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1 Understanding retention and metabolization of aroma compounds using

2 an *in vitro* model of oral mucosa

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8 Abstract

The mechanism leading to aroma persistence during eating is not fully described. This study aims 9 at better understanding the role of the oral mucosa in this phenomenon. Release of 14 volatile 10 compounds from different chemical classes was studied after exposure to in vitro models of oral 11 mucosa, at equilibrium by Gas-Chromatography-Flame Ionization Detection (GC-FID) and in 12 dynamic conditions by Proton Transfer Reaction- Mass Spectrometry (PTR-MS). Measurements at 13 equilibrium showed that mucosal hydration reduced the release of only two compounds, pentan-2-14 one and linalool (p<0.05), and suggested that cells could metabolize aroma compounds from 15 different chemical families (penta-2,3-dione, trans-2-hexen-1-al, ethyl hexanoate, nonan- and decan-16 2-one). Dynamic analyses for pentan-2-one and octan-2-one evidenced that the constituents of the 17 mucosal pellicle influenced release kinetics differently depending on molecule hydrophobicity. This 18 19 work suggests that mucosal cells can metabolize aroma compounds and that non-covalent interactions occur between aroma compounds and oral mucosa depending on aroma chemical 20 structure. 21

22 Keywords

Aroma persistence, oral mucosa, mucosal pellicle, TR146/MUC1 cells, aroma retention, aroma

24 metabolism, aroma release, in vitro model

26 **1. Introduction**

When consuming food, most aroma notes are perceived almost instantly after placing food in the 27 28 mouth and they dissipate rapidly after swallowing, while some continue to be perceived for a longer 29 time. This phenomenon, called aroma persistence or "long lasting aroma" contributes to the quality of food. However, the biological and physicochemical mechanisms responsible for persistence are 30 not fully understood. Aroma perception during eating is a complex process, initiated by the release 31 of odorants from the food into the oral cavity and their transport via the retronasal route to the 32 33 olfactory receptors in the nose. The main hypothesis for explaining aroma persistence is that aroma compounds adsorb at the surface of the oral mucosa, before being progressively desorbed and 34 released into the oral cavity after the equilibrium has changed due to food swallowing (Buettner, 35 Beer, Hannig, Settles, & Schieberle, 2002; Esteban-Fernandez, Rocha-Alcubilla, Munoz-Gonzalez, 36 Moreno-Arribas, & Pozo-Bayon, 2016). This implies that aroma compounds bind through non-37 covalent interactions to the mucosal surface, as reported for tannins in astringency perception 38 (Ployon et al., 2018). Some in vivo experiments have evaluated the ability of the oral and pharyngeal 39 mucosae to retain aroma compounds, for example by the Spit-Off Odorant Measurement method, 40 41 i.e. quantification of odorants remaining in aqueous samples (Buettner et al., 2002; Hussein, 42 Kachikian, & Pidel, 1983) and wine (Esteban-Fernandez et al., 2016) after expectoration. Other in vivo measurements followed in-mouth release of aroma compounds using the Buccal Odor 43 Screening System (Buettner & Welle, 2004) or more recently an intra oral Solid phase 44 Microextraction (SPME) fiber (Esteban-Ferñandez, Munoz-Gonzalez, Jimenez-Giron, Perez-45 Jimenez, & Pozo-Bayon, 2018; Esteban-Fernandez et al., 2016) and Proton-Transfer-Reaction 46 Mass Spectrometry (PTR-MS) (Muñoz-Gonzalez, Canon, Feron, Guichard, & Pozo-Bayon, 2019; 47 Sanchez-Lopez, Ziere, Martins, Zimmermann, & Yeretzian, 2016). It emerges from those studies 48 49 that the physicochemical properties of aroma compounds do not fully explain in-mouth persistence. For example, a decrease of intra-oral persistence with aroma compounds polarity was noted 50 (Buettner & Schieberle, 2000) but guaiacol, a highly polar compound, has high intra-oral retention 51 52 (Esteban-Fernandez et al., 2016) and higher persistence than less polar compounds (Muñoz-Gonzalez et al., 2019). Also globally the most persistent compounds are hydrophilic and the least 53 persistent ones are hydrophobic, but there are many exceptions and compounds with similar 54

hydrophobicity may have very different persistence behaviors (Esteban-Fernandez et al., 2016; 55 Linforth & Taylor, 2000). This may be explained by the different natures of the non-covalent 56 interactions involved. Another mechanism to consider when studying persistence is that some 57 58 aromas may be enzymatically converted to new compounds in the oral cavity by salivary enzymes (Buettner, 2002a, 2002b; Pagès-Hélary, Andriot, Guichard, & Canon, 2014) or by cellular enzymes 59 as demonstrated in the nasal cavity (Robert-Hazotte et al., 2019; Schoumacker, Robert-Hazotte, 60 Heydel, Faure, & Le Quere, 2016). The outer part of the oral mucosa is composed of an epithelium 61 62 onto which is anchored the mucosal pellicle, a hydrated layer of epithelial and salivary proteins (Bradway, Bergey, Jones, & Levine, 1989). Mucins are very abundant at the mucosal surface, 63 specifically the salivary mucins MUC5B and MUC7 (Gibbins, Proctor, Yakubov, Wilson, & Carpenter, 64 2014). Because mucins have a well-documented capacity to interact with aroma compounds (Friel 65 & Taylor, 2001; Muñoz-González, Feron, & Canon, 2018; Pagès-Hélary et al., 2014; Ployon, Morzel, 66 & Canon, 2017), the involvement of the mucosal pellicle in aroma persistence arouses the interest 67 of food scientists (Canon, Neiers, & Guichard, 2018). The purpose of this work was to evaluate the 68 capacity of the oral mucosa to interact with aroma compounds, to describe the respective role of the 69 70 cell surface and the mucosal pellicle in this phenomenon and to identify the nature of the interactions involved. The strategy was to measure aroma release in presence of an *in vitro* model of oral mucosa 71 previously developed (Ployon, Belloir, Bonnotte, Lherminier, Canon, & Morzel, 2016). As any 72 73 simplified model, this system presents some limitations. For example, the preserved cells surface 74 integrity of the model differs from the cell status in the superficial layer of mouth mucosa, and interactions between aromas and other food matrix constituents are not considered. However, the 75 use of an *in vitro* model allowed controlling the experimental parameters (e.g. air flow, volume of the 76 77 system, pellicle composition) and avoided human inter-individual variability. First, static headspace 78 measurements were performed to investigate the capacity of the model mucosa (without and with a pellicle) to retain aroma compounds at thermodynamic equilibrium, using gas-chromatography -79 flame ionization detection (GC-FID). Then, a real-time monitoring method by PTR-MS was 80 developed to study the dynamic of aroma compounds release from the model mucosa. The ability 81 of the model mucosa to metabolize aroma compounds was also investigated in both static and 82 dynamic approaches. 83

