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1 Peptide release from SEDDS containing
2 hydrophobic ion pair therapeutic peptides measured
3 by Taylor dispersion analysis

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15 KEYWORDS. Taylor dispersion analysis, therapeutic peptides, SEDDS, hydrophobic ion
16 pairing, drug release, diffusion coefficient, hydrodynamic radius, pharmaceutical excipients.

17 **ABSTRACT.**

18 Therapeutic peptides are facing an increasing interest as drugs for the treatment of many
19 diseases. The challenge in the administration of such drugs, due to inherent properties of these
20 peptides, is to make them bioavailable. Self-emulsifying drug delivery systems (SEDSS) are
21 considered a suitable and promising strategy to deliver the peptides and increase their
22 bioavailability. However, to enter into the SEDSS nanodroplets, the peptides must be made
23 hydrophobic by complexation with surfactants (formation of hydrophobic ion pair, HIP). The
24 aim of this work is to assess the possibility to quantify the amount of released peptides and of the
25 remaining docusate / peptide HIP in the nanodroplets by Taylor Dispersion Analysis (TDA) on
26 two therapeutic peptides (leuprorelin and desmopressin). It also clearly demonstrates that the
27 $\log P$ value of the peptide has a strong influence on the extent of HIP inside of the SEDSS
28 nanodroplets. For instance leuprorelin-docusate complex ($\log P = 3$) was 100 % inside of the
29 nanodroplets at low ionic strength, while for desmopressin-docusate complex ($\log P = 0.5$) only
30 30 % were able to enter the nanodroplets. It was also shown that an increase in the ionic strength
31 of the release media allowed to increase the amount of released peptide up to 80 % for
32 leuprorelin and 100 % for desmopressin, at physiological ionic strength. **TDA experiments**
33 **allowed to determine the partitioning coefficient, $\log D$ value, of the peptide between the SEDSS**
34 **and continuous aqueous phases.** In conclusion, this work demonstrates that TDA is a rapid,
35 straightforward and useful technique for developing SEDSS formulations.

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39 **1. Introduction**

40 Therapeutic peptides are a class of drugs meeting an increasing interest in the pharmaceutical
41 industry, mainly because of its non-immunogenicity and its low cost production as compared to
42 other biological drugs such as proteins or antibodies. However, naturally occurring peptides
43 suffer from certain inherent limitations. For instance, they have short plasma half-life due to the
44 presence of peptidases and of excretory mechanisms leading to their cleavage and elimination
45 (Lau and Dunn, 2018). Using peptide analogs and synthetic peptides are strategies that help to
46 increase the plasma half-life (Di, 2015). Another limitation is their low oral bioavailability due to
47 the presence of digestive enzymes in the gastrointestinal tract and to their high polarity limiting
48 their permeability through the intestinal membrane (Lau and Dunn, 2018). For these reasons
49 most therapeutic peptides are injectable drugs and only few oral peptide drugs exist on the
50 market (Fosgerau and Hoffmann, 2015). A strategy to increase the bioavailability of these highly
51 polar peptides without modifying their chemical structure or synthesizing analogs, is the use of
52 lipidic self-emulsifying drug delivery systems (SEDDS) (Leonaviciute and Bernkop-Schnürch,
53 2015). SEDDS can protect the peptide drugs from the digestive enzymes and can efficiently
54 permeate the intestinal barrier to transport the drug to its target. Nevertheless, because of their
55 hydrophilicity, peptides cannot be directly loaded in SEDDS microdroplets and the formation of
56 hydrophobic ion pair, between the peptide and a surfactant, is required (Meyer and Manning,
57 1998). This strategy allows the increase in the lipophilic character of the peptide drugs ($\log P$ or
58 $\log D$) by several orders of magnitude (Griesser et al., 2017; Hintzen et al., 2014; Morgen et al.,
59 2017; Zupančič et al., 2016).

