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Oral Behaviour of Emulsions Stabilized by Mixed Monolayer

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ABSTRACT

Controlled flavour release is highly important for formulation design of food emulsions. However, manipulating oral behavior and maintaining the stability of flavoured emulsion is quite challenging. Hence, the objective of the study was to investigate the effect of emulsion stability and oral behaviour using mixed emulsifiers of different nature for their controlled flavour release. Orange oil flavoured (0.1 % orange oil + 10 % sunflower oil) oil-in-water emulsions were prepared by microfluidization through modified starch (MS) and whey protein isolate (WPI) with different mass ratios (0.5:0.5, 0.5:1, 1:0.5, 1:1, 1:0 and 0:1) of emulsifiers. The fabricated emulsions were less than 0.134 μm in size (d_{32}) with stable oil droplets having strong negative charges. Increase in mean droplet size d_{32} (1.124 and 0.937 μm) was observed for 0.5:0.5 and 0:1 emulsions during storage at 10^{th} day in 28±1 ºC; but the emulsions stored at 4±0.1 ℃ were found to be stable. All the emulsions exhibited Newtonian flow; however once mixed with artificial saliva they displayed shear thinning behaviour for 1:0.5 and 1:0. During oral processing, in-vitro and in-vivo showed flocculation and coalescence; subsequently structural deformation was observed with increase in size (d_{32}) and weak negative charge in 1:0.5 and 1:0 emulsions. Backscattering profile revealed more destabilization for 1:0 and less for 1:0.5 emulsions. Contrarily, other emulsions were did not show any changes. Therefore, oral processing of emulsion results suggested that 1:0 had quick destabilization and 1:0.5 changed gradually. Thus, mixed emulsifier monolayer contributed significantly to the behavior of emulsion once mixed with saliva and it can be useful for controlled flavour release.

Keywords: Emulsion; Food oral processing; Modified starch; Storage stability; Oral behaviour; Emulsion-saliva interaction.

1. Introduction

Emulsions play an essential role in the food supplementation, pharmaceutical and nutraceutical applications. Previously, many research studies have been reported on the methods of preparation (i.e. high and low energy emulsification), stability (i.e. physicochemical) and properties (i.e. thermal, functional and structural) of emulsions (Karthik & Anandharamakrishnan, 2016a; Qian, Decker, Xiao, & McClements, 2012). Presently, there has been more attention on designing emulsions which can improve the nutritional and functional aspects of foods (Ezhilarasi, Karthik, Chhanwal, & Anandharamakrishnan, 2013; Mao & Miao, 2015; Roohinejad, Greiner, Oey, & Wen, 2018). Food emulsions are involved into different stages of oral processes such as oral shearing, salivation, bolus formation and swallowing during consumption (Mao, Roos, Biliaderis & Miao). During oral processing, the emulsion is destabilized which is resulted in very different microstructure that leads to textural and sensorial experiences entirely different from that of a stable emulsion system (Chen, 2015). However, the effect of different combination of emulsifier on the behavior of emulsion during oral processing is little known. To understand the underlying mechanism of emulsion interacting with saliva is more important for manipulating of their stability and oral behaviour. Hence, the emulsion based system can be used for studying the oral behavior of food emulsions using suitable emulsifiers.

Emulsions are colloidal systems comprising two immiscible liquids, with one phase being dispersed into another phase in the form of fine droplets that can be either oil or water. Emulsions are extensively used as an important vehicle for delivering the flavour molecules (Anandharamakrishnan, 2014). However, during the delivery of flavour in the oral digestive system, many physiochemical factors are involved into the release of flavour molecules; furthermore it is associated with properties of emulsifier monolayer. Hence, it is essential to know about the flavour release from emulsions prepared by different combination of emulsifiers during oral processing. Food oral processing is a dynamic process in which food will be broken down structurally for easy transportation to the stomach and for the sensory experience (Chen, 2015). Once a food emulsion enters into the mouth, the emulsion is subjected to a wide range of physical and biochemical conditions, i.e. mixing with saliva and air, equilibrium to body temperature and shear between the epithelial surfaces of the tongue and the oral palate. Moreover, it is also exposed to salivary enzymes, various biopolymers such as mucins, changes in the ionic environment due to presence of electrolytes and a change in pH (Bardow, Moe, Nyvad, & Nauntofte, 2000; Chen, 2009; de Wijk, Prinz, Engelen, & Weenen, 2004; Glantz, 1997). Mixing food with saliva facilitates food manipulation and bolus formation in the oral cavity (Prinz & Lucas, 1997), which also influences flavour release (van Ruth & Roozen, 2000) and can be responsible for taste and flavour molecules to become diluted (van Ruth, Roozen, Nahon, Cozijnsen, & Posthumus, 1996; Christensen, 1985). These flavour molecules are then either diffused through the salivary media to the taste buds or released to the air. Hence, saliva plays a significant role in the food oral processing and sensory perception (Chen, 2015). During oral

processing of oil/fat, saliva can also function as an emulsifier to give a coarse emulsion (Glumac, Qin, Chen, & Ritzoulis, 2018).

