma recovered (relative area) by LLE-GC/MS
2.0e-02
1.5e-02
1.0e-02
5.0e-03
0.0e+00
B)
Aroma partitioning of MP samples by HS-GC/MS (expressed as percentage relative to control)
log P
C)
BP (ºC)

Figure 2.

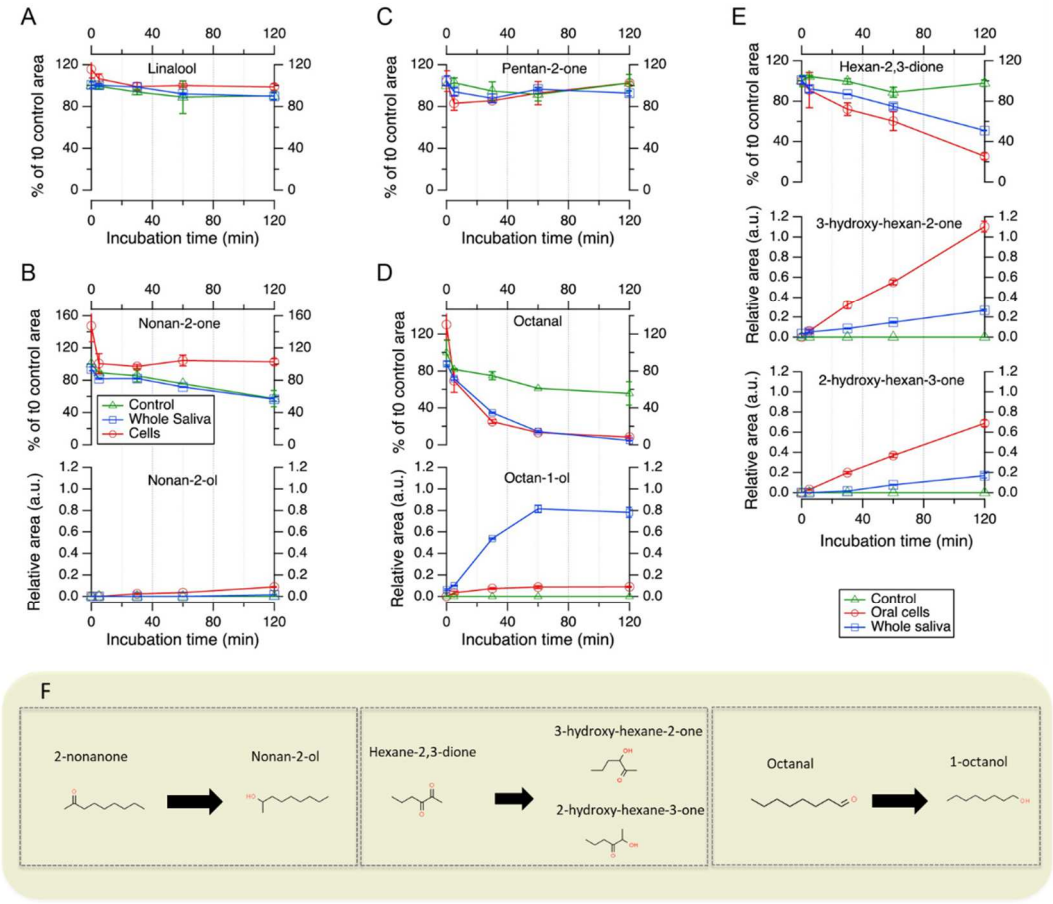
A
Linalool
% of t0 control area
Incubation time (min)
B
Nonan-2-one
Control
Whole Saliva
Cells
Nonan-2-ol
Relative area (a.u.)
Incubation time (min)
C
Pentan-2-one
D
Octanal
Octan-1-ol
E
Hexan-2,3-dione
3-hydroxy-hexan-2-one
2-hydroxy-hexan-3-one
Control
Oral cells
Whole saliva
F
2-nonanone
Nonan-2-ol
Hexane-2,3-dione
3-hydroxy-hexane-2-one
2-hydroxy-hexane-3-one
Octanal
1-octanol

Figure 3.