60 The study of the release of the peptide drugs from SEDDS is normally studied by membrane
61 diffusion methods (Bernkop-Schnürch and Jalil, 2018), where the oily droplets are separated
62 from the release medium through filters (Griesser et al., 2018) or dialysis membranes (Hetényi et

63 al., 2017); or by sample-and-separate methods where the oily phase is separated from the
64 continuous medium by centrifugation. Aforementioned methods can lead to non-reliable results
65 (Bernkop-Schnürch and Jalil, 2018), either because the membrane diffusion data are difficult to
66 interpret or because the application of high external energy (centrifugation) can alter the drug
67 release behavior by destabilizing the SEDDS. On the contrary, *in situ* methods were found to be
68 efficient methods to study the release from SEDDS. They are based on the use of specific
69 detectors for the drugs *e.g.* specific drug electrodes (Tan et al., 2007) or on the measurement of a
70 change in a physico-chemical parameter (such as *e.g.* pH) in the release medium due to the
71 release of the free drug (Mora et al., 2009). Even though *in situ* methods give reliable results,
72 they are not general methods as they strongly depend on a certain specific property of the
73 released drug.

74 In this work, Taylor dispersion analysis (TDA) was investigated as a general method to study
75 the release of peptide drugs from SEDDS. Two therapeutic peptides were studied: (i) leuporelin
76 (leuprolide acetate), a synthetic peptide analogue of gonadotropin releasing hormone (GnRH)
77 mainly used in the treatment of sex hormone related disorders such as prostate cancer,
78 precocious puberty and endometriosis (Plosker and Brogden, 1994); and (ii) desmopressin, a
79 synthetic peptide analogue of the human hormone arginine vasopressin used in the treatment of
80 diabetes insipidus, primary nocturnal enuresis, hemophilia A, Type I von Willebrand's disease
81 and also to improve human memory function (Wang et al., 2002). The hydrophobic ion pair
82 (HIP) of these two peptides with sodium docusate was described by Griesser *et al.* (Griesser et
83 al., 2017) showing a significant increase in the lipophilicity of the peptides allowing their loading
84 into SEDDS. Elsewhere, TDA was shown to be a useful method to monitor the size of SEDDS
85 microdroplets and their evolution under digestive conditions (Chamieh et al., 2015; Chamieh et

86 al., 2016; Chamieh et al., 2018). TDA is based on the broadening of a marked SEDDS plug in a
87 capillary tube under laminar Poiseuille flow, allowing the determination of the molecular
88 diffusion coefficient (and the hydrodynamic radius) of the SEDDS microdroplets.

89 The aim of this study is to quantify by using TDA the free fraction of therapeutic peptides in
90 HIP-SEDDS formulations for leuprorelin and desmopressin, and to study the influence of the
91 ionic strength of the dilution media on the release of the peptides.

92 **2. Materials and methods**

93 **2.1 Materials**

94 Capryol™ 90 (Batch 166619) and Transcutol® HP (Batch 165296) were provided by Gattefossé
95 SAS (Saint-Priest, France). Kolliphor® RH 40 (Lot BCBV1401), docusate sodium salt (Lot
96 SLBV3090), 1-Methylnaphthalene (Lot STBD3222V), N,N-Dimethylformamid 99% and
97 phosphate buffered saline tablets (Lot SLBW3999) were purchased from Sigma-Aldrich (St
98 Quentin Fallavier, France). Desmopressin acetate (Batch 062FKE) and leuprorelin acetate (Batch
99 620PBH) were from Glentham Life Science (United Kingdom). Sodium chloride (Batch
100 13F130010) and hydrochloric acid 37% (Batch 13J180522) were from VWR Chemicals,
101 Belgium. All chemicals were used as received, without any further purification. All solutions
102 were prepared by dissolving the appropriate amount in ultra-pure water (18 MΩ.cm) purified on
103 a Milli-Q system from Millipore (Molsheim, France).