Conversely, emulsifier is also greatly involved in producing stable emulsion, as it adsorb on the interface between oil and water phase. Thus, reducing interfacial tension prevents the instability mechanism (e.g. flocculation and coalescence) that occurs by generating protective layer between the oil droplets through steric and/or electrostatic stabilizing mechanisms (McClements, 2015, Karthik, Ezhilarasi, & Anandharamakrishnan, 2017). Usually, food proteins are widely used as emulsifiers in food industry due to their advantages in high nutrition, and excellent surface activities (Chen, et al. 2018). However, the proteins like whey protein isolate stabilized emulsions are sensitive to temperature, pH, salt, environmental stresses, etc., which also influence the flocculation, coalescence, creaming and phase separation (Lam & Nickerson, 2013; Dickinson, 2010). On the other hand, modified starch has become commendable for food formulations due to their emulsification properties, accessibility and economical (Lin, Liang, Zhong, Ye, & Singh, 2018). Synthesize of octinyl succinic anhydride (OSA)-modified starch is achieved by an esterification reaction between the hydroxyl groups of native starch and OSA (Sweedman, Tizzotti, Schafer, & Gilbert, 2013). The hydrophobic octenyl side chains in the OSA groups attached to the hydroxyl groups of starch yields the emulsifying property to the starch (Torres, Tena, Murray, & Sarkar, 2017). The modified starch can change the digestion behavior of the emulsions as well as the release of flavours, nutrients and bioactives in the oral cavity. Once the OSA-starch stabilized emulsion interact with saliva, the α-amylase presented in the salivary phase initiates the starch hydrolysis and it turns to reduce the solidity of layer in the emulsion

droplets; therefore resulting in destabilization of starch emulsion (Chen, 2007; Chiu et al. 2017; Ettelaie, Holmes, Chen, & Farshchi, 2016). This is the basis for creating mixed monolayer emulsion, which contains starch and protein emulsifier system for manipulating oral behaviour.

Hitherto, Vingerhoeds, Blijdenstein, Zoet, & van Aken, (2005) studied physicochemical effects of saliva on protein-stabilized food emulsions and observed the aggregation phenomena. Presumably, Sarkar, Goh, & Singh, (2009) investigated milk-protein stabilized emulsions mixed with artificial saliva to study the colloidal stability. It reported that emulsions stabilized by β-lactoglobulin were stable but showed depletion flocculation at higher mucin levels. Contrarily, emulsion stabilized by lactoferrin exhibited aggregation in the presence of salts but in the absence of mucin. However, droplet aggregation observed at higher mucin. Recently, Chiu et al. (2017) reported on OSA-starch stabilized emulsion for enhancing saltiness perception during oral cavity. Likewise, WPI-pectin stabilized flavoured emulsion showed release of volatility increased with increasing salt and addition of artificial salivas (Mao, Roos, O'Callaghan, & Miao, 2013). However, very limited studies are carried out in the manipulation of oral behavior on food emulsions by comparing in-vitro and in-vivo methods; therefore, it is required to study extensively. Thus, the present work aimed to reveal the impact of mixed monolayer characteristics on the stability and oral processing of emulsion for the flavour release application. In addition, this study postulated that mixed monolayer emulsion may yield a unique route towards food oral processing that can be further helpful in the design of food products for desirable oral experience for controlled flavour release.

The objective of this study is to evaluate the role of mixed emulsifier monolayers (modified starch and whey protein isolate) on the stability and oral behaviour of flavoured emulsions produced by microfluidization. The emulsions oral behaviour by in-vitro and in-vivo is examined in terms of size, morphology, droplet charge, rheology and turbiscan (TSI and % BS). Moreover, emulsion stability against storage condition also studied.

2. Materials and Methods

2.1. Preparation of emulsion

Modified starch (Purity gum ultra, modified from waxy maize starch, Ingredion Incorporated, Shanghai, China) was dispersed into aqueous solution and mixed using a high-speed homogenizer (T25 digital Ultra Turrax, IKA, USA) at 3,600 rpm for 1 hr in room temperature (28±1 ºC) to form continuous phase. The prepared solution was stored at overnight in refrigerated condition (4±0.1 ºC) for complete hydration. On the other hand, whey protein isolate (Glanbia Nutritionals Inc.) was mixed into aqueous phase through high-speed homogenizer at 5000 rpm for 5 min in room temperature to produce protein emulsifier solution. In this study, different concentrations (%, w/w) of MS and WPI emulsifier (i.e. 0.5:0.5, 0.5:1, 1:0.5, 1:1) were mixed to produce mixed monolayer emulsion. In addition, individual emulsifier such as 1:0 (MS) and 0:1 (WPI) were also used as control for preparation of emulsion. Table 1 shows the composition of the six emulsions prepared. For the preparation of oil phase, orange oil (0.1 %, w/w; now essential oils, IL, USA) was blended with sunflower oil (10 %, w/w; local

supermarket, Hangzhou, China). The orange oil was used to mimic the model flavour oil-in-water emulsion system.

