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ADDRESSING VARIOUS CHALLENGES RELATED TO FOOD BOLUS AND NUTRITION WITH THE AM² MASTICATION SIMULATOR

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Abstract

Preparing a food bolus for swallowing is the first and major goal of oral processing ensured by mastication. To fulfill this goal, mastication is accurately adjusted to the structure and texture of the food. Since the bolus is the main outcome of food oral processing, there is growing interest in improving understanding the food bolus and its features at the end of the masticatory sequence and throughout its formation. Although this step is trivial, its execution is somewhat more complex than it appears. Until now, the food bolus produced has not subjected to full analysis, mainly due to the lack of accessibility during *in vivo* oral processing.

To overcome this difficulty, we developed a masticator apparatus, named AM², on the basis of *in vivo* compression and shear stresses applied on food during oral processing. The apparatus was validated against particle size analyses of boluses produced by individuals presenting normal mastication. The present paper provides a comprehensive overview of the various potential uses of the AM² apparatus to produce food boluses for a large range of scientific needs.

The food bolus contains much valuable information regarding the progress of mastication, the completion of the function and its role in food disruption, the oral release of food compounds, changes and bioaccessibility, and finally its impact on nutrition. The AM² apparatus can also be used to simulate mastication in specific populations, such as the elderly and children, and to address various objectives related to this initial step of digestion.

Keywords

artificial mastication, food bolus, oral release, oral bioaccessibility, oral digestion, nutrition

Highlights

- The mastication simulator provides food bolus for many foods and various purposes.
- Food disruption, the oral release of food compounds, and digestion can be analyzed.
- Food bolus analysis provides pertinent knowledge on deficient mastication.
- AM² reveals *in vitro* bolus features (dynamics, formation) in various oral conditions.
- AM² is a very attractive model for mechanistic digestion/nutrition studies.

1. Introduction

Food oral processing and more specifically the ready-to-swallow food bolus have become major topics of interest in food and dental science (Engelen, Fontijn-Tekamp, & Bilt, 2005) and in the field of nutrition (Bonnet, Batisse, Peyron, Nicolas, & Hennequin, 2018; Chen, 2009, 2015; Engelen, Fontijn-Tekamp, & Bilt, 2005; Ng et al., 2017). Food bolus analysis is at the crossroads between food structure (Gao, Wong, Lim, Henry, & Zhou, 2015), food formulation, the oral perception of food, food oral processing (Hwang et al., 2012; Jalabert-Malbos, Mishellany-Dutour, Woda, & Peyron, 2007; Koç et al., 2014; Shiozawa, Kohyama, & Yanagisawa, 2003) and the further stages of digestion (Hoebler, Devaux, Karinthe, Belleville, & Barry, 2000; Hoebler et al., 1998), including oral digestion and related nutrient bioaccessibility (Ellis et al., 2004; Grundy et al., 2015). However, the mere fact that food bolus formation occurs inside the mouth and that access inside the mouth is not possible during mastication hampers most study designs. Collecting the ready-to-swallow bolus in normal or deficient subjects is not a simple procedure. Among other reasons, the bolus is

76 often partly swallowed before the final swallow event (Hiemae et al., 1996), the bolus itself
77 is mixed in an unknown proportion with saliva, making it impossible to determine the
78 proportion of saliva, food fluids and solid nutrients present within the bolus during the
79 masticatory process. During chewing, several physico-chemical processes are at work,
80 transforming the characteristics of the final bolus in an uncontrolled way. In addition, asking
81 volunteers to masticate is sometimes impossible for several reasons such as the use of
82 “experimental” or non-natural food, the presence of food contaminants, the chewers studied
83 are disabled, young children or elderly people, or simply because it is too time consuming.
84 Therefore, *in vitro* mastication using a mastication simulator is a valuable alternative to an *in*
85 *vivo* approach for the experimental production and analysis of food boluses. This kind of
86 simulator also provides solutions for multiple analyzes of food bolus properties at the time of
87 swallowing or of the kinetics involved during its formation, and it provides a more realistic
88 oral simulation than that frequently ~~done~~ performed before (Funami, 2016; Hedrén, Diaz, &
89 Svanberg, 2002; Hoebler et al., 2002; Minekus et al., 2014). The possible questions of interest
90 include saliva impregnation, the oral release of nutrients and oral digestion assessment,
91 investigations on the impact of food formulation on the food bolus and its characteristics, for
92 example, in the design of new foods for targeted populations (the elderly, children, non-
93 cooperative human subjects, and even animals). It can also be used to examine the oral release
94 of nutrients and of active, toxic substances and food contaminants from the matrix being
95 chewed, for example, to evaluate the role of the oral cavity in the balance between the oral
96 bioaccessibility and bioavailability of nutrients. Using a mastication simulator is also very
97 useful for the production of specific food boluses to address the fate of the food in the
98 digestive tract. Numerous studies are conducted with a simulated bolus produced by mincing,
99 mixing or crushing a food sample without any control of particle size distribution and saliva
100 action in the pre-swallow food bolus before its subjection to artificial digestion (Bornhorst &