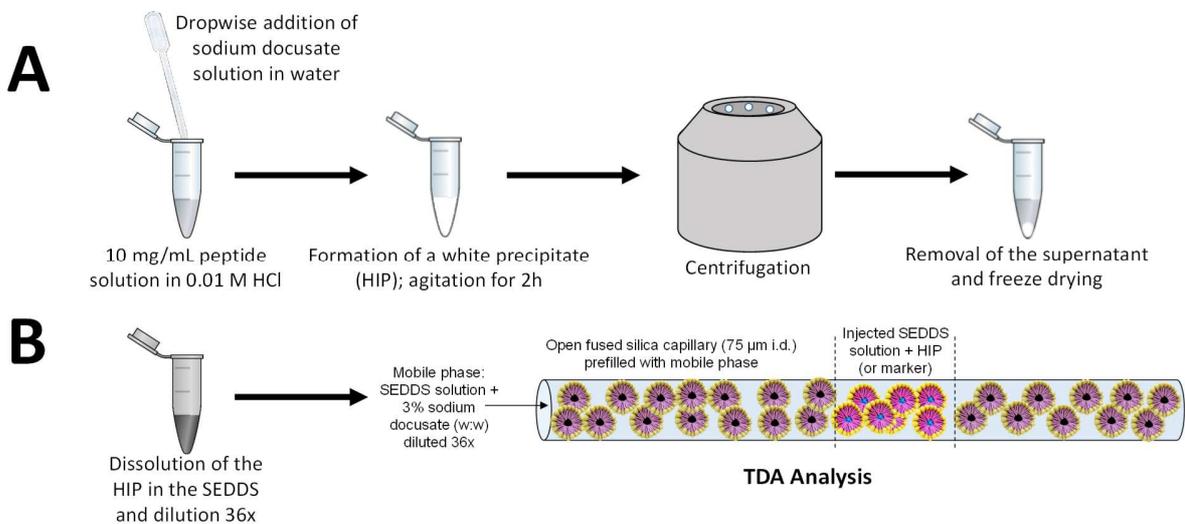
104 **2.2 Hydrophobic ion pairing**

105 The preparation of the HIP was realized following the procedure described by Griesser *et al.*
106 (Griesser et al., 2018; Griesser et al., 2017) and schematically depicted in Figure 1A. Briefly,
107 several aliquots of 5 mg of peptide were dissolved in 0.5 mL of a 0.01M HCl solution. In acidic
108 media, both peptides are positively charged (two positive charges for leuprorelin and one for

109 desmopressin). Then, a sodium docusate solution (anionic surfactant) was added dropwise under
110 vigorous stirring at room temperature. The complex was formed and appeared as a white
111 precipitate. The concentration of the sodium docusate solution depended on the peptide: 2:1
112 (resp. 1.5:1) docusate-peptide molar ratio in the case of leuprorelin (resp. desmopressin). The
113 aliquots were left shaking for 2 h. Then, they were centrifuged at 10620 g (10000 rpm) for 10
114 min at 25°C using a Sigma 302K centrifuge. The supernatant was discarded and the precipitate
115 was washed with 1 mL of 0.01M HCl solution and centrifuged again at 10620 g (10000 rpm) for
116 10 min at 25°C. Finally, after removal of the supernatant, the precipitate was freeze dried using a
117 CRIOS-80 freeze drier from Cryotec (France). The aliquots were kept at -20°C for further use.

118 **2.3 Preparation of SEDDS, TDA mobile phase and samples for TDA analysis**

119 The placebo SEDDS was prepared by mixing 40 % (v:v) Capryol 90, 25% (v:v) Transcutol HP
120 and 35% (v:v) Kolliphor RH 40 (Griesser et al., 2018). In this placebo SEDDS, HIP were
121 dissolved at a concentration of 10% in weight (representing 7% of peptide and 3% of docusate)
122 before 36-fold dilution in the studied medium (see Figure 1B): water, saline solutions having
123 different NaCl concentrations (5, 10, 50, 100 and 150 mM) or 10 mM PBS buffer pH 6.8 (ionic
124 strength 158 mM). For the TDA mobile phase, 3% in weight of sodium docusate were added to
125 the placebo SEDDS before 36-fold dilution in the studied medium (see Figure 1B). The size of
126 the microdroplets was obtained by injecting a sample containing a 0.1% (w:w) of 1-
127 methylnaphthalene (marker) in the TDA mobile phase. **Compositions of the different solutions**
128 **are summarized in the supporting information, Table S1.**



129

130

131 **Figure 1:** Schematic representation of the experimental protocol for the analysis of HIP in
 132 SEDDS by TDA. A. Formation of the hydrophobic ion pair between the peptide and sodium
 133 docusate in acidic media, the precipitate is isolated by centrifugation and freeze dried. B.
 134 Dissolution of the HIP in SEDDS, dilution by a factor of 36 followed by TDA analysis. More
 135 details in sections 2.3 and 2.4.