The coarse emulsion was prepared by mixing these two phases using high-speed homogenizer at 6000 rpm for 5 min. The obtained coarse emulsion was further homogenized by microfluidizer (Microfluidics M-110P, Westwood, Massachusetts, USA). The operation condition of microfluidizer was chosen at 200 bar of 5 cycles for preparation of MS and MS mixed with WPI emulsions; whereas 250 bar of 5 cycles was used for WPI emulsion. In the preliminary study, emulsification condition was optimized in order to achieve consistent droplet size distribution for different emulsions. After the preparation of flavoured emulsions, the samples were stored at refrigeration (4±0.1 ºC) and room (28±1 ºC) condition for studying the stability and oral processing.

2.2. Preparation of artificial saliva

The artificial saliva was prepared according to Davis, Hartman, & Fincher, 1971; Sarkar et al., 2009 and their compositions are shown in Table 2. After preparation of artificial saliva, the pH was adjusted to pH 6.8 using 1M HCl solution.

2.3. Droplet size measurements

The droplet size distribution and span value of all the emulsions were measured by laser light diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments, Worcestershire, UK). Refractive indices of 1.46 for oil and 1.33 for dispersant medium were used to determine the particle size. The absorbance value was set at 0.001. Emulsion droplet size study was performed at different time intervals (1, 5, 10 and 15

183 days) and storage conditions i.e. refrigeration (4±0.1 ℃) and room (28±1 ℃).
184 Measurements were done in triplicates. Similarly, the mean droplet size (d_{32}) of
185 emulsion mixed with artificial saliva was determined by the same instrument.
186
187 2.4. Determination of zeta-potential
188 The electrical charge (ζ-potential) of orange oil flavoured emulsions prepared
189 with different concentration of emulsifiers was determined using Malvern Zetasizer
190 (Nano-ZS90; Malvern Instruments, U.K.). Emulsions ζ-potential was examined under
191 different storage intervals i.e. on the 1, 5, 10 and 15th day and measurements were
192 made in triplicates. Similarly, the ζ-potential of emulsion mixed with artificial saliva was
193 examined using the same procedure.
194
195 2.5. Rheological characteristics
196 Rheological characterization of all the emulsions were performed using a shear
197 rheometer (Discovery HR-2, TA Instruments, New castle, USA) with a double gap cup
198 and bob geometry attachment. The shear rate was gradually increased from 1 to 100 s^{-1}
199 at a controlled temperature of 25 ºC. The rheological measurements were conducted in
200 triplicate. The flow behavior of emulsion mixed with artificial saliva was studied at
201 temperature of 37 ºC using the same measurement setup.
202
203 2.6. Morphology
204 Morphology of emulsions was observed by optical microscope (Leica DMC2900,
205 Heidelberg, Germany) with a 100X oil immersed objective lens and images were

206 captured using LAS v4.6 software. Similar experimental procedure was applied to find
207 out the microstructure of all the emulsions for in-vitro and in-vivo salivary studies.
208
209 2.7. Creaming stability at different storage condition
210 Orange oil flavoured emulsions (15 mL) were kept into a measuring glass tube
211 with a stopper. Creaming was measured at different time intervals (1, 5, 10, and 15
212 days). Creaming was examined by determining the height of the cream layer on top
213 (HC) and the height of total emulsion (HE) in the emulsion stored tube (Huimin et al.,
214 2014). Measurements were done in duplicates. Emulsion creaming stability in terms of
215 creaming index (CI %) was calculated by Eq. (1)

216 $$\text{Creaming Index}\,(\text{CI}\,\%) = \left(\frac{\text{H}_\text{C}}{\text{H}_\text{E}}\right)\times 100 \qquad (1)$$

217 Similarly, phase separation and sedimentation were monitored during the storage for all
218 the emulsions.
219
220 2.8. Oral processing by in-vitro method
221 In-vitro emulsion stability studies were carried out by mixing emulsion with
222 artificial saliva (as explained in the section 2.2). The initial emulsion was mixed with
223 artificial saliva in a ratio of 1:1. In order to avoid the early destabilization of emulsion
224 structure, the mixture of solution was gently stirred using glass rod. The ratio of 1:1 was
225 chosen based on the consideration that flavoured emulsion is a mouth-feel and olfactory
226 sensation perceived while receiving a small amount of liquid. Rituja and Chen, (2019)
227 stated that emulsions like liquid foods have short oral residence times and saliva flow

rates of about 1-5 g/s would be required to get a 1:1 mixing ratio. In addition, Brossard, Cai, Osorio, Bordeu, & Chen, (2016); Cai, Li, & Chen (2017) have also reported that 1:1 ratio is probably a good approximation and it was found to be acceptable for studies. After mixture of artificial saliva with emulsion the pH was adjusted to pH 6.8 using 1 M HCl and incubated at 37 ºC in shaking water bath at a rotation speed of 100 rpm. The emulsion samples were withdrawn at different time intervals such as 10, 300, 600, and 1,200s and immediately characterized through different analytical methods. In the preliminary study, in-vitro oral processing was optimized by varying the time interval. However, longer time interval was chosen to mimic agitation in the mouth (Chang and McClements, 2016). Generally, this time is longer than a liquid food would spend in the mouth; but it was used to match up with the in-vivo oral processing. In order to compare the effect of α-amylase interaction with mixed emulsifier, individual emulsifier was also used as reference.