2. Material and methods

85 2.1. Saliva collection

The study was performed in accordance with the guidelines of the declaration of Helsinki. 86 Participants provided written informed consent when enrolling into the study. Saliva was obtained 87 from fifteen volunteers who declared to be in good oral condition. Volunteers refrained from smoking, 88 89 eating or drinking for at least two hours before saliva collection. Subjects donated saliva during 90 approximately 1 h by spitting out at their own rhythm saliva accumulating spontaneously in their 91 mouth into plastic vessels. Over the whole collection time, plastic vessels were kept on ice in order 92 to limit alteration and bacterial development. All samples were subsequently pooled and centrifuged at 14 000 g for 20 min at 4 °C. The resulting pool of clarified saliva was aliguoted and immediately 93 frozen at - 80 °C. 94

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2.2. Cell-based model of oral mucosa

The TR146/MUC1 cell line was used in this study. Cells were seeded at a density of 4.10⁴ cell/cm² in 10 ml modified headspace vials coated with Cell-TakTM (Corning Life Sciences, New York, NY, USA). Cells were cultured during 5 days as previously described (Ployon et al., 2016). In order to form a mucosal pellicle, clarified saliva diluted into growth medium (1:1) was deposited onto 5-days cells subcultures for 2 h. After incubation, samples were washed twice with PBS in order to eliminate the non-adsorbed saliva. Exposure to aroma compounds and subsequent analyses were performed immediately after washing with PBS.

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2.3. Aroma compounds

Aroma compounds were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Compounds used in this study and their relevant chemical properties are listed in table 1. Stock solutions were prepared in water at concentrations below the solubility threshold, and kept at 4°C. In order to evaluate the effect of molecule hydrophobicity, we studied a series of linear methyl ketones from C5 to C10, having a log *P* value, which refers to the molecule hydrophobicity, ranging from 0.91 to 3.73. The effect of the position of the chemical functional group was probed by comparing different methyl ketones having their ketone function in position 2 and 3.

2.4. Toxicity assessment

The toxicity of aroma compounds on TR146/MUC1 cells was evaluated. The cells were seeded in 112 96-well plates. Confluent cells were incubated with 200 µl of aroma solutions at 10⁻⁴ mol/l in PBS 113 (except for guaiacol prepared at 10⁻³ mol/l in PBS) for 1 h à 37 °C. The concentrations in mg/l are 114 listed in table 1. Molecule toxicity was assessed using the Neutral Red assay (Rat, Korwinzmijowska, 115 Warnet, & Adolphe, 1994). Briefly, after incubation with aroma compounds, cells were incubated for 116 3 h at 37 °C with 200 ml of medium containing neutral red at 50 mg/ml, washed twice with PBS and 117 then incubated at room temperature for 1 h in neutral red eluent (ethanol:H₂O:acetic acid, 50:49:1) 118 with gentle agitation. Reading of fluorescence was performed with Victor3V microplate reader 119 (PerkinElmer) with excitation and emission wavelengths fixed at 544 nm and 595 nm, respectively. 120 Assays were performed in duplicates. Viability of cells was above 90% for all aromas, confirming 121 their non-cytotoxicity. 122

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2.5. Evaluation of residual water retained on cell surfaces

After rinsing with PBS, the cells' surface remains covered by a thin layer of residual PBS. Since this 124 residual liquid phase may affect aroma retention and release, the PBS volume remaining onto the 125 cells' surface after rinsing was estimated. Six vials containing the model mucosa were washed with 126 127 PBS and immediately weighed. Open vials were evaporated for 30 min at room temperature and weighed again. The amount of PBS remaining onto cell surface was estimated as the difference 128 between the two weights and was calculated to be 24.9 ± 5.6 mg. In order to take into account the 129 hydration of mucosa in the experiments, 25 µl of PBS were added to the control vials (without 130 131 mucosa): this condition is referred to as "hydrated control (HC)".

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2.6. Static equilibrium headspace analysis

Single aroma solutions at 10⁻⁴ mol/l in PBS were prepared from stock solutions (cf table 1), except for guaiacol and pyzarines for which 10⁻³ mol/l solutions were prepared because preliminary work revealed that in our conditions, the molecules were not detected at 10⁻⁴ mol/l by GC-FID headspace analysis. pH of the solutions was set at 7.4. In order to avoid competition between aroma compounds, each molecule was tested individually. 300 µl of a single aroma solution were added to the vial, which was then sealed with silicone septum in magnetic caps (Supelco, Bellefont, PA, USA).