136

137 2.4 Taylor dispersion analysis: experimental conditions

138 TDA was performed on an Agilent 7100 CE instrument (Waldbronn, Germany) using μ -SIL-
 139 DNA neutral coated fused silica capillary (Agilent technologies, Waldbronn, Germany), having a
 140 length of 45 cm (36.5 cm to detector) \times 75 μ m I.D.. The carrousel was thermostated at 37°C
 141 using an external circulating water bath 600F from Julabo (Germany). Between each analysis,
 142 capillaries were rinsed with the corresponding mobile phase (10 min). Samples were injected
 143 hydrodynamically at the inlet end of the capillary (50 mbar, 2 s, 16-22 nL). The temperature of
 144 the capillary cartridge was set at 37°C. The solutes were monitored at 193 nm and 226 nm. The
 145 taylorgrams were recorded with the Chemstation software, and exported to Microsoft Excel for
 146 subsequent data processing.

147 2.5 Taylor dispersion analysis: data treatment

148 The temporal variance σ^2 of the elution profile from the TDA experiment is calculated by fitting
149 the elution peak by a Gaussian function, using equation (1):

$$150 \quad S(t) = \frac{S_0}{\sigma\sqrt{2\pi}} \exp\left[-\frac{(t-t_0)^2}{2\sigma^2}\right] \quad (1)$$

151 where $S(t)$ is the absorbance signal, t_0 is the average elution time, σ^2 is the temporal variance of
152 the elution profile and S_0 is a constant that depends on the response factor and the injected
153 quantity of solute. t_0 , σ^2 and S_0 are the three adjusting parameters obtained by nonlinear least
154 square regression using Microcal Origin. The sample hydrodynamic radius R_h , and diffusion
155 coefficient D of the solute, are related to σ^2 via equation (2) and equation (3) respectively:

$$156 \quad D = \frac{R_c^2 t_0}{24\sigma^2} \quad (2)$$

$$157 \quad R_h = \frac{k_B T}{6\pi\eta D} \quad (3)$$

158 where R_c is the capillary radius (m), k_B is the Boltzmann constant, T the temperature (K) and η
159 the viscosity of the carrier liquid (Pa.s). The viscosities of the 36-fold diluted SEDDS solutions
160 were measured comparing the mean elution time of the media compared with the value for pure
161 water at the same temperature (Bello et al., 1994). In order to find out the mean elution time, the
162 media solution was doped with 0.1% (v:v) DMF used as a marker. The capillary was filled with
163 the non-doped solution and then the marked solution was continuously injected at 50 mbar. The
164 DMF was detected at 200 nm. The viscosity, being proportional to the elution time, is given by
165 equation (4) with about 3% precision (Bello et al., 1994) by:

$$166 \quad \eta = \frac{t_{DMF}}{t_0} \eta_0 \quad (4)$$

167 where η_0 is the viscosity of water at the same temperature, t_{DMF} is the mean elution time of DMF-
168 marked solution and t_0 the elution time for water. The measured viscosities are presented in
169 supporting information (Table S2).

170 In this work, TDA conditions were verified by calculating the τ and the Pe number for each run
171 using equations (5) and (6) (Chamieh and Cottet, 2014; Cottet et al., 2014; Taylor, 1954):

$$172 \quad \tau = \frac{Dt_0}{R_c^2} \geq 1.25 \quad (5)$$

$$173 \quad Pe = \frac{uR_c}{D} \geq 40 \quad (6)$$

174 where τ is an adimensional characteristic time, Pe is the Péclet number and u is the linear mobile
175 phase velocity (m/s). In this work, τ varied between 2.6 and 45, while Pe varied between 220 and
176 3785.