Singh, 2013; Mandalari et al., 2018; Minekus et al., 2014). A food bolus produced by a mastication simulator is more realistic, closer to the material passing through the esophagus and arriving in the stomach, and it can be subjected to artificial, static or dynamic digestion more pertinently. Indeed the oral step of food destructurement by mastication is very often badly simulated in digestion studies and sometimes not considered at all, even when remarkable *in vitro* digestion models are used (Dupont et al., 2018; Hoebler et al., 2002; Kong & Singh, 2008b; Minekus et al., 2014; Shani-Levi et al., 2017). Since the AM² can produce a realistic food bolus, its association with digestion equipment such as the DIDGI® apparatus (Ménard et al., 2014; Sayd, Chambon, & Santé-Lhoutellier, 2016) makes it possible to assess the role of mastication in the subsequent steps of digestion. From this perspective, studies could be performed on the role of oral microbiota in bolus formation, nutrient release and digestion when mixed with the intestinal microbiota.

As already reviewed, several artificial mastication simulators have been developed but most of them are unable to produce a food bolus for further analysis or use (Morell, Hernando, & Fiszman, 2014; Peyron & Woda, 2016). The Artificial Masticatory Advanced Machine (AM²) was developed and validated against *in vivo* data to address multiple issues regarding in-mouth food processing and the resulting food bolus (Woda et al., 2010a). Its purpose was to simulate the result of mastication, *i.e.* to produce a food bolus with properties similar to those produced by natural mastication, taking into account food oral management as described in the literature (Chen, 2009). The anatomical features of the teeth and mouth were not duplicated. On the contrary, the basic characteristics of jaw movement and other oral parameters at work during chewing were mimicked so that the production of a pre-swallow food bolus with the AM² obeys natural physiological laws (Lund & Kolta, 2006; Woda, Foster, Mishellany, & Peyron, 2006). Consequently, after correctly selecting the programming, the granulometry of the food boluses obtained at different times during the

chewing process were similar *in vitro* and *in vivo*, thus providing the basis for validating the apparatus (Mishellany-Dutour et al., 2011; Woda et al., 2010a). Until now, very few of the results obtained with this tool have been published (Peyron, Santé-Lhoutellier, François, & Hennequin, 2018). This article presents an overview of several types of original results obtained with the AM² in the fields of food and nutrition science related to oral processing. The results presented in Fig.12 are the only ones adapted from the paper published previously (Peyron et al., 2018). Other versions of the AM² masticator apparatus could be produced to demand. Designing modified prototypes is also undoubtedly a realistic goal in view to satisfying specific needs to answer various potential scientific questions.

2. The masticator apparatus AM²

2.1 Description of the masticatory apparatus AM²

The complete description of the apparatus and its validation were provided in previous papers (Mishellany-Dutour et al., 2011; Woda et al., 2010a). Briefly, this tool is composed of a cylindrical cavity that constitutes a masticatory chamber (Fig. 1). The two ends of the cylindrical chamber are formed by a fixed masticatory **disk** (=“maxillary disk”) on one side, and a mobile masticatory **disk** (=“mandibular disk”) on the other, to simulate the dental arch. These masticatory disks have surfaces whose shape differs from human dental anatomy but are similar to the contact surface area at work in human mastication. They are designed to produce the same types of shear and compression stresses as those exerted *in vivo*. The mobile masticatory **disk** is moved like a piston by two motors imparting translational and rotational movements like those described during food management in the mouth during mastication. A spring is located behind the fixed maxillary disk. The force generated for food compression

during mastication depends on the stiffness of the spring which is chosen as a function of food resistance (Mishellany-Dutour et al., 2011; Woda et al., 2010a). The actions of shearing food material, as well as gathering particles and transporting them to the masticatory surface are performed by the rotational movement of the mandibular disk, as the tongue would do. Water or a validated artificial saliva (Roger-Leroi, Mishellany-Dutour, Woda, Marchand, & Peyron, 2012) can be injected at the beginning and / or by staggered injections during the masticatory sequence.

2.2 Programming of the masticatory apparatus AM²

Whatever the objective of the study, a common procedure is used for all foods when programming the AM² masticatory apparatus. It is always based on preliminary *in vivo* data collected both from subjects presenting the oral conditions selected for the experiment (healthy oral condition, edentate, etc.) and from the granulometric characteristics of the food bolus collected (Jalabert-Malbos et al., 2007; Ngom, Diagne, Aïdara-Tamba, & Sene, 2007; van der Bilt, Abbink, Mowlana, & Heath, 1993). The cumulative particle size distribution curves obtained are used for comparing *in vivo* and *in vitro* food boluses. The median particle size d50, extracted from the cumulative curve by extrapolation and projection on the x-axis, and defined as the theoretical sieve size through which 50% of the bolus mass can pass, is also used for comparisons. To obtain the most efficient programming, comparisons are made after successive adjustments of the AM² parameters until the *in vivo* and *in vitro* curves and d50 values match each other.