For each molecule, equilibrium headspace analysis was performed in 4 conditions: an empty vial 139 named dry control (DC), an empty vial with 25 µl of PBS named hydrated control (HC), a vial 140 containing the TR146/MUC1 cells (T) and a vial containing the model mucosa: TR146/MUC1 cells 141 142 with the mucosal pellicle (TP). Headspace analysis of an empty vial with 25 µl of PBS + 150 µl of clarified saliva + 150 µl of aroma solution at 2.10⁴ mol/L (i.e. final concentration of aroma compounds 143 is 10⁴ mol/L) (CS) was also performed to determine the effect of the clarified saliva on aroma release. 144 Static headspace sampling (SHS) experiments were performed using GC-FID. Vials were placed 145 146 into the incubator of an automatic sampler (GERSTEL MPS2, Gerstel Inc., Mülheim an der Ruhr, Germany) and incubated at 37 °C for 40 min. Preliminary experiments confirmed that the 147 thermodynamic equilibrium was reached after this duration in the control condition (HC). 148

100 µl of the headspace were sampled automatically using a syringe preheated at 42 °C and 149 analyzed in splitless mode by a gas chromatograph coupled to a FID detector (Agilent 7890B, Agilent 150 Technologies, Santa Clara, CA, USA). A 250 µl liner was used. Injector temperature was set at 151 240 °C and detector temperature was set at 250 °C. A DB-WAX column (30 m, 0.32 mm i.d., 0.5 µm; 152 Agilent Technologies) was used with helium as carrier gas at a velocity of 21 cm/s. For each 153 154 compound, the oven temperature was set to values leading to a retention time between 2 and 4 min (Table 1). Each condition was tested in triplicate, repeating the analysis sequence DC, HC, T and 155 TP three times. For each aroma compound, a calibration curve was established by GC/FID in the 156 157 same analytical conditions as reported above and using a 1 µl liquid injection of a solution of aroma 158 compounds in CH₂Cl₂ using OpenLab (Agilent Technologies, Santa Clara, CA, USA) (Supplementary Material S2). The calibration curves were used to determine the concentration of 159 160 each aroma compound in the gas phase.

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2.7. Analysis of compounds degradation by GC-MS.

GC-MS analyses were performed to determine whether bioconversion occurred and to identify the resulting metabolites. The degradation of molecules was tested only for molecules for which a decrease in headspace concentration was observed (i.e pentan-2,3-dione, trans-2-hexen-1-al, ethyl hexanoate, nonan-2-one, and decan-2-one). For those molecules, 5 vials containing the model mucosa without pellicle (T) were incubated 40 min at 37 °C with 300 µl of aroma solution at 10⁻⁴ mol/l

in PBS. After incubation, supernatants were pooled (i.e. 1.5 ml) and aroma compounds were 167 extracted with 750 µl of CH₂Cl₂. As a control, the same volume of aroma solution (1.5 ml) non 168 exposed to cells was extracted with 750 µl of CH₂Cl₂. 1 µl of extract was analyzed by GC-MS. A 169 170 6890A gas chromatograph coupled to a 5973N mass selective detector (Agilent Technologies) was used. For electron ionization (EI), analyses were done at an electron energy of 70 eV at a rate of 4 171 scans/s, covering the m/z range of 29-350 with a source temperature of 230 °C. The injector 172 temperature was set at 240 °C. A DB-WAX column (30 m, 0.32 mm i.d., 0.5 µm; Agilent 173 Technologies) was used with helium as carrier gas at a velocity of 44 cm/s. The initial oven 174 temperature was set at 40 °C for 5 min then increased to 240 °C at a rate of 5 °C/min. The 175 compounds present in the extract were identified by comparison of their MS spectra to an internal 176 mass spectra database (INRAMASS) and to mass spectra databases (NIST 2008, Wiley 138). 177

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2.8. Conversion rate calculation

For each compound, we determined its concentration in the liquid phase from its partition coefficient in the buffer (HC) and concentration in the gas phase (C_{gas}). First, we calculated the concentration of aroma compounds in the gas phase in the different conditions from the peak areas using the calibration curves (Supp. Material S2). Then, the partition coefficient of each compound was determined in the buffer condition by the formula $K_{HC} = C_{gasHC}/C_{liqHC}$.

Then, the liquid phase concentrations C_{liq} in the T and TP conditions were calculated using the formula $C_{liq(T \text{ or } TP)} = C_{gas (T \text{ or } TP)}/K_{HC}$. Conversion rates were calculated in T or TP condition as following: $r = (C_{liq(T \text{ or } TP)}(t0) - C_{liq(T \text{ or } TP)}(t40))/40$.

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2.9. Dynamic aroma release monitoring by PTR-ToF-MS

In this part, two aroma compounds that did not appear metabolized by the cells were tested: pentan-2-one (MH⁺ m/z = 87.14) and octan-2-one (MH⁺ m/z = 129.22). Aroma solutions at 10⁻⁵ mol/l in PBS ([pentan-2one] = 0.86 mg/l and [octan-2-one] = 1.28 mg/l) were prepared from stock solutions. 300 µl were injected in the vial using an automatic liquid dropper. Three biological replicates were analyzed per aroma compound. Compounds were analyzed separately in order to avoid competition using proton transfer reaction – mass spectrometry (PTR-MS). This technique allows the ionization of a volatile molecule through a proton transfer from [H₂O+H]⁺ ions to the volatile depending on its proton

affinity. For most volatile organic compounds their proton affinity is above that of water. The 195 instrument used in this study includes a time-of-flight analyzer providing high resolution and high 196 speed of acquisition. Thus, this instrument allows real time monitoring on a large range of m/z of 197 volatile organic compounds, such as aromas. Furthermore, this more sensitive technique allows 198 using lower aroma concentrations: this presents the advantage of limiting the risk of saturating the 199 mucosa. The experimental device is illustrated in figure 4A. A PTR-ToF-MS (PTR-ToF-MS 8000, 200 Ionicon Analytik, Innsbruck, Austria) was used with a scanning speed of 108 ms/spectrum for a mass 201 202 range from 0 to 250 u. Calibration was performed following ions at m/z 21.022086 u ($[H_2^{18}O + H]^+$); 39.03265 u $[H_2O+H_2^{18}O + H]^+$ and 59.042141 [acetone + H]⁺. $[H_2O + H]^+$ was used as reacting ion. 203 Analyses were performed under a drift tube pressure of 2.3 mbar, at 80 °C, a voltage of 490 V and 204 a ratio E/N of 110 Td. The air flow at the entrance of the system was set at 100 ml/min. 205