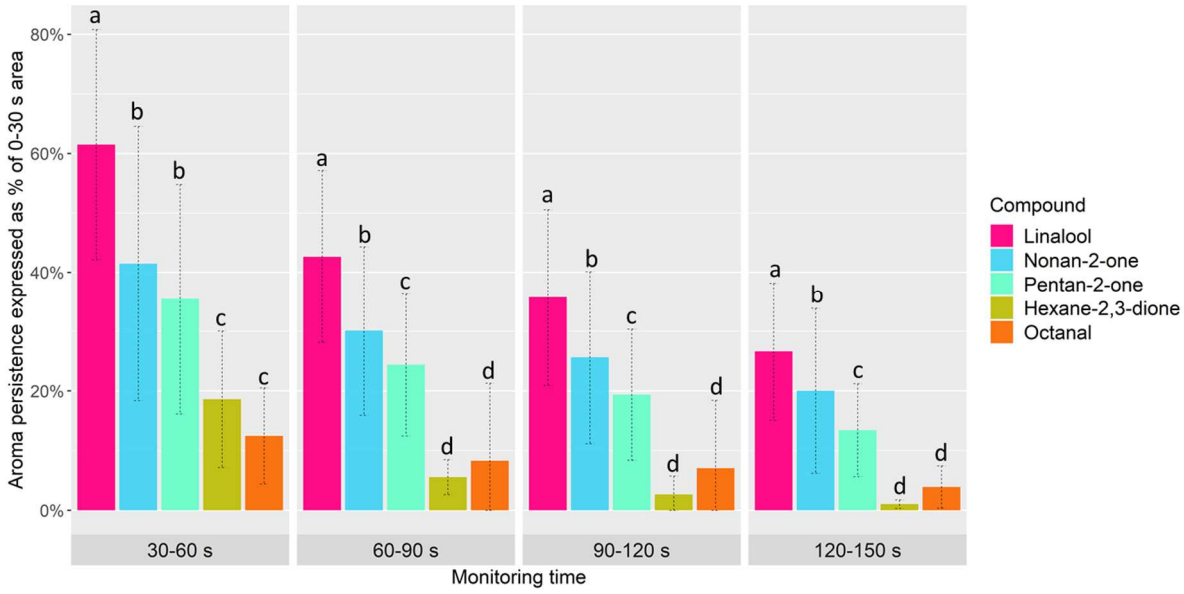
Aroma persistence expressed as % of 0-30 s area
80%
60%
40%
20%
0%
a
b
b
c
c
a
b
c
d
d
a
b
c
d
d
a
b
c
d
d
30-60 s
60-90 s
90-120 s
120-150 s
Monitoring time
Compound
Linalool
Nonan-2-one
Pentan-2-one
Hexane-2,3-dione
Octanal

Figure 4.

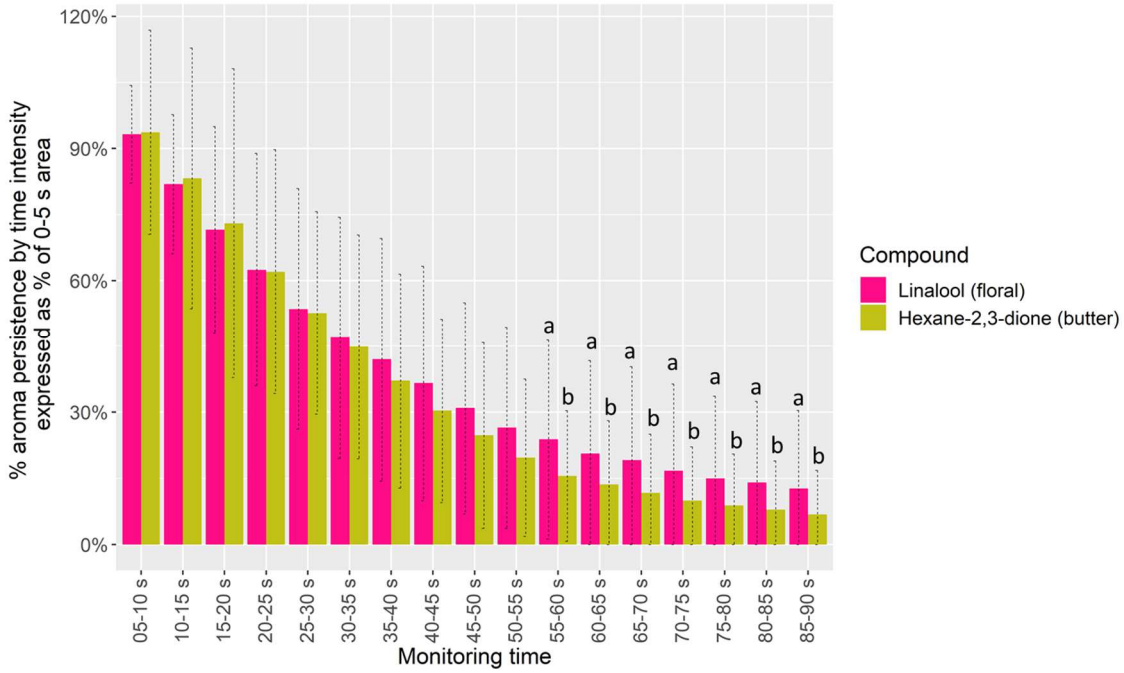
120%
90%
60%
30%
0%
% aroma persistence by time intensity expressed as % of 0-5 s area
a
a
a
a
a
a
a
b
b
b
b
b
b
b
05-10 s
10-15 s
15-20 s
20-25 s
25-30 s
30-35 s
35-40 s
40-45 s
45-50 s
50-55 s
55-60 s
60-65 s
65-70 s
70-75 s
75-80 s
80-85 s
85-90 s
Monitoring time
Compound
Linalool (floral)
Hexane-2,3-dione (butter)

Figure 5.