The adjustments made to the mechanical parameters can be of many types and depend on the food masticated.. Briefly, the range of forces to be applied will depend on the spring chosen according its stiffness (from 10.38 to 35.2 N/mm). As examples derived from

experimental tests, the mastication of a French baguette sample would be done with the 10.38 N/mm spring and a raw carrot sample with the 25 N/mm spring. The number of masticatory cycles is always obtained from the *in vivo* measurements of mastication of the food considered. A large angle (around 200°) is chosen for a “fibrous” food, such as meat needing more shear stress to be disrupted compared to a brittle food such as cookies for which an angle of 90° is enough. Various rotational angles are also tested for gathering particles. The temperature of the masticatory chamber can be regulated at 36°C if the oral processing studied is temperature dependent. Water, human or artificial saliva can be used for mastication. The flow and composition of artificial saliva with respect to mucin and enzyme contents (amylase and lipase, for example) can be chosen according to the scientific issue to be addressed (Roger-Leroi et al., 2012). Fig. 2 shows an example of the programming approach in which two numbers of masticatory cycles and two levels of spring-induced forces were sequentially changed in the programming of the masticator apparatus to find the best fit with the *in vivo* particle size distribution curve in meat bolus analyzed at swallowing time. As shown in Fig. 2, by performing the same number of masticatory cycles as observed *in vivo*, the stiff spring (23.5 N/mm) overly-disrupted the meat bolus compared to the softer spring (18.2 N/mm). Finally, after progressive adjustment, the mean particle size distribution curves of the meat boluses obtained *in vivo* and *in vitro* in the conditions selected were identical at swallowing time (Fig. 2).

Similar *in vivo* - *in vitro* agreements have also been reported for pork meat and green olive (Peyron & Woda, 2016). Several examples are shown in Fig. 3 for gherkins, a mouthful of beef, minced beef, Frankfurt sausages, coconut and a pasta product. *In vivo* - *in vitro* agreements were also observed when mastication was interrupted at different times while chewing carrots or peanuts (Mishellany-Dutour et al., 2011a) and with pork meat in Fig. 4 (A and B). Fig. 4c shows that the difference observed in the particle size distributions, as well as

in d50 values, between two types of bread *in vivo* can be completely reproduced *in vitro*.

A complete validation of the apparatus should also be performed by considering other physical properties of the food bolus, for example, using an instrumental Texture Profile Analysis (TPA) test consisting in applying double compression on the food bolus. Despite its empirical nature, this test is useful to compare the properties of food boluses collected at different times of the masticatory sequence, for different food matrixes or in different oral conditions, both for *in vivo* or *in vitro* food boluses (Peyron et al., 2011). Hardness, recoverability, adhesiveness and springiness values can be extracted from each TPA curve for bolus mechanical characterization. As an example of this interesting use, Fig. 5A illustrates that a piece of French bread (baguette) is softened, rendered less elastic and less cohesive as the *in vivo* masticatory sequence progresses. Physical characteristics obtained for *in vivo* and *in vitro* boluses can be compared to attest the effectiveness of the masticator apparatus (Fig. 5B). The possible addition of water, or human or artificial saliva in the AM² is undoubtedly a key parameter in preparing a food bolus for instrumental measurements since it provides the specific rheological environment of the food after mastication. A comparison of the physical characteristics of the *in vitro* food boluses obtained with these different fluids would be informative regarding the role of the specific components such as enzymes and proteins. Nevertheless, great care must be taken in the treatment of TPA test variables which are not absolute values, they are often misinterpreted, and they can only be used in a comparative way (Nishinari & Fang, 2018).

All the *in vivo* data used to validate or to program the masticator apparatus were obtained after collecting the informed consent of each subject and experiments were performed in accordance with different local and national ethical agreements (CE-CIC-GREN-10/06-#5044; DREAM-2013-A00096-39).

3. Substance release in the mouth

Chewing a solid matrix causes the release of numerous substances within the mouth, normally resulting in a satisfactory sensory experience from food containing juice or tasty molecules, for example. It can also favor nutrient extraction as early as the oral step. Conversely, such release must be avoided when the foods or the non-nutritive products contain toxic or pollutant substances as can happen, for example, when a child is chewing or gnawing something containing a non-edible or toxic substance. However, obtaining the delivery of an active substance could be the objective pursued, for example, with medicated chewing-gums (Konar, Palabiyik, Toker, & Sagdic, 2016).