A vial containing the model mucosa, without (T) or with (TP) a mucosal pellicle, was closed by a 3-206 way cap with silicon septum. A first way was connected to a Tedlar® bag containing wet air. A second 207 way was connected to the PTR-MS. Aroma injection was performed through the third way. Two 3-208 209 way automatic valves were used to direct the airflow way through to two parallel circuits. The circuit 210 connected to the glass vial with the model mucosa is called "indirect", while the second circuit, directly connected to the Tedlar® bag, is called "direct". The experiment started with the circuit in 211 direct position. Aromatized gas was injected into the vials by the third way of the vial cap and exposed 212 213 to the model mucosa for 1 min. Then, the circuit was turned to the indirect position and the air flow 214 from the Tedlar® bag swept the glass vial headspace to the PTR for 3 minutes. The composition of the gas was analyzed by PTR-MS analysis. Area under the curve of the ions $[C_5H_8O_+H]^+$ (m/z = 215 87.14) and $[C_8H_{16}O+H]^+$ (m/z = 129.22) were extracted from the mass spectra as a function of time 216 (Supplementary Material S1a). Then the average noise signal during the first 60 sec of acquisition 217 218 was subtracted. The resulting curves of each of the peak area of the two ions as a function of time 219 were established (Supplementary Material S1b). From these curves, the maximum intensity (Imax) 220 and cumulated area (CA) as a function of time were determined for each condition (Supplementary Material S1c). Data were extracted using IgorPro 6.36 (Igor Pro Wavemetrics, USA). 221

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2.10. Statistical analysis

- 223 For each aroma compound, partition coefficients measured by GC-FID in the DC and in the HC
- 224 conditions (i.e. K_{DC} and K_{HC}), and in the HC and the CS (ie K_{HC} and K_{CS}) were compared using a
- 225 Student t-test (alpha = 0.05). HC was used as control for all further experiments. Partition coefficients
- measured in the different conditions (i.e. K_{HC}, K_T or K_{TP}) were submitted to univariate analysis of
- variance (ANOVA) followed by a Tukey multiple comparison test (significance for p<0.05).
- Aroma conversion rates calculated in the T and in the TP conditions were compared using a Student
- t-test (alpha = 0.05). Conversion rates of all aroma compounds for each condition (T or TP) were
- submitted to univariate analysis of variance (ANOVA) followed by a Tukey multiple comparison test
- 231 (significance for p<0.05).
- 232 For PTR-MS analysis, Imax and CA in the three conditions HC, T or TP were compared by ANOVA
- followed by a post-hoc Tukey multiple comparison test (significance for p<0.05).

234 3. Results and discussion

235

3.1. Effect of mucosal hydration on aroma partitioning

The mucosal pellicle is a lubricating layer containing mucins anchored at the surface of the epithelial 236 237 cells. Mucins have hydrophilic regions with the ability to form H-bonds and electrostatic interactions (Bansil & Turner, 2006). As a consequence, the mucosal surface is wet and this property has to be 238 considered as a factor that impacts on aroma retention (Déléris, Saint-Eve, Saglio, Souchon, & 239 Trelea, 2016). As described in the materials and methods section, we determined that the surface of 240 241 the model mucosa retains on average 24.9 ± 5.6 mg of buffer. In order to evaluate how this surface wetness impacts aroma partitioning, the partition coefficients (K) in the dry control (DC) and hydrated 242 control (HC) conditions were measured and the ratio between the two partition coefficients was 243 calculated for each studied molecule (figure 1A). A ratio below 100 % indicates that the considered 244 aroma compound is significantly retained by the residual liquid. The signal/noise ratios for pyrazine 245 and 2'3-dimethylpyrazine were below the limit of quantification, therefore it was not possible to 246 establish the impact of the cells' wetness on their release. For the other compounds, we observed 247 that the residual buffer significantly retained pentan-2-one (-18 ± 7 %) (p<0.05) of the 2-methyl 248 ketone series, while hexan-2-one (-19 \pm 3 %), and octan-2-one (-10 \pm 4 %), heptan-2-one (-8 \pm 5 %), 249 nonan-2-one (-6 ± 7 %) and decan-2-one (-5 ± 9 %) were also retained but not significantly. 250 Regarding other compounds, they tended to be retained by the presence of the buffer (except 251 252 guaiacol), however this effect was only significant for linalool (p<0.05). This global tendency, despite being significant only for pentan-2-one and linalool, suggests that a part of aroma compounds is 253 transferred into the buffer according to the thermodynamic laws and to their affinity for the liquid 254 phase, decreasing the amount of aroma in the headspace. This could for example explain the high 255 persistence of hydrophilic compounds such as pyrazines (Buffo, Rapp, Krick, & Reineccius, 2005; 256 257 Linforth et al., 2000; Wright, Hills, Hollowood, Linforth, & Taylor, 2003) or small alcohols (ethanol, propan-2-ol) previously observed (Linforth et al., 2000). 258

259 Since the presence of residual buffer on cells surface affected the partitioning of several compounds, 260 the HC condition was used in the rest of the study as a control to test the effect of the cells and the 261 mucosal pellicle.

3.2. Aroma partitioning in presence of oral cells and/or a mucosal pellicle

Partition coefficients (K) were measured by static headspace analyses (SHS) in the hydrated control (HC), TR146/MUC1 (T) and TR146/MUC1 + mucosal pellicle (TP) conditions after 40 min of incubation at 37 °C. Results are presented in figure 1B, as the ratio between the K values in the condition T or TP (K_T or K_{TP}) and the partition coefficient in the control HC (K_{HC}). A ratio lower than 100% indicates that aroma release is lower in the vial containing the cells alone (T) or the model mucosa with a mucosa pellicle (TP) than in the control vial (HC).

The partitioning ratios calculated for hexan-3-one, guaiacol, octan-3-one and the four other linear ketones pentan-2-one, hexan-2-one, heptan-2-one and octan-2-one indicate that there were no significant differences in the K_T and K_{TP} of these compounds compared to the K_{HC} (control condition). Thus, the release of these compounds was not affected by the model mucosa with and without pellicle at equilibrium.