2.9. Turbidity scan

Destabilization mechanism of emulsions mixed with artificial saliva was evaluated using a vertical scan analyzer TurbiscanLab (Formulaction, Toulouse, France) at 37 ºC. Emulsions mixed with artificial saliva (about the volume 20 mL, height 42 mm) was placed in a cylindrical glass tube and scanned from the bottom to top with a laser light source (λ = 850 nm) for 1 hr. The scan was repeated every three minutes, each time giving a single curve and at the end of the experiment all curves were superimposed on the resultant graph to show the overall destabilization of the emulsion system.

2.10. Oral processing by in-vivo method

10 human subjects (6 females, 4 males, aged between 25-33, non-smokers) were recruited for the in-vivo oral behavior study. Emulsions sample name was coded randomly with three digits and kept in a random order. Initially, subjects were requested to ingest 2 mL of emulsions to the mouth without swallowing. Afterwards, the emulsion was gently stirred and mixed up with the subject's saliva for 3, 10, 30 and 60s. Further, the subjects were requested to spat out the samples into a clean container for the analysis. Before and after moving to the every individual test, subjects were asked to use mouth-wash and warm water to clear residual taste and wash their mouth with clean water for three times. Approximately, 3 to 5 min was given to the subject between 2 tests. The samples collected from 10 subjects were immediately characterized in terms of size, morphology and zeta-potential. The average values of 10 subjects were considered for this study. Written consent form was taken from the entire participants and a financial compensation was offered for their participation. Ethical approval was obtained from the Zhejiang Gongshang University (2018030106).

2.11. Statistical analysis

Results were expressed statistically as mean value ± standard deviation of experiments performed in either duplicates or triplicates. Statistical analysis was carried out by analysis of variance (ANOVA) using SPSS statistical software version 16. Comparison of means was performed by Tukey's post hoc test. The level of significance used was $p<0.05$ for all the statistical tests.

3. Results and discussion

3.1. Effect of storage temperature on mean droplet size

In the purpose of commercial applications, the flavoured emulsions long-term stability that is emulsion remains stable throughout their shelf-life is the most important factor. Hence, the influence of temperature and storage time on the emulsion stability was investigated. The mean droplet size distribution of MS and WPI mixed emulsifier (i.e. 0.5:0.5, 0.5:1, 1:0.5, 1:1, 1:0 and 0:1) are illustrated in Fig. 1. All the emulsions exhibited monomodal and uniform droplet size distribution. The mean droplet size of emulsions stored at different temperature (4±0.1 ℃ and 28±1 ℃) is show n in the Table 3 and Fig. 2A (i and ii). The emulsions prepared with different concentration of MS and WPI i.e. 0.5:1, 1:0.5, 1:1, and 0:1 showed smaller size (0.10, 0.11, 0.10 and 0.10 µm) than those prepared with 0.5:0.5 and 1:0 (0.13 and 0.13 µm) at initial day. On the other hand, span value of 0.5:0.5, 1:0.5 and 1:0 emulsions depicted lower values (2.71, 2.76 and 2.47, respectively) as compared to 0.5:1, 1:1, and 0:1 (3.02, 2.98 and 2.88) emulsions (Table 3). This lower span value leads to much narrower droplet size distributions respectively.

During 5 days of storage, the emulsions kept at 4±0.1 ℃ and 28 ±1 ℃ did not show any change in their mean droplet size (Fig. 2A). However, a significant ($p<0.05$) increase in mean droplet size d_{32} (1.12 and 0.93 µm) was observed in 0.5:0.5 and 0:1 emulsions stored at 28±1 ºC from the 10^{th} day onwards (Fig. 2A, ii). The mean droplet size of 0.5:0.5 emulsion increased with increase in storage temperature and this may influence oil droplet flocculation and coalescence. On the contrary, there was no