An artificial device is mandatory when the precise concentration of a compound released during mastication has to be determined. Fig. 6A shows the release curve along the mastication of a non-alimentary product containing two different concentrations of a toxic substance. The kinetics and level of release could be determined and used for toxicity risk analysis. This approach is obviously applicable for quantifying the release of toxic or contaminant compounds from any alimentary product.

Most often the substances analyzed released from food or from any non-nutritive product during chewing are dissolved or dispersed in an oral liquid composed of a mix of saliva and liquids expressed in the food matrix. Determining the true oral concentration of the substance of interest implies the separation of these two types of liquids. In usual conditions, the volume of saliva cannot be determined because the saliva flow undergoes a large number of unpredictable variations and due to frequent intermediate swallows (Hiemae & Palmer, 1999). AM² can be used to distinguish between saliva and the juice extracted from food.

In the examples shown in Fig. 6 (B and C), mango juice was collected *in vivo* after chewing with the fruit placed within an impermeable plastic bag (Fig. 6B) and *in vitro* with

the fruit in the AM² (Fig. 6C). The weight of the mango juice collected *in vitro* after 24 masticatory cycles was similar to the amount obtained after *in vivo* chewing performed with 24 cycles. With this AM² based design, the juice released from the matrix disruption was estimated to be about 20% of the initial weight of the mango sample (Fig. 6Bb). Without the bag, the *in vivo* mastication produced a liquid phase composed of juice and saliva, representing about 40% of the bolus weight. As expected, the amount of mango juice extracted also depended on the number of chewing cycles (Fig. 6C).

Fig. 7 shows the kinetics of glucose release in saliva during *in vivo* and *in vitro* mastication of a chewing gum. In this experiment, the gum was weighed before and after 1, 3, 6, 10 and 12 min chewing. The gum weight was expressed as the cumulative course of weight loss observed along the masticatory sequences (Fig. 7A). The glucose was determined in saliva samples at the same time points of the masticatory sequence (Fig. 7B). It was obtained with a glucometer using the electrochemical determination of glucose oxidase activity compared to the pre-established standard curve for glucose in saliva. The results were expressed as the cumulative time course of the amount of glucose measured in saliva at the different time points of the masticatory sequences. Mastication occurred either *in vivo* with 8 food bolus samples obtained from five volunteers or *in vitro* with 8 repetitions performed with the masticator apparatus. The greater proportion of glucose release occurred during the three first minutes of mastication and the gum weight decreased mostly at the same time. Weight loss and glucose release changes had similar time courses for *in vivo* and *in vitro* gum chewing.

This kind of experiment can be conducted, for example, for the development of a medicated chewing gum designed to provide a slow steady release of the medicine contained in it, favoring the release of a substance in saliva and its progressive absorption. A relevant time-curve would be obtained with the AM² apparatus.

4. Oral bioaccessibility of nutrients, digestion studies

The role of mastication and considerable food matrix disruption in the absorption of nutrients is important (Pennings et al., 2013; Rémond et al., 2007). Sieving through a tissue mesh enables the separate collection of liquid and solid phases of the food bolus, giving access to the role of oral processes in nutrient bioaccessibility and digestion. This method has been successfully applied in *in vitro* food boluses. Fig. 8 (ABC) shows free-iron release from ~~the~~ a meat matrix during the mastication of either a beef sample or its minced counterpart. Although the bolus of minced meat was normally chewed in fewer masticatory cycles than the same meat not minced (10 *versus* 32 cycles), it produced a bolus composed of more small particles (Fig. 8A). Free-iron release from the matrix (Fig. 8B and C) rose significantly in both saliva and bolus material when the meat had been minced before mastication (12 replicates for iron determination). Fig. 8D shows that the amount of β -carotenes released in saliva during the *in vivo* mastication of raw carrot increased along the masticatory process, obviously due to matrix disruption. This kind of measurement is totally feasible *in vitro* with the masticatory simulator. The analysis of the bioaccessibility of carotenoids could undoubtedly be performed *in vitro* with the AM², since the release of nutrients from plant foods is largely dependent on food breakage during mastication and could be decisive for their bioavailability (Faulks & Southon, 2005; Hedrén et al., 2002; Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011). Oral bioaccessibility is also a relevant issue for other macro and micronutrients such as in the case of lipids in almonds (Cassady, Hollis, Fulford, Considine, & Mattes, 2009; Ellis et al., 2004; Grundy et al., 2015).