In contrast, a significant decrease (p<0.05) of aroma partitioning was observed in both conditions T 274 and TP compared to the HC control condition, for pentan-2,3-dione (-73 ± 16 %, for T and - 83 ± 1 275 % for TP), trans-2-hexen-1-al (-69 \pm 3 % for T and - 75 \pm 1 % for TP), ethyl hexanoate (-16 \pm 3 % for 276 277 T and - 19 ± 6 % for TP), nonan-2-one (- 16 ± 3 % for T and - 17 ± 3 % for TP) and decan-2-one (-37 \pm 6 % for T and - 34 \pm 6 % for TP). There was no significant difference measured between the 278 conditions with and without pellicle, except for trans-2-hexen-1-al, which was significantly less 279 280 released in presence of the mucosal pellicle. The effect of diluted clarified saliva on the partition 281 coefficient of these aroma compounds was also measured by SHS: there was no retention effect of saliva (CS) compared to the control condition of aroma diluted in the buffer without saliva (HC) (figure 282 1C). Interestingly, even though a strong effect of the model oral mucosa was observed on penta-283 2,3-dione partitioning, it did not impact the partitioning of the mono ketone pentan-2-one. The 284 285 position of the ketone group on hexan-2 or 3-one and octan-2 or 3-one (i.e. in C2 or C3) did not 286 modify the effect of the model mucosa.

Aroma compounds exhibit a large range of hydrophobicity. This physico-chemical characteristic can be at the origin of their behavior. The hydrophobicity of a molecule can be measured by determining its octanol/water partition coefficient, abbreviated log *P*. The higher log *P* value of a compound, the higher the compound's hydrophobicity.

Ketones belonging to the 2-methyl ketone series differ only by the length of their aliphatic chain, 291 which is correlated to the molecule hydrophobicity. Thus, in order to probe the effect of molecule 292 hydrophobicity, the ratios K_T/K_{HC} and K_{TP}/K_{HC} were expressed as a function of log *P* values (figures 293 294 2A and 2B). Partitioning of molecules when exposed to the model mucosa appeared to be negatively correlated with log *P* ($R^2 = 0.87$ and $R^2 = 0.96$ respectively for T and TP), meaning that molecules 295 were less readily released from the model mucosa as their hydrophobicity increased. A negative 296 correlation between linear methyl ketones partitioning in presence of salivary proteins and molecules 297 298 hydrophobicity was previously reported (Pagès-Hélary et al., 2014). Here, the slopes of the curves were comparable (-0.161 and -0.167 respectively for T and TP), indicating that there is no effect of 299 the pellicle on the partitioning of methyl ketones for cells expressing MUC1 at their surface. When 300 plotting K values of the 13 molecules as function of their log P, no correlation was found between 301 molecule hydrophobicity and retention by the oral mucosa (R = 0.09, data not shown). The two 302 compounds for which partitioning was the most reduced in the presence of the model mucosa, pent-303 2,3-dione (log P = -0.85) and trans-2-hexen-1-al (log P = 1.58), have very different hydrophobicity. 304 Therefore, it appears that the functional group has also a strong impact on the effect of the model 305 306 mucosa on aroma release.

In order to explain the effect of the model mucosa on aroma release, two main hypotheses can be formulated. The first one is that aromas bind to the surface of the cells in presence or not of the mucosal pellicle. The second one postulates that the cell line is able to metabolize the studied aroma compounds.

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3.3. Compounds degradation by model mucosa

In order to explore the hypothesis that modification of the release of aroma compounds in presence of the model of oral mucosa results from their metabolization by cells, the composition of the liquid phase of aroma solutions incubated in presence of the model mucosa without pellicle was characterized by GC–MS after extraction with dichloromethane for pentan-2,3-dione, trans-2-hexen-1-al, ethyl hexanoate, nonan-2-one and decan-2-one. The example of nonan-2-one extracts analyses is presented in figure 3. A decrease in the initial concentration of nonan-2-one (eluted at 13.1 min) was observed in the same order of magnitude of the one observed in GC-FID. A new

compound eluted at 16.5 min (figure 3B) in the presence of cells compared to the hydrated control 319 extract (figure 3A). MS spectrum extracted at 16.5 min is provided in figure 3C. Comparison with MS 320 databases allowed identifying the new compound as nonan-2-ol. The chromatograms and MS 321 spectra of the five compounds studied are given in supplementary material S3. For the five aroma 322 compounds, a decrease in initial compounds amount in comparison to the control condition was 323 associated with the presence of new compounds in the T condition. The identified molecules and 324 the conversion rate of initial compounds in the T and TP conditions are reported in table 2. The 325 326 decrease in nonan-2-one and decan-2-one concentrations was associated with the production of the corresponding alcohols, namely nonan-2-ol and decan-2-ol, respectively. Decrease of pentan-2,3-327 dione solution concentration was associated with the production of two reduced forms of the 328 molecule: 2-hydroxy-pentan-3-one and 3-hydroxy-pentan-2-one. Ethyl hexanoate was hydrolyzed 329 into hexanoic acid, and trans-2-hexen-1-al was oxidized into its corresponding acid hexenoic acid. 330

331 Although conversion rates of aroma by the model mucosa were in the same order of magnitude, small differences were observed between aroma compounds. Pentan-2,3-dione and trans-2-hexen-332 1-al were converted significantly (p<0.05) faster. Concerning the impact of the mucosal pellicle, a 333 significant difference between the oral mucosa with and without the mucosal pellicle was observed 334 only for trans-2-hexen-1-al. These observations indicate that the TR146/MUC1 cell line is able to 335 metabolize the molecules reported in table 2. The ability of epithelial cells to metabolize organic 336 volatile compounds from different chemical families has already been observed on primary cells 337 338 cultures of human nasal mucosa or rat olfactory mucosa. Zaccone et al (2015) reported oxidation of two diketones into monoketones (diacetyl and pentan-2,3-dione to 3-hydroxybutanone and 2-339 hydroxy-3-pentanone, respectively) in a culture of bronchial/tracheal human epithelial cells (Zaccone 340 et al., 2015). Microsomal and cellular fractions obtained from rat olfactory mucosa exhibited ability 341 342 to metabolize guinoline (heterocycle) and coumarin (lactone) into oxygenated metabolites, and isoamyl acetate (ester) into isoamylic alcohol (Thiebaud et al., 2013). Although the respective 343 contributions of the mucus and the epithelial cells was not determined, ex vivo rat olfactory mucosa 344 converted ethyl acetate into ethanol (Schoumacker et al., 2016) and pentan-2,3-dione into 2-345 hydroxy-pentan-3-one and 3-hydroxy-pentan-2-one (Robert-Hazotte et al., 2019). These reactions 346