297 significant ($p<0.05$) increase in mean droplet size observed for all the emulsions stored
298 at 4±0.1 ℃ till 15 days. Similar trend of stability was observed in the DHA algae oil
299 emulsion when stored at lower temperature than ambient condition (Karthik &
300 Anandharamakrishnan, 2016b). Also, it was reported that emulsion stability is more
301 related to the type and concentration of emulsifiers. Thus, the obtained result suggested
302 that emulsion stored at low temperature and addition of more starch emulsifier to the
303 emulsion formulation had higher storage stability. Moreover, 0.5:1, 1:0.5, 1:1 and 1:0 of
304 emulsifier stabilized emulsion droplets covered the oil droplets considerably and these
305 emulsion systems can provide better stability in the different environment conditions.
306
307 3.2. Effect of storage temperature on droplet charge
308 Droplet charges of emulsions are shown in Table 3 and Fig. 2B (i and ii). In this
309 study, zeta potential values of all the emulsions were found to be of high negative
310 charge in the range of −41 to −46 mV. The strong negative charge on all the emulsions
311 can mainly be attributed to the presence of modified starch and whey protein molecules
312 at around the oil droplet surfaces. The different magnitude of charges on emulsions is
313 due to the existence of negative charge group in protein molecules. In this study, the
314 zeta-potential value of all the emulsions stored at refrigeration condition showed slight
315 reduction of their negative charge. Moreover, at 15^{th} day of storage all the emulsions
316 found to be not much difference and stability was maintained during the studies. On the
317 other hand, the emulsions stored at 28±1 ℃ were resulted extreme decrease of zeta
318 potential. Initially, 0.5:0.5, 0.5:1, 1:1 and 0:1 emulsion had showed higher zeta potential
319 value of −42.8, −45.66, −41.46 and −41.2 mV; however, at 15^{th} day it was found −20.03,

320 −18.7, −18.86 and -28 mV respectively. On contrast, 1:0.5 and 1:0 emulsions exhibited
321 −30.75 and −37.10 mV which confirms the high stability during storage at 28±1 ℃ (F ig.
322 2C, ii). The instability of emulsion systems at 28±1 ℃ may be due to the more
323 concentration of WPI in the emulsion formulation and it did not provide enough stability
324 once it's exposing for longer storage period. When emulsion is exposed to the different
325 environmental condition, the functional properties of emulsifiers may change
326 significantly; further this may not cover interface of oil droplets efficiently (Karthik &
327 Anandharamakrishnan, 2016a). Stachurski & Michalek, (1996) indicated that increase in
328 surface charge can significantly improve emulsion stability. This is due to the surface
329 charges that can produce repulsive forces between oil droplets against flocculation and
330 coalescence (Liu, Sun, Li, Liu, & Xu, 2006). Therefore, this present study suggested
331 that emulsion produced by 1:0.5 and 1:0 emulsifier systems showed higher physical
332 stability in terms of storage conditions (4, and 28 ºC).
333
334 3.3. Effect of storage temperature on creaming
335 Creaming of emulsion is formed due to the gravitational separation. The
336 creaming stability of emulsions is shown in Fig. 2C (i and ii). Creaming was not
337 observed for the emulsion stored until 3 days kept at 4±0.1 ℃ and 28±1 ℃. Further, the
338 emulsion stored at refrigeration condition exhibited very thin layer formation of creaming
339 and there was no difference observed within the emulsion systems (2C, i). On the other
340 hand, 0.5:0.5 emulsion stored at 28±1 ºC showed more increase of creaming index (%).
341 Whereas, 0.5:1, 1:0.5, 1:1, 1:0 and 0:1 emulsions showed very little creaming and
342 maintained throughout the storage (Fig. 2C, ii). The instability of 0.5:0.5 emulsion is

because of the MS and WPI mixed monolayer may not covered the oil droplets properly and this may influenced to rupture the interfacial layer when stored at 28±1 ºC. In addition, the lower concentration of mixed monolayer may also be the reason for the formation of emulsion instability. Moreover, droplet size is also one of the important aspects for maintaining the stability of emulsions against creaming (Desrumaux & Marcand, 2002). According to Stokes' law, the emulsions creaming rate (terminal velocity) is directly proportional to the square of diameter of the oil droplets (Joshi et al. 2012). Furthermore, the obtained result had more correlation with the inferences derived from mean droplet size (Fig. 2A). Besides, the primary instability of oil in water emulsion system is creaming, which impacts the macroscopic phase separation into two separate observable regions of cream and serum (Dickinson, 2003). In this study, there was no indication of phase separation and sedimentation in the emulsions stored at both the temperature conditions throughout the storage.

3.4. Rheological characteristics

Stability of emulsion and rheological characteristics are more subjected to the interactions between oil droplets and the interfacial layer of oil/water in the emulsion system (Dickinson, 1999). The rheological characteristics of emulsions are shown in Table 3 and Fig. S1. All the formulated emulsions exhibited almost similar rheological behavior during increase in the shear rate (s^{-1}). The presence of a linear relationship between shear stress and shear rate in all the emulsified system shows a Newtonian flow behavior. However, the emulsion prepared with 1:0.5 emulsion exhibited more viscous than the other emulsions. Similarly, 1:0.5 emulsion found to be of higher