Fig. 9 illustrates the first step of digestion occurring in the mouth during mastication and quantified by the production of maltose after the oral hydrolysis of starch by salivary amylase. Pieces of different breads (2.5 g) were introduced in the AM². Mastication was carried out (20

masticatory cycles) either with water (Volvic®) or human saliva (1.6 mL as estimated by weighting *in vivo* food boluses collected during the programming of the AM² apparatus). Logically, as expected, the concentration of maltose was higher with human saliva than with water, but also differed regarding the type of bread chewed. The mastication of pieces of white bread led to significantly higher hydrolysis of maltose than the mastication of whole wheat bread of the same weight. This observation confirmed the initiatory role of mastication in digestion by disintegrating food into smaller particles that are impregnated by saliva (Hoebler et al., 1998; Pentikäinen et al., 2019; Tournier, Grass, Zope, Salles, & Bertrand, 2012).

Although the major mechanical reduction of food occurs during mastication, it is completed in the stomach (Kong & Singh, 2011). This continuum of activity is exemplified in Fig. 10a, which shows the granulometric characteristics measured first in the *in vitro* food bolus of pasta obtained with the AM² just before swallowing, and then in the gastric compartment after *in vitro* digestion. Moreover, this example also highlighted that the stomach cannot totally overcome the failure in food reduction during deficient mastication resulting in a bolus with numerous large particles. This must be taken into account, considering that the level of disintegration of a food in the stomach depends on the structure of the matrix (Kong & Singh, 2008a) and impacts gastric emptying (Pera et al., 2002), thus assigning a specific role to mastication and the quality of the food bolus arriving in the stomach. Large particles have also been shown to lengthen gastric emptying and to produce lower glycemic and insulin blood responses than smaller particles (Ranawana, Clegg, Shafat, & Henry, 2011). This could be an acceptable explanation for the lengthening of the whole digestive process and the delay in nutrient postprandial appearance observed in edentulous people (Rémond et al., 2007).

Fig. 10B shows the kinetics of protein concentration measured *in vitro* in the stomach

simulating digestion of an elderly person (in terms of pH kinetics and pepsin amount) after the oral ingestion of meat. Using the masticator apparatus AM² with different programmings to simulate either *in vitro* normal or deficient mastication, combined with an instrumental gastric system, it appeared that large food fragments constituting the food bolus after deficient *in vitro* mastication resulted in a significant deficit in protein release in elderly digestive conditions.

5. Biochemical modifications during oral processing

The mastication simulator offers the possibility of exploring the chemical role of saliva and modification of the food compounds occurring within the mouth while the food is being transformed into a bolus ready to be swallowed. The various roles of saliva can be explored since it can be added or not in the apparatus for *in vitro* mastication. For example, human saliva is assumed to protect food compounds against oxidation (Moore, Calder, Miller, & Rice-Evans, 1994). Thus the AM² allows testing the role of saliva by using *in vitro* no-saliva, human saliva or any composition of artificial saliva containing enzymes or not, for example. Fig. 11 (A and B) shows the effect of chewing on the oxidation of beef meat when the artificial mouth was devoid of saliva. Lipid and protein oxidation in the food bolus was assessed by changes in TBARS (ThioBarbituric Acid Reactive Substances) and by the quantification of carbonyl groups (aldehydes and ketones), respectively. Three meat samples were measured before chewing (raw meat, cooked meat, cooked + minced meat) and 12 boluses were obtained after *in vitro* mastication (just before swallowing). Without saliva, significantly higher lipid oxidation was observed when the meat sample was chewed in minced format rather than as a non-minced meat (Fig. 11A). The oxidation of proteins occurring during chewing without saliva was also significant although less marked than for

lipids (Fig. 11B). These results confirm the potential occurrence of lipid and protein oxidation and underline the essential antioxidant role of saliva in protecting food compounds from oxidation (Qu et al., 2016). The more a food is chewed the more likely it will be impregnated by saliva and the nutrients protected against oxidation. This aspect should be thoroughly studied for various foods since it may have serious consequences for certain nutritional issues as nutrient oxidation has known consequences on intestinal absorption (Bax et al., 2012).

6. Effect of deficient mastication on food bolus processing

The AM² can also simulate different dental states related to oral diseases, healthy childhood and ageing conditions. Fig. 12 indicates the effects of different modalities of impairment on the granulometry of poultry meatball boluses (Peyron et al., 2018). The *in vivo* boluses were collected from seven subjects at swallowing threshold after normal mastication (NM) composed of 17 masticatory cycles. As usual, preliminary *in vivo* experiments allowed determining the main settings for *in vitro* mastication, and the simulation of deficient mastication was performed with modifications of certain parameters. This introduced specific deteriorations of the masticatory sequence in terms of the force and motility of oral elements, as illustrated in Figure 2 for the choice of spring-induced forces. Two levels were set for deficient force by using the spring of lowest stiffness together with an increasing offset between the jaws at closing (Def Force 1 and Def Force 2) and deficient motility simulated by two different low values of rotation angle (compared to normal programming) applied during jaw confrontation (Def Motil 1 and Def Motil 2). A considerable decline in masticatory force (Def Force 2 in Fig. 12) significantly changed the particle size distribution in the meatball boluses towards a greater proportion of large particles ($p < 0.001$). Motility deficiency also significantly limited food fragmentation, with an impact corresponding to the level of the