are catalyzed by a range of enzymes named Odorant Metabolizing Enzyme (OME) (Heydel, Hanser, 347 Faure, & Neiers, 2017) that belong to the xenobiotic metabolism enzymes (XMEs) family. Overall, 348 XMEs are specialized in the catabolism of exogenous compounds in order to facilitate their 349 350 elimination by the organism (Croom, 2012). Xenobiotics elimination results from three mains steps. During the first phase, nonpolar and reactive compounds are converted into more polar and less 351 reactive compounds through different reactions, such as epoxidation, hydroxylation, desalkylation, 352 oxidation or reduction. This step involves enzymes such as the cytochrome P450 or 353 354 carboxylesterase (Thiebaud et al., 2013). In the second phase, conjugate enzymes, such as glutathione transferase, catalyze reaction of conjugation with polar compound such as glutathione, 355 glucuronic acid or a sulfate (Heydel et al., 2019). Finally, metabolites can be easily excreted via 356 transporter proteins. The OMEs family includes a large variety of enzymes. The reduction of pentan-357 2,3-dione into 2-hydroxy-pentan-3-one and 3-hydroxy-pentan-2-one, previously observed in 358 presence of bronchial/tracheal human epithelial cells and rat nasal mucosa has been attributed to 359 the dicarbonyl/L-xylulose reductase (DCXR) (Robert-Hazotte et al., 2019; Zaccone et al., 2015). 360 Hydrolysis of isoamyle acetate into its corresponding acid has been previously attributed to a 361 362 carboxylesterase (Thiebaud et al., 2013). Thus, we hypothesized that the conversion of ethyl hexanoate into acid hexanoic is catalyzed by a carboxylesterase. Regarding the conversion of 363 ketones into alcohols, such activity has been previously reported in presence of saliva and was 364 365 proposed to be due to an aldo-keto reductase (Muñoz-González, Feron, Brulé, & Canon, 2018). The 366 oxidation of aldehyde (Trans-2-hexen-1-al) into carboxylic acid (hexenoic acid) could result from the activity of aldehyde dehydrogenases. Moreover, the presence of aldo-keto reductases (AKR1C3, 367 AKR1C2, AKR7AC2, AKR1C1, SPR and KCNAB2) DCXR, carboxylestease (CES1, CES2, CES3, 368 CES4A) and aldehyde dehydrogenases (ALDH9A1, ALDH1B1, ALDH3A1, ALDH3B2, ALDH4A1, 369 370 ALDH5A1, AGPS) in the oral mucosa has been previously reported using specific antibodies (Uhlén et al., 2015). All these enzymes have been reported to be present in the oral mucosa at different 371 concentrations (Uhlén et al., 2015), which could explain the difference of metabolization between 372 the different affected compounds. RNA encoding for cytochrome P450 have been detected in 373 salivary glands (Kragelund et al., 2008) and human oral mucosa (Vondracek et al., 2001). 374 Carboxylesterase activity has already been observed in rats and mice's oral cavity (Robinson, 375

Bogdanffy, & Reed, 2002). The presence of these enzymes in the oral mucosa indicates that this 376 latter has the potential to metabolize xenobiotics. Indeed, like the nasal cavity, oral mucosa is a 377 tissue exposed to exogenous and potentially toxic compounds, which have to be eliminated. From a 378 379 sensory point of view, it was recently reported that metabolic activity in the nasal and oral cavities impacts on perception (ljichi et al., 2019). This work demonstrates for the first time the importance 380 of aroma conversion activity in oral mucosal cells. This activity is probably due to different enzymes, 381 which have different enzymatic activities (kinetics, affinity,...) on aroma compounds as a function of 382 their structure. 383

384

3.4. Effect of oral mucosa on kinetics of *in vitro* aroma release

In-mouth aroma release is a dynamic process. Static headspace (SHS) experiments require the establishment of the thermodynamic equilibrium, which takes approximatively 20 min (Pagès-Hélary et al., 2014). To obtain information on earlier phases, we studied the kinetic release of two aroma compounds unaffected by the presence of the epithelial cells, pentan-2-one and octan-2-one, during the first 2.5 minutes using Proton-Transfer-Reaction Mass Spectrometry (PTR-MS).

390 The maximum of intensity (Imax) and the cumulated area (CA) of aroma release at t= 5, 15, 30, 60,

391 90, 120 and 160 sec in the 2 conditions T and TP are presented in figure 4.

392 There was no significant difference in the Imax for octan-2-one between the three conditions, i.e. in buffer (HC) or in presence of the model mucosa with (TP) and without pellicle (T). However, for 393 pentan-2-one the Imax was significantly higher in presence of the cells with the mucosal pellicle (TP 394 vs HC), but was not significantly different between T and TP conditions. This observation suggests 395 396 that the rate of transfer of molecules pentan-2-one from the gas phase to the liquid phase is affected 397 by the presence of cells plus the mucosal pellicle. The cells could indeed alter the capacity of water to solubilize the molecules and provoke a salting-out effect explaining the increase of the intensity 398 of the release in presence of the cells plus the mucosal pellicle for pentan-3-one. The absence of 399 400 effect for octan-2-one could be explain by non-covalent interactions between the compound and the 401 cells with or without the mucosal pellicle, decreasing the result of this salting-out effect.

For pentan-2-one, a significant decrease in cumulated area values was observed for all times after
60 sec for the TP conditions. Furthermore, a significant difference was measured between the two

404 conditions at 160 sec, with a significantly lower release from the model with a mucosal pellicle 405 compared to the cells-only model (figure 4C). In other words, the reduced release was observable 406 more rapidly when cells were lined by a mucosal pellicle.