366 viscosity (2.19 mPa.s), whereas other emulsions showed comparatively less viscosity
367 such as 0.5:0.5 (1.83 mPa.s), 0.5:1 (1.55 mPa.s), 1:1 (1.62 mPa.s), 1:0 (1.66 mPa.s)
368 and 0:1 (1.22 mPa.s) (Table 3). The increase in emulsion viscosity may be due to the
369 increase in concentration of modified starch in the emulsifier formulation. Contrarily, the
370 emulsion viscosity reduces when increasing the concentration of WPI. The emulsion
371 rheological behavior was affected by changing the concentration of MS and WPI
372 emulsifiers in the emulsion system. Thus, the higher concentration of MS emulsifier in
373 the mixed monolayer can yield viscous and stable emulsions. Also, it provides for a
374 longer shelf-life against flocculation, creaming and coalescence due to their rheological
375 behavior and this was confirmed by storage stability studies (Fig. 2A, B and C).
376
377 3.5. Oral stability of flavoured emulsions during in-vitro studies
378
379 3.5.1. Structural characteristics and droplet size distribution
380 The structural characteristics and mean droplet size (d_{32}) of mixed emulsifiers
381 stabilized emulsions treated with artificial saliva during in-vitro oral processing are
382 shown in Fig. 3 and Fig. 5a. Initially, all the emulsions droplets were observed spherical
383 in shape with no flocculation and coalescence. However, the morphology of 1:0.5 and
384 1:0 emulsions were changed subsequently once artificial saliva mixed with emulsions.
385 Thus, the emulsion prepared with only MS (1:0) was illustrated flocculation and
386 coalescence at 10s; later structural deformation (300s) was observed and this process
387 continued until the end of 1200s (Fig. 4a). On the other hand, 1:0.5 emulsion exhibited
388 little coalescence at 10s; further it increased gradually. At 1200s, it displayed more

389 coalescence and structural deformation (Fig. 4c). In contrast, the morphology of 0.5:0.5,
390 0.5:1, 1:1 and 0:1 stabilized emulsion was not exhibited any major changes until 1200s.
391 Likewise, the mean droplet size (d_{32}) of 0.5:0.5, 0.5:1, 1:1 and 0:1 mixed
392 monolayer emulsions did not show any changes and it continued throughout the in-vitro
393 oral processing (1200s) (Fig. 3a). However, the 1:0 emulsion exhibits drastic changes of
394 its mean droplet size (d_{32}) at beginning stage onwards. Also, the mean droplet size (d_{32})
395 yielded in 0.526±0.08 μm within 10s; further the emulsion size increased extensively
396 during the process (1.465±0.07 μm). Whereas, 1:0.5 emulsion mean droplet size (d_{32})
397 was started fairly from 0.162±0.002 μm at 10s and it steadily increased throughout the
398 oral process. Further, mean droplet size of 1:0.5 emulsion showed 4 fold increases
399 when compare to 0.5:0.5 emulsion at end of the in-vitro process. This result was well
400 correlated with structural changes of all the emulsions analyzed during in-vitro oral
401 processing (Fig. 3). From this study, it is postulated that substantial change in size and
402 morphology of 1:0 emulsion suggested quick release of flavour molecules during oral
403 processing. Henceforth, enhanced and controlled orange oil flavour release can be
404 achieved through 1:0.5 emulsion due to the gradual change in size and morphology.
405 Therefore, this obtained mixed monolayer emulsifier concept can be used for
406 manipulating oral behavior of flavoured food emulsion.
407
408 3.5.2. Zeta-potential
409 The zeta-potential data of the artificial saliva mixed with emulsions are shown in
410 Fig. 5c. The reason behind measurement of zeta-potential in the emulsion treated with
411 saliva is to know about the information in alteration of interfacial electrical properties

during oral processing. Once treated with artificial saliva, all the emulsions zeta-potential value was sharply reduced from a higher negative to a lower value. The decrease in zeta-potential value was changed based on the mixed monolayer concentration. In this study, 1:0 emulsion showed extreme decrease in zeta-potential within 10s (−6.44±0.37 mV) and it continued till 1200s (−6.29±0.22 mV). Conversely, other emulsions also exhibited reduction in negative charge; however they resulted slightly higher zeta-potential as compared to 1:0 emulsion. This decrease in negative charge may be due to electrostatic screening by mineral ions present in the artificial saliva (Israelachvili, 2011), or it may have been due to interactions of the mucin molecules (Zhang et al. 2015), or enzymatic hydrolysis of starch emulsifier by α-amylase with the oil droplet surfaces.

3.5.3. Flow behavior

All the emulsions were showed Newtonian flow behavior in the absence of saliva (as explained earlier). In case of emulsions mixed with artificial saliva, 1:0 emulsions exhibited shear thinning behavior (Fig. S2). In contrast, 1:0.5 emulsion did not show prominent flow difference and this may be due to very slow digestion behaviour of this mixed emulsifier system. The 1:0 emulsion revealed higher apparent viscosity at initial shear rate; later on it decreased with increase in shear rate during oral process. The reason for the increase in viscosity at low shear-rates is due to the presence of emulsion droplet aggregates that resides more volume than the non-aggregated oil droplets. Further, while increasing the shear-rates this aggregate leads to break up into smaller ones, which yield decrease in effective droplet volume and therefore emulsion

viscosity (Barnes & Walters, 1989). In addition, α-amylase hydrolyzed the MS emulsifier very quickly and it influenced the aggregation in the emulsion system. This can be seen by the flow behavior of 1:0 emulsion. In this study, very little flow difference was observed for 0.5:0.5; however it was not found much difference when compare to 0.5:0.5, 0.5:1, 1:0.5, 1:1 and 0:1 emulsions.