deficiency ($p < 0.001$). This difference was even more dramatic when the masticatory force and motility deficiencies (Def Motil & Force) were added together during mastication compared to other conditions ($p < 0.001$). The description of the impact of gradual and cumulative deficiencies on the physical properties of boluses obtained with poultry meatballs has been described elsewhere (Peyron et al., 2018) and the results agreed with those obtained *in vivo* (Mishellany-Dutour, Renaud, Peyron, Rimek, & Woda, 2008; Woda et al., 2010b).

7. Measurement of forces applied during mastication

The AM² can measure forces developed during mastication with a strength sensor (Fig. 1). The specific approach described in Figs. 13 and 14 is mandatory. The large artifacts recorded from the force sensor included in the AM² came from frictions induced by the mobile jaw's forward and backward movements. This implied stopping all the movements for one or two seconds during which the force was measured. Fig. 13A shows the method used to calibrate the force sensor to a null force with an empty mastication chamber to overcome the artifacts. Fig. 13B shows an experiment on force measurements performed with gelatin samples as models (10 mm high, 20 mm diameter) presenting three levels of hardness but the same recoverability—(Peyron, Lassauzay, & Woda, 2002). The forces recorded during five successive cycles differed according to the level of hardness of the food models.

Fig. 14A shows an experiment where the forces ~~used~~ during a long sequence of 36 cycles were recorded during the chewing sequence of a banana sample (10 mm high and 20 mm diameter). During this experiment the moving jaw compressed the sample, while the fixed jaw moved back due to spring compression, leading to a partial deformation of the sample. The masticatory forces progressively decreased from 25.42 ± 0.81 N—at the first bite to near zero N at the last bite (5 replicates). A comparable force value (26.99 ± 1.08 N) was measured

with the Instron machine (Fig. 14B) performing a single compression test at 65% deformation (the same deformation range applied on the banana sample by the AM²) on two banana samples taken from the same bunch as the sample used for the experiment presented in Fig. 14A. The resistance force obtained with the Instron machine when the sample was compressed ($26.99 \pm 1.08\text{N}$) was comparable to that obtained with the AM² at the first bite. In another experiment presented in Fig. 14 (C and D), the AM² was used to mimick testing machine mode to test the effect of fruit ripening. The ripe and firm banana samples were subjected to five successive compressions. The resistance was higher ($7.93 \pm 1.22\text{N}$) for the firm banana than for the ripe one ($4.28 \pm 1.04\text{N}$).

8. Conclusion

For various reasons ranging from financial to ethical considerations, but also because it is not possible to access the interior of the mouth during mastication, the AM² masticator apparatus offers a unique approach in numerous fields of research in food science, including food design, oral health and nutrition issues. It facilitates experiments in particular when large samples of subjects or high numbers of replicates would be required *in vivo*. Also, the number of repetitions can be much smaller because the variations between them are due only to mechanical hazards that are much easier to control than biological intersubject variations. Finally, no ethical requirements are needed with *in vitro* experiments.

The AM² has certain limits. Contrary to many other artificial masticators (Salles et al., 2007; van Ruth & Buhr, 2004), it does not currently offer the possibility of collecting volatile aromatic compounds during food disruption within the masticatory chamber. This limit, however, could be overcome technically if needed. At present, the choice of masticatory programming is based on several *in vivo* recordings followed by the adjustment of the AM²

parameters. In the future, it may be possible to introduce a retrocontrol based on neural networks in the AM² which would allow adaptation to ongoing food bolus transformation, as is the case in *in vivo* mastication

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Legends

Figure1: Schematic representation of the AM² artificial masticator with insert of an open view of the mastication chamber in which the fixed and mobile masticatory **disks** are shown. The whole apparatus is 130 cm long and 30 cm in height. The masticatory chamber (19.5 x 9.5 cm) is located in the continuation of the shaft holding the mobile masticatory **disk**.

Figure 2: Example of AM² programming for meat mastication. Mean particle size distribution curves are expressed as cumulative weight percentages of particles passing through each sieve during manual sieving. The force generated for food compression during mastication depends on the stiffness of the spring which must be chosen as a function of food resistance. The curve obtained with the spring 3 (18.2 N/mm of spring stiffness; n = 4) and 22 masticatory cycles gave the best fit with the mean *in vivo* curve (n = 8). Spring 3 with only 20 cycles gave a higher median value (= *d50*, theoretical sieve letting pass 50% particle mass), indicating a poorly prepared food bolus with larger particles (n = 4). Spring 4 (23.5 N/mm of spring stiffness) was harder and gave a lower *d50* value when used with 22 masticatory cycles, indicating excess in food bolus disruption (n = 4). For the sake of readability, standard deviations are not drawn.