407 Regarding octan-2-one release, a significant decrease of octan-2-one release occurred after 120 sec for the condition T compared to the control condition (figure 4D). Thus, the reduced release was 408 greater for cells with the mucosal pellicle for pentan-2-one, while it was greater for cells without the 409 salivary proteins forming the pellicle for octan-2-one. In these experiments, we used the 410 411 TR146/MUC1 cell line which expresses at its surface the extracellular domain of the mucin MUC1/Y-LSP (Zhang, Vlad, Milcarek, & Finn, 2013). The presence of this domain at the cells' surface 412 increases the anchoring of the salivary proteins (Ployon et al., 2016), while modifying the cell surface 413 properties. Atomic Force Microscopy (AFM) experiments using functionalized tip and conducted on 414 the TR146/MUC1 cell line revealed first that the surface of the cells present both highly hydrophobic 415 and hydrophilic domains due to the expression of MUC1/Y-LSP, and second that the anchoring of 416 salivary proteins decreases the number of these highly hydrophobic or hydrophilic domains, 417 suggesting that the anchoring of salivary proteins involves these domains (Aybeke et al., 2019). 418 419 Thus, the surface of the model mucosa is less hydrophobic in presence of the mucosal pellicle (TP) 420 than without it (T). Octan-2-one differs from pentan-2-one by the length of the aliphatic chain making this molecule more hydrophobic. Previous investigations on the effect of mucin on aroma release 421 422 have revealed that mucin can retain aroma compounds through non-covalent interactions involving 423 hydrophobic effect (Pagès-Hélary et al., 2014). Therefore, it can be hypothesized that the most hydrophobic compounds are more prone to interact with the most hydrophobic cell surface (cells 424 without a mucosal pellicle T), which is the case for octan-2-one. Conversely, pentan-2-one, which is 425 less hydrophobic, is significant more retained in presence of the salivary proteins forming the 426 427 mucosal pellicle. This result suggests that pentan-2-one is more prone to interact with salivary proteins than octan-2-one. This observation could be explained by the hypothesis that the presence 428 429 of salivary proteins increases the number of pentan-2-one binding sites, while the longer aliphatic chain of octan-2-one precludes its access to these binding sites due to steric hindrance. The nature 430 of the non-covalent interaction involved remains unknown. 431

432 To summarize, the anchoring of salivary proteins to MUC1 changes the cell surface properties and

433 the nature of the exposed aroma binding sites. As a result, it modifies the ability of the mucosa to interact with aroma compounds depending on their structure, leading to either a decrease or an 434 increase of the binding of some aroma compounds and to a modification of the release of aroma 435 compounds through time depending on their structure. The present study suggests that both 436 compounds are less released in presence of the epithelial cells both with or without the mucosal 437 pellicle. Moreover, this latter decreases the release of pent-2-one while it does not significantly affect 438 439 octan-2-one. As a result, the mucosal pellicle seems to play a role in aroma persistence as a function 440 of aroma compounds structure.

441 **4. Conclusion**

The study allowed deciphering the respective impact of hydration, the epithelial cells and the mucosal proteins on the release of several aroma compounds. First, hydration of the mucosal surface modified the release of only two aroma compounds over 13 studied (here, pentan-2-one and linalool), suggesting that it does not play a prominent role in the impact of the oral mucosa on aroma release.

The model mucosa impacted both the partitioning of pentan-2,3-dione, trans-2-hexen-1-al, ethyl 447 hexanoate, nonan-2-one and decan-2-one at equilibrium and the release of the pentan-2-one and 448 octan-2-one (the only two aroma compounds studied in dynamic condition). This impact appears to 449 result from the ability of oral cells to metabolize aroma compounds (herein methyl ketones, 450 451 aldehydes and esters) depending on their structure. The ability of cells to metabolize aroma compounds results from the activity of the enzymes that they express. Enzymes are biological 452 catalysts that accelerate specific chemical reactions as a function of their three-dimensional structure 453 and the structure of the metabolized compound. OMEs which specifically metabolize different 454 455 families of aroma compounds, are present in the oral mucosa at different concentrations to detoxify reactive aroma compounds as a function of their structure. The metabolic activity observed here 456 457 could result from the activity of DCXR for pentan-2,3-dione, aldo-keto-reductases for nonan-2-one and decan-2-one, carboxyesterase for ethyl hexanoate and aldehyde dehydrogenase for trans-2-458 459 hexen-1-al. Moreover, the difference of activity observed between the different compounds could result from either a difference in the enzymatic reaction as a function of the affinity of the enzyme -460 aroma compound couple and/or differences of enzyme concentrations as previously reported in the 461 oral mucosa. Thus, no generalization could be drawn and each compound is a specific case that will 462 be metabolized as a function of its structure and the composition of the oral epithelial cell proteome. 463 The dynamic study was only performed on two compounds (here two 2-methyl ketones) and 464 suggested that other phenomena such as non-covalent interactions between the two studied aroma 465 compounds and mucosa (cells and mucosal pellicle) also occur. As a result, times after 60 s were 466 467 significantly affected for both molecules, with a decrease of their release in presence of the cells and the pellicle mucosal. The mucosal pellicle significantly affected the release of pentan-2-one in the 468

TR146/MUC1/Y-LSP cell line. Thus, this model of mucosa appears as a promising tool to study the effect of the oral mucosa on aroma release as a function of aroma compounds structure and/or pellicle composition. It may also aid in further researches on the role of the mucosa on aroma metabolization. In the future, it would be of interest to perform real time monitoring of aroma release and persistence in nasal and/or oral cavities after *in vivo* consumption of aroma solution in order to establish a comparison with *in vitro* experiments.

To conclude, this paper, by demonstrating that the oral mucosa both impacts the kinetic of aroma compounds release and metabolizes aroma compounds, opens new avenues of research on the

role of the oral mucosa in aroma persistence and aroma perception.