3.5.4. Turbidity scan

Turbidity scanning index (TSI) and backscattering (%, BS) profile of artificial saliva treated emulsions are shown in Fig. 6, 7 and S3. The destabilization mechanism (i.e. flocculation, aggregation, creaming, sedimentation and phase separation) of emulsion mixed with saliva during oral processing is constantly monitored with respect to droplet size alteration. There was a strong increase (4.3) in TSI curve observed in 1:0 emulsion which confirmed more instability behavior during oral processing (6a). Conversely, the 1:0.5 emulsion showed gradual increase in TSI curve that reveals the slow destabilization mechanism. This may be due to the α-amylase interacting with emulsions and destabilizing the modified starch and thus influencing the flocculation, aggregation, creaming and releasing of oil droplets. In contrast, other emulsions like 0.5:0.5, 0.5:1, 1:1 and 0:1 emulsions did not show any noticeable colloidal destabilization.

Likewise, backscattering (%) profile of 1:0.5 emulsion exhibited gradual increase of instability due to the formation of slow enzymatic hydrolysis (Fig. 7a). This result is in line with the expectations as the low degree of flocculation which is greatly demonstrated by the emulsion and the time scale of the experiment. Whereas, 1:0

458 emulsion BS (%) profile resulted in more aggregation and creaming instability (Fig. 7b).
459 Therefore, oil droplets coalesced as it can be observed by the decrease in BS (%)
460 values, subsequently reduction in BS (%) is strongly influenced by emulsion droplet size
461 (Mengual et al. 1999). This change in oil droplet size can impact decrease in the
462 attractive forces acting among the droplets resulting in less emulsion stability. In
463 addition, this was expected because the 1:0.5 and 1:0 emulsions had change in mean
464 droplet size (d_{32}) and unstable oil droplets (Fig. 3 and Fig. 5a). In contrast, other
465 formulated emulsions did not show any differences observed from BS (%) profile during
466 the measurement (Fig. S3).
467
468 3.6. Oral stability of flavoured emulsions during in-vivo studies
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470 3.6.1. Structural characteristics and droplet size distribution
471 The structural characteristics and mean droplet size (d_{32}) of mixed monolayer
472 emulsion during in-vivo oral processing are illustrated in Fig. 4 and Fig. 5b. In this study,
473 1:0.5 mixed monolayer emulsion showed aggregation at 3s and later formation of
474 coalescence was observed at 10s. Further, the coalescence increased slowly and it
475 became structural deformation at 60s (Fig. 4c). On the other hand, 1:0 emulsion
476 exhibited extreme change in the morphology. At this point, the emulsion droplets were
477 coalesced followed by structural deformation occurred (3s to 60s). In addition, the
478 interfacial layer rupture was clearly observed at 30s (Fig. 4e). Likewise, mean droplet
479 size (d_{32}) of 1:0.5 emulsion was changed from 0.113±0.006 μm to 0.127±0.004 μm (3s
480 to 60s) which resulted in the sustainable increase in emulsion mean size (Fig. 5b). In

comparison with 1:0.5, the 1:0 emulsion revealed extreme change in mean droplet size (d_{32}) i.e. 0.843±0.52 μm to 3.694±0.78 μm (3s to 60s). In contrast, there was no change in morphology and mean droplet size observed in other emulsions. Therefore, the mean droplet size was highly interrelated with the morphology during the oral process (Fig. 4). Overall, the obtained in-vivo oral processing results are more consistent as that observed from in-vitro studies.

3.6.2. Zeta-potential

The zeta-potential values of all the emulsions treated with human salivary phase during in-vivo oral processing are illustrated in Fig. 5d. These attained results are very reliable as that experiential from the in-vitro method. In this study, zeta-potential values further confirmed the gradual reduction for 1:0.5 emulsion and drastic reduction for 1:0 emulsion respectively. The reason behind the reduction in negative charge is destabilization of emulsions due to the various salivary proteins, ions and minerals present in human salivary fluid (as explained earlier). Furthermore, the 1:0.5 emulsion showed slightly strong negative charge reduction i.e. −46.46 mV to −12.43 mV than the result observed from in-vitro oral processing studies (Fig.5d). This may be due to the change in human saliva composition as well as biophysical properties in every individual subject.