177 3. Results and discussion

178 3.1 Optimization of the TDA conditions

179 In previous studies, we have shown that TDA can be used to determine the size of SEDDS by
180 injecting a plug of microdroplets solution doped with a hydrophobic marker and by mobilizing
181 with non-marked microdroplets solution (Chamieh et al., 2015; Chamieh et al., 2016; Chamieh et
182 al., 2018). By this way, the equilibrium of the microdroplets was conserved and the
183 hydrodynamic radius of the marker followed the size of the droplet as far as the marker remained
184 in the droplets. In this work, the size of the SEDDS has been first determined in the absence of
185 peptide. For that, a hydrophobic chromophore (1-methylnaphthalene) was used as a marker. This
186 marker was found to have a very low solubility in water allowing thus the determination of the
187 microdroplets size according to equation (7):

188 $R_h = f R_{h,m} + (1-f) R_{h,w}$ (7)

189 where R_h is the apparent average hydrodynamic radius which is expressed as the sum of the
190 hydrodynamic radius of the free marker, $R_{h,w}$, and of the marked microdroplets, $R_{h,m}$, weighted
191 by their molar fraction. f is the molar fraction of marker inside the micelles ($f \sim 1$, for 1-
192 methylnaphthalene marker (Burkey et al., 1984; Chamieh et al., 2015)). The hydrodynamic
193 radius corresponding to the SEDDS microdroplets, using 1-methylnaphthalene as a marker, was
194 derived from the diffusion coefficient D using Stokes-Einstein equation: $R_h = 11.10 \pm 0.27$ nm
195 ($n=3$) (Figure S1).

196 To solubilize the peptides in the SEDDS, HIPs between the positively charged peptides and the
197 sodium docusate were prepared and dissolved in SEDDS (see experimental part). After 36-fold
198 dilution with water, the HIP in SEDDS formulation was injected in the capillary for TDA
199 experiment and mobilized with the 36-fold diluted in water SEDDS solution (without HIP).
200 Unfortunately, non-Gaussian peaks were obtained and were difficult to interpret (see Figure S2).
201 Deformed elution profiles with typical negative contribution close to the peak apex were
202 observed due to a composition mismatch between the sample and the mobile phase (Latunde-
203 Dada et al., 2015) . The addition in the mobile phase of 3 % w:w of sodium docusate to the
204 SEDDS before the 36-fold dilution with water leads to symmetrical Gaussian peaks, in these
205 conditions the final docusate concentration is of 1.98 mM. The sodium docusate addition to the
206 mobile phase did not significantly affect the size of the microdroplets, as verified by using 1-
207 methylnaphthalene as marker ($R_h = 11.58 \pm 0.29$ nm; $n=5$).

208 Before studying the HIP formulation in SEDDS, the stability of SEDDS was evaluated, at 37°C,
209 every 5 min for two hours, by injecting SEDDS marked with 1-methylnaphthalene for
210 subsequent TDA analysis. The results demonstrated that the SEDDS were stable at least during

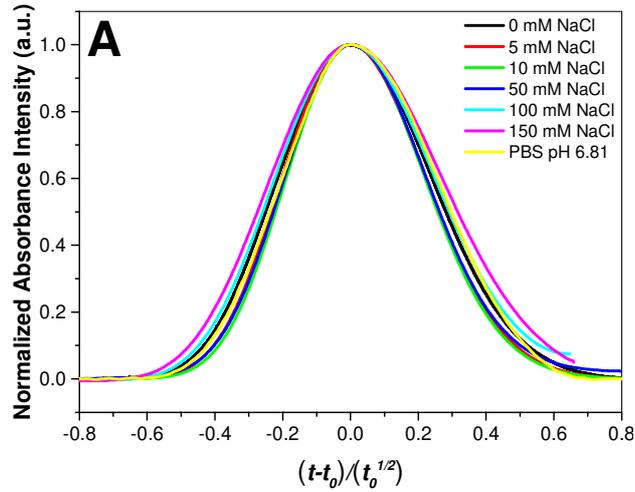
211 two hours at 37°C as the Taylorgrams were perfectly overlaid (see Figure S3 in supporting
212 information).

213 In a second time, it was verified that the peptides in their free form (*i.e.* without docusate) are
214 unable to enter in the microdroplets. For that, the peptides were dissolved in PBS buffer (or in
215 36-fold diluted SEDDS with PBS buffer) before TDA. The results showed non-significantly
216 different hydrodynamic radii in buffer and in SEDDS for both peptides (see supporting
217 information, Figures S4 and S5) demonstrating that the peptides remained in the continuous
218 phase and did not enter in the droplets. The R_h of free Leuprorelin in SEDDS was 1.05 ± 0.03 nm
219 ($n=5$), as for free desmopressin a R_h of 0.78 ± 0.02 nm ($n=5$) was obtained.

