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

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

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1. Introduction

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

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

In spite of this well-known inter-individual variability on saliva composition, the possible effects of this variability on aroma compounds (release, metabolism) by *ex-vivo* approaches have received very little attention and very few studies have tackled this question

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

With this background, the aim of the present study was to investigate the effects of human saliva composition on aroma compounds. In order to reach this goal, an HS-GC method adapted to low volumes of saliva (200 μL) was firstly developed, validated and then independently applied to 17 key aroma compounds in the presence of saliva from three individuals and submitted to different treatments (whole *vs* clarified). The selected aroma compounds belonged to three different chemical families (ketones, aldehydes and alcohols), possessed different structures (carbonyl position, presence of double bonds, linear *vs* branched) and a range of physicochemical properties (hydrophobicity, volatility). Control samples with water were employed to evaluate the extent of the saliva effect. The relationship between some saliva compositional parameters and HS-GC data was examined. Finally, HS-SPME-GC/MS and LLE-GC/MS analyses allowed us to investigate the nature of the observed effects.

2. Material and methods

2.1. Aroma compounds

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

2.2. Saliva samples

2.2.1. Saliva collection

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

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

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

2.2.2. Saliva biochemical analyses

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

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

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

2.3. HS-GC analyses

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

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