4. Conclusions

The influence of different concentration of mixed monolayer (MS with WPI) on emulsions stability and oral stability behaviour were investigated through in-vitro and in-vivo conditions. Better stability was achieved in the emulsions stored at 4 ºC in terms of droplet size, charge and creaming. In contrast, 0.5:0.5 and 0:1 emulsions exhibits unstable while storing at 28±1 ºC. The stability of emulsions found to be more dependent on storage temperature and concentration of mixed emulsifiers. In comparison with all the emulsions, 1:0.5 and 1:0 emulsions morphology showed flocculation, aggregation and coalescence; further structural destabilization was observed during oral behavioural studies. Similarly, change in size and weak negative charge was found in 1:0.5 and 1:0 emulsion. Turbidity scanning index demonstrated more destabilization of emulsion for 1:0 and less for 1:0.5, respectively. Likewise, backscattering (%) profile revealed more increase in creaming rate for 1:0 and gradual increase for 1:0.5 with time during oral processing. Hence, the degradation of modified starch by α-amylase interaction could be the most deciding factor for the oral stability of mixed monolayer emulsion. Further, the obtained results were highly correlated between in-vitro and in-vivo oral behavior. Moreover, the oral behaviour study suggested that 1:0.5 emulsion can be used for controlled flavour release; whereas 1:0 emulsion can be suitable for quick flavour release. Therefore, different concentration of mixed emulsifier stabilized emulsions may behave differently in oral cavity. Thus, the 1:0.5 emulsion system can enhance the controlled flavour release and designing of food and pharmaceutical products with desirable oral experience. Further research is required to design in-vitro and in-vivo experiments with aroma release measurements

525 and sensory perception, in order to understand the mixed monolayer emulsions
526 behaviour more precisely.
527
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534
535 **Appendix A. Supplementary data**
536
537 **References**
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Table 1 Composition of emulsion formulations.

Emulsion Formulation (MS:WPI)	Composition (%, w/w)			
	Sunflower oil + Orange oil	MS	WPI	Aqueous phase
0.5:0.5	10+0.1	0.5	0.5	88.9
0.5:1	10+0.1	0.5	1	88.4
1:0.5	10+0.1	1	0.5	88.4
1:1	10+0.1	1	1	87.9
1:0	10+0.1	1	0	88.9
0:1	10+0.1	0	1	88.9

695 **Table 2** Compositions of artificial saliva.

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Chemicals	Content (g/L)	Grade	Manufacturers
Sodium Chloride	0.111	AR	Qiangshun Chemical, China.
Potassium Chloride	1.492	AR	KeLong Chemical, China.
Sodium Bicarbonate	3.948	AR	KeLong Chemical, China.
Calcium Chloride	0.278	AR	Merck, China.
Magnesium Chloride Hexahydrate	0.096	AR	KeLong Chemical, China
Mucin from porcine stomach type II	1.5	AR	Sigma-Aldrich, U.S.A.
α-amylase (4000 U/g 1G)	2	AR	MAYA Reagent, China.

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Table 3 Measurement of mean droplet size, span value and zeta-potential of emulsions.

Emulsion Formulation (MS:WPI)	Mean droplet size d_{32} (μm)	Span value	Zeta-potential (mV)	Viscosity (mPa.s)
0.5:0.5	0.13±0.007	2.71± 0.03	-42.7±0.43	1.83±0.015
0.5:1	0.10±0.001	3.02± 0.21	-41.76±0.20	1.55±0.005
1:0.5	0.11±0.001	2.76± 0.08	-40.66±0.25	2.19±0.030
1:1	0.10±0.001	2.98± 0.04	-42.56±0.11	1.62±0.030
1:0	0.13±0.001	2.47± 0.02	-39.13±1.11	1.66±0.056
0:1	0.10±0.001	2.88± 0.04	-40.56±0.92	1.22±0.041

Figure captions

Fig. 1. Droplet size distribution of different concentration of MS and WPI stabilized emulsions on the day of preparation.

Fig. 2. (A) Mean droplet size, (B) Zeta-potential measurement and (C) Creaming storage stability studies of emulsions: i) stored at refrigeration (4±0.1 ºC); ii) stored at room (28±1 ºC): Error bar represents standard deviation of the measurements (n=3). Different alphabets letters are significantly different from control ($P < 0.05$) according to Tukey's multiple comparisons test. The lowest value in the same line indicated with a, and the increase in value was indicated with b and c.

Fig. 3. Morphology of different concentration of MS and WPI emulsions during in-vitro oral processing: (a) 0.5:0.5, (b) 0.5:1, (c) 1:0.5, (d) 1:1, (e) 1:0 and (f) 0:1. Structural changes are mentioned in the micrograph.

Fig. 4. Morphology of different concentration of MS and WPI emulsions during in-vivo oral processing: (a) 0.5:0.5, (b) 0.5:1, (c) 1:0.5, (d) 1:1, (e) 1:0 and (f) 0:1. Structural changes are mentioned in the micrograph.

Fig. 5. Different concentration of MS and WPI stabilized emulsions during oral processing: (a) Mean droplet size by in-vitro (n=10), (b) Mean droplet size by in-vivo (n=10), (c) Zeta-potential by in-vitro (n=9) and (d) Zeta-potential by in-vivo (n=9). Error bar represents standard deviation of the measurements.

Fig. 6. Turbidity scanning index (TSI) of the destabilization of emulsions reacted with artificial saliva.

Fig. 7. Turbiscan backscattering (BS) profile of artificial saliva treated with emulsions: (a) 1:0.5 (MS:WPI) stabilized emulsion and (b) 1:0 (MS:WPI) stabilized emulsion.