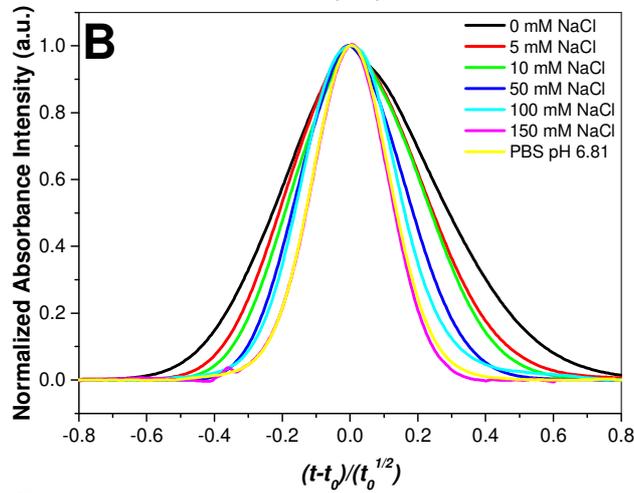
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221 **3.2 Application of Taylor dispersion analysis to the determination of free peptides**
222 **in SEDDS solutions**

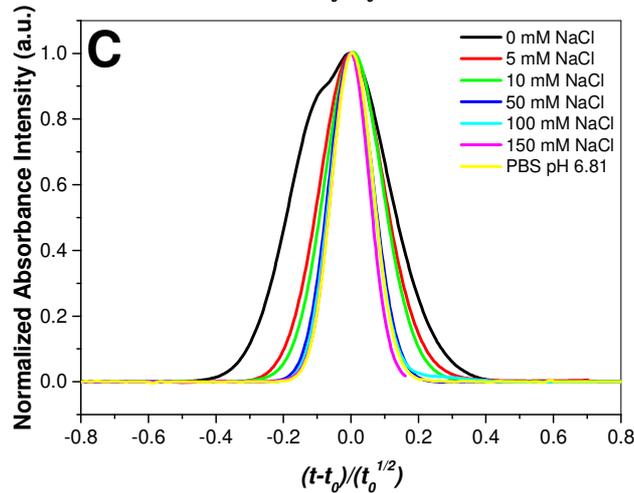
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224



225



226 **Figure 2:** Overlay of Taylorgrams obtained at different ionic strength. TDA experimental
227 conditions: μ -SIL-DNA neutral coated fused silica capillary, 45 cm (36.5 cm to detector) \times 75
228 μ m i.d.. Mobile phase: SEDDS + 3% (w:w) sodium docusate, diluted 36 times in water or in
229 NaCl solutions of different concentrations (5, 10, 50, 100 and 150 mM) or in PBS buffer pH 6.8.
230 Mobilization pressure: 50 mbar. Hydrodynamic injection: 50 mbar for 2 s. Injected samples: A:

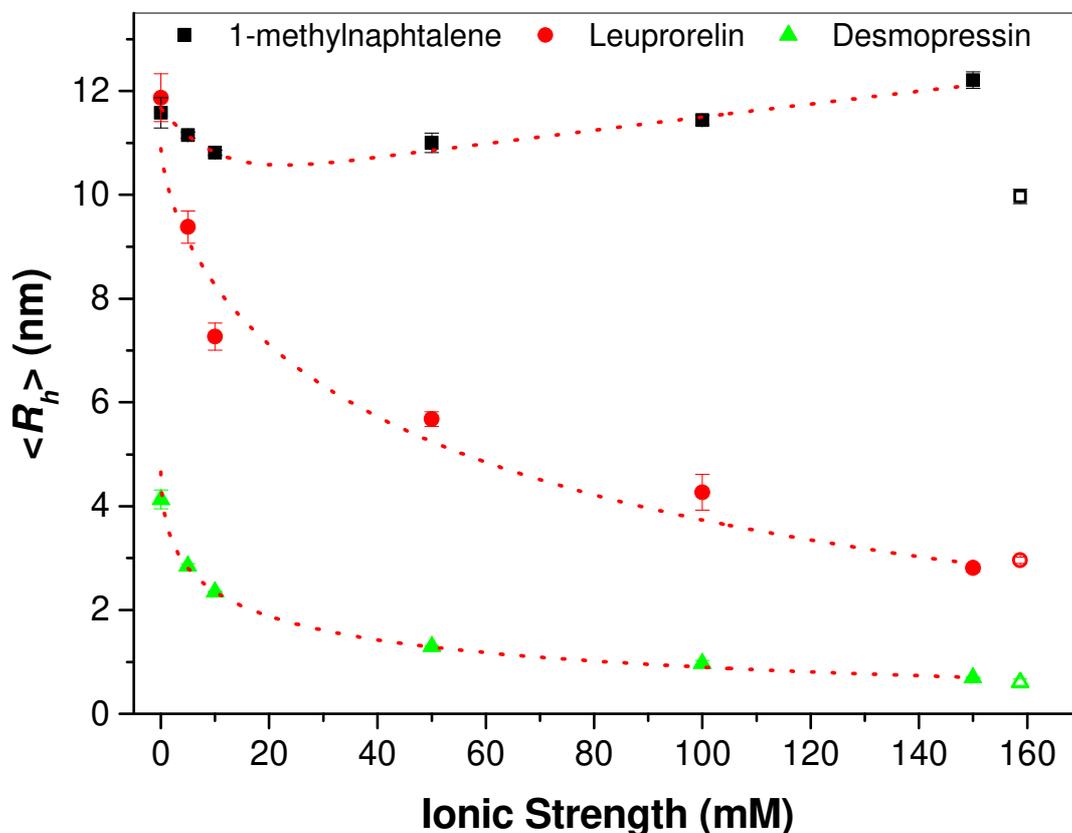
231 0.1 % 1-methylnaphthalene in mobile phase. Temperature: 37°C; UV detection at 226 nm; B:
232 Leuprorelin-docusate HIP in mobile phase (7% w:w of peptide + 3% w:w of sodium docusate in
233 SEDDS diluted by a factor of 36 as for the mobile phase); C: Desmopressin-docusate HIP in
234 mobile phase (7% w:w of peptide + 3% w:w of sodium docusate in SEDDS diluted by a factor of
235 36 as for the mobile phase). Temperature: 37°C; UV detection at 193 nm. The *x*-axis was
236 changed from *t* to $x = (t - t_0) / \sqrt{t_0}$ for correction from viscosity changes, while the *y* axis was
237 normalized for better visual comparison. For detailed compositions of the TDA samples and
238 mobile phase see experimental section or Table S1.

239
240 Figure 2A shows the overlay of taylorgrams obtained for 1-methylnaphthalene + 3% sodium
241 docusate in SEDDS diluted 36x in all the studied media (as indicated on the Figure) at 226 nm, a
242 specific wavelength for 1-methylnaphthalene. The *x*-axis was changed from time to
243 $x = (t - t_0) / \sqrt{t_0}$ to correct from the viscosity changes, as described elsewhere (Chamieh et al.,
244 2016), while the *y*-axis was normalized for better visual comparison. As can be seen from the
245 Figure 2A, a slightly significant difference was observed for the microdroplets size as a function
246 of ionic strength. The R_h values varied between 10 and 11.6 nm (Figure 3). This result was
247 expected since the SEDDS are composed of nonionic surfactants which are not sensitive to the
248 ionic strength. In all cases, a Gaussian shaped profile was obtained suggesting a fast exchange
249 rate of the surfactant unimers from one droplet to another compared to the time-scale of the
250 experiments (a few minutes). In these conditions, the Gaussian taylorgram is representative of a
251 weight average hydrodynamic radius on all the different microdroplets at equilibrium in the
252 sample.

253 To study the release of the therapeutic peptides in the SEDDS-HIP formulation, the peptide
254 formulation was injected and mobilized by the SEDDS containing 3% docusate and diluted 36
255 times with the appropriate aqueous solution. Figure 2B shows the overlay of the normalized
256 