478 **Conflict of interest statement**

479 The authors declare no conflict of interest.

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617 Figure 3





Compound	CAS number	MW ª (g/mol)	Log P ^b	Sol ^c (mg/l)	Pvap ^d (mmHg)	GC Oven Temp (°C)	Stock solution conc. (mg/l)	Final conc. tested for toxicity test and GC-FID-HS analyses (mg/l)
Pentan-2-one	107-87-9	86	0.91	2.1.10 ⁴	39.4	70	86.0	8.6
Hexan-2-one	591-78-6	100	1.38	7.7.10 ³	11.6	80	100.0	10
Heptan-2-one	110-43-0	114	1.98	2.1.10 ³	3.86	95	114.0	11.4
Octan-2-one	111-13-7	128	2.37	884.2	1.35	100	128.0	12.8
Nonan-2-one	821-55-6	142	3.14	170.6	0.62	125	142.0	14.2
Decan-2-one	693-54-9	156	3.73	46.43	0.27	135	31.2	15.6
Hexan-3-one	589-38-8	100	1.24	1.0.10 ⁴	13.90	80	100.0	10.0
Octan-3-one	106-68-3	128	2.22	1.2. 10 ³	2.00	95	128.0	12.8
Pentan-2,3- dione	600-14-6	100	-0.85	6.2.10 ⁵	31.1	80	100.0	10.0
Linalool	78-70-6	154	2.97	1.5.10 ³	0.16	135	154.0	15.4
Guaiacol	90-05-1	124	1.32	2.8.10 ⁴	0.10	Grad ^e	1.24.10 ³	124.0
Trans-2-	6728-26-	98	1.58	1.6.10 ⁴	4.72	95	98.0	9.8
Hexen-1-al	3							
Ethyl hexanoate	123-66-0	144	2.83	629	1.8	95	144.0	14.4
Pyrazine	290-37-9	80	-0.26	2.2.10 ⁵	10.8	100	800.0	80.0
2,3- dimethylpyra zine	5910-89- 4	108	0.54	3.8.10 ⁴	2.74	100	1.08.10 ³	108.0

^a Molecular weight ^b partition coefficient octanol/water Episuit ^c Solubility in water at 25 °C ^d Vapour pressure

^e Temperature gradient for guaiacol analysis: 120°C to 150°C at 8°C/min then 150°C to 200°C at 5°C/min.

626

Table 2: GC-MS identification of new compounds generated after aroma exposure to the

629 *model mucosa and their conversion rates. Conversion rates are expressed as mean value* ± SD.

630 Different letters indicate a significant difference (Tukey test, α =0.05) between aroma compounds.

- 631 Different numbers indicate a significant difference (p< 0.05) between T and TP condition (p< 0.05).
- 632

Initial aroma compounds	New compounds identified	Calculated conversion rate (T) (mM/L/min)	Calculated conversion rate (TP) (mM/L/min)	
Pentan-2,3-dione	2-hydroxy-pentan-3-one	1.87 ± 0.38 ^{1,a}	2.11 ± 0.02 ^{1,a}	
,	+ 3-hydroxy-pentan-2-one			
Trans-2-hexen-1-al	Hexenoic acid	1.74 ± 0.07 ^{1,a}	1.90 ± 0.03 ^{2,a}	
Ethyl hexanoate	Hexanoic acid	0.42 ± 0.07 ^{1,b}	0.51 ± 0.15 ^{1,b}	
Nonan-2-one	Nonan-2-ol	0.53 ± 0.07 ^{1,bc}	0.55 ± 0.07 ^{1,bc}	
Decan-2-one	Decan-2-ol	1.00 ± 0.15 ^{1,c}	0.93 ± 0.13 ^{1,c}	

634 Figure captions

Figure 1: (A) Partition coefficient of aroma in hydrated control (HC) K_{HC} expressed as percentage 635 of the value in dry control DC (K_{DC}). Mean values are reported with their standard deviation (SD). 636 637 Asterisks indicate a ratio significantly lower than 100% (ANOVA, ** p < 0.01). (B) Partition coefficient of aroma after exposure to model mucosa without (K_T) and with mucosal pellicle (K_{TP}) . Values are 638 expressed as the percentage of the HC value K_{HC}. Different letters indicate significant difference 639 between the conditions HC, T and TP (Tukey test, α =0.05). (C) Partitioning of aroma in clarified 640 641 saliva (CS): K_{CS}. Values are expressed as the percentage of the HC value K_{HC}. All results are presented as the mean value ± SD. 642

Figure 2: Partition coefficient of 2-methyl ketones (K) as a function of molecules hydrophobicity (log *P*) after exposure to oral mucosa without a mucosal pellicle (K_T) (**A**) or with a mucosal pellicle (K_{TP}) (**B**). Values are expressed as the percentage of the HC value K_{HC}. Mean values are reported with their standard deviation (SD). Asterisks indicate a ratio significantly lower than 100% (ANOVA, * p < 0.05, ** p < 0.01)

Figure 3: Data supporting the hypothesis of metabolization of nonan-2-one by oral epithelial cells.
(A) GC-MS chromatogram of nonan-2-one extract in hydrated control (HC) (B) GC-MS
chromatogram of supernatant extract of TR146/MUC1 cells (T) after exposure to nonan-2-one (C)
MS spectrum at elution time of the new compound generated in T condition (t=16.5 min).

Figure 4: Effect of model mucosa without (T) or with (TP) a mucosal pellicle on dynamic release of pentan-2-one and octan-2-one. **(A)** Experimental set-up for the real-time measurements of aroma release from model mucosa **(B)** Imax = maximum aroma release intensity, CA(t) = Cumulated Area of pentan-2-one **(C)** and octan-2-one **(D)** release at different times. Results are presented as the mean value \pm SD. For each parameter, different letters indicate significant difference between the conditions HC, T and TP (Tukey test, α =0.05).

Table 1: list of compounds used in the study with their main physicochemical parameters

- Table 2: GC-MS identification of new compounds generated after aroma exposure to the model
 mucosa and their conversion rates.
- Supplementary Material S1: (a) real time monitoring of m/z ion intensity (b) curve of release of ion
 m/z (c) curve of cumulated area under the curve.
- 663 **Supplementary Material S2:** GC-FID calibration curves of aroma compounds.
- **Supplementary Material S3:** Data supporting the hypothesis of metabolization of aroma compounds by oral epithelial cells. (Left) GC-MS chromatograms of initial compounds extract in hydrated control (HC) (Middle) GC-MS chromatogram of supernatant extract of TR146/MUC1 cells (T) after exposure to aroma solution (Right) MS spectra at elution time of the new compound generated in T condition.