Figure 3: Comparisons between mean particle size distribution curves of boluses obtained *in vivo* and *in vitro* from different food types. Piece of beef: cooked samples, 2cm square and 1cm thick. Minced beef: same cooked meat, molded in the same sample shape as non-minced meat. Frankfurt sausage: cylindrical samples of 6.5g. Pasta products: cooked Fusilli pasta in 7g bite. Coconut: 1cm square and 1cm thick sample. Gherkins: cylindrical sample of 3.5g. Each point represents the weight of the particles (in %) retained in the corresponding sieve size added to the sum of all the preceding points (cumulative curve). The similarity required between *in vivo* and *in vitro* bolus granulometry was not limited to the median particle size (*d50=50% on cumulative curve*) but sought for the whole mean particle size distribution curves. In these examples, better fitting could be obtained for minced meat and gherkins if further studies were carried out. For the sake of readability, standard deviations are not drawn.

Figure 4: Comparisons between mean particle size distribution curves for boluses obtained *in vivo* (A) and *in vitro* (B). The masticatory sequence of pork meat (2cm cubes) was interrupted

669 after one, two, three and four quarters of the *in vivo* (A) and *in vitro* (B) chewing sequences
 670 (0.25, 0.5, 0.75 and 1; n=10). In C, particle size distributions were obtained for two types of
 671 bread (white and whole wheat breads, sample = 1 /4 of a 2cm thick slice cut in a French
 672 baguette) after *in vivo* and *in vitro* mastication (n=10). The difference observed *in vivo*
 673 between the two types of bread can be completely reproduced *in vitro*. For the sake of
 674 readability, standard deviations are not drawn.

675 Figure 5. A: Evolution of four physical characteristics of bread boluses produced *in vivo* from
 676 a French baguette after one, two or three thirds of the complete chewing sequence (1/3, 2/3,
 677 swallowing; n = 10 subjects x 2 repetitions; sample = 1 /0 of a 2cm thick slice). B:
 678 Comparison of hardness and recoverability of pasta boluses produced *in vivo* and *in vitro* (n =
 679 5 subjects or replicates; cooked Fusilli pasta in 7g bite). The food bolus was subjected to a
 680 double compression test during a Texture Profile Analysis test (TPA) performed with an
 681 Instron machine equipped with a flat piston head (Ø 28 mm) moved at a constant
 682 displacement rate of 50 mm.min⁻¹, a cylindrical container (int. Ø 35 mm, ext. Ø 40 mm) and a
 683 500 N load cell. The bolus was spread at the bottom of the cylindrical container and subjected
 684 to 65% deformation of its initial height. In this example, hardness (N), adhesiveness (N.s),
 685 elasticity and recoverability (both unitless) were extracted from the TPA curves (mean and
 686 SD). Physical characteristics can be obtained in the same manner for *in vitro* boluses
 687 produced with the masticator apparatus AM².

688 Figure 6. A: Curve of a contaminant released in saliva along the mastication of a non-
 689 alimentary product. The two curves were obtained for the same product containing two
 690 different concentrations of the same toxic substance. B: Liquid content in a bolus of fruit (in
 691 % of bolus weight, mean and SD) after 24 *in vivo* masticatory cycles performed in normal
 692 conditions and with the fruit sample placed in a bag while chewing to separate juice from

693 saliva (n=10; mean and SD). C: Percentage of juice extracted from fruit after 15 and 24 *in*
 694 *vitro* masticatory cycles with AM² (n = 10, mean and SD).

695 Figure 7. Chewing a non-alimentary gum. Cumulative loss of gum weight (A; in %, mean and
 696 SD) and cumulative concentration of glucose released in saliva (B; in g, mean and SD) were
 697 obtained from different time point measurements along 12 minutes of *in vivo* and *in vitro*
 698 mastication of the gum sample (n=8).

699 Figure 8. Impact of food structure on free-iron and carotene release in saliva. A: Bolus of
 700 minced meat analyzed after normal mastication consisting of 10 cycles while the meat sample
 701 (2x2x2 cm) needed 32 masticatory cycles to produce swallowable boluses (mean and SD). B
 702 and C: At the end of mastication, free-iron release was greater for minced than for mouthful
 703 meat, both in the solid part of the bolus and in saliva. D: Examples of the oral release of
 704 carotene measured by HPLC in the salivary part of the bolus. For readability, spectra of beta-
 705 carotenes determined in three saliva samples were superimposed according to the elution time
 706 on the x-axis. It clearly shows an increase of beta-carotene release in saliva with the progress
 707 of the masticatory sequence from the boluses collected at the beginning, in the middle of the
 708 masticatory sequence, and when the bolus was ready to be swallowed.

709 Figure 9. Production of maltose in the mouth during the artificial mastication of whole wheat
 710 and white bread samples, with the addition of water or saliva as the oral liquid (n=10; mean
 711 and SD). All differences were significant.

712 Figure 10. A: Mean particle size distribution curve in the swallowable bolus after *in vitro*
 713 mastication of pasta and in the gastric compartment after *in vitro* gastric digestion. B: Kinetics
 714 of protein appearance in a simulated aged gastric compartment after normal and deficient
 715 mastication simulated with the AM² (n = 3; mean and SD). Protein concentration was

716 significantly lower (*) from 90 min digestion in deficient mastication compared to normal
 717 mastication.

718 Figure 11. Lipid and protein oxidation during *in vitro* mastication in the absence of saliva. A:
 719 Lipid oxidation during *in vitro* mastication without saliva, measured by malondialdehyde
 720 appearance (MDA) as thiobarbituric acid reactive substances (TBARS). B: Protein oxidation
 721 during *in vitro* mastication without saliva measured by the quantity of carbonyl groups
 722 (aldehydes and ketones present in the food bolus (reaction with dinitrophenylhydrazine
 723 (DNPH).

724 Figure 12. Effects of two levels of deficient force (Def Force 1 & 2) and motility (Def Motil 1
 725 & 2), and their combination, on the granulometry of poultry meatball boluses obtained with *in*
 726 *vitro* mastication (n=10; mean and SD). *d*50 value (in mm) is the median value of the particle
 727 size distribution in the food bolus and defined as the theoretical sieve size through which 50%
 728 of the mass can pass. Comparison was performed with normal mastication (NM). Significant
 729 differences against NM *d*50 values were observed from Def Force 2 condition. *Data adapted*
 730 *from Peyron et al, 2018*

731 Figure 13. Calibration (A) and measurement (B) of the resistance to compression of three
 732 gelatine products (Ø 2 cm; height 1 cm) of different harnesses. Forces are measured with the
 733 strength sensor along the shaft actuating the mobile masticatory **disk**. A: the AM² is activated
 734 with a pure translation movement, with an empty chamber and for five successive cycles. At
 735 the end of each cycle, the mobile (mandibular) jaw is stopped at initial contact, and contact
 736 for two seconds, without applying forces. Forces recorded in the translational axis with the
 737 strength sensor are in Newtons (N). The whole procedure lasted 23 seconds (s). The force
 738 sensor records four types of events: ① friction of the mobile jaw against the mastication
 739 chamber wall while the jaw is moving towards the useful range of movements; ② forces

740 resulting from the air compressed by the progress of the mobile jaw in the chamber; ③ forces
 741 resulting from negative air pressure while the jaw moves backwards; ④ null force while the
 742 jaw is stopped for 2 seconds with no pressure against the fixed jaw. B: Measuring the force
 743 during compression of three gelatines of different hardnesses. The events are the same as
 744 those described above except for event 4 which displays the forces in Newtons (N) applied
 745 during the mastication of the three gelatines differing in hardness. The translational
 746 movement was performed at 100 mm/s.

747 Figure 14. Forces recorded with the strength sensor of the AM² (Fig. 1) while chewing banana
 748 samples with 1.8 ml saliva, at 100 mm/s translational speed. A: Successive bites performed in
 749 2 minutes chewing. The progressively decreasing force—from $25.42 \pm 0.81\text{N}$ to almost zero, is
 750 highlighted by black circles and illustrates that the banana sample was progressively crushed
 751 and less resistant with the successive bites. B: Measures with an Instron machine performing
 752 a single compression test in replicates on 2 banana samples (2 cm diameter, 1 cm high) with a
 753 cylindrical piston head of 2.8 cm diameter. The values of forces in Newtons ($26.99 \pm 1.08\text{ N}$)
 754 were similar to the value obtained during the first bite with AM² ($25.42 \pm 0.81\text{ N}$). C and D:
 755 Measures of forces developed during a five-bite compression to test the effect of banana
 756 ripening on sample resistance with the AM² mimicking a testing machine mode (no saliva,
 757 spring of lowest stiffness, mobile disk actuated at 100mm/s).

PROOFREADING CERTIFICATE

To whom it may concern,
the document bearing the title:

ADDRESSING VARIOUS CHALLENGES RELATED TO FOOD BOLUS AND NUTRITION

WITH THE AM² MASTICATION SIMULATOR

was proofread and corrected by a native English speaker working for Accent Europe on 13 June 2019.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

A

B

Figure 10

Figure 11

Figure 12

Figure 13

Chewing of banana sample in AM² in 36 cycles

B Instron: compression test; 2 banana samples

Five-bite compression of ripe and firm bananas samples in AM²

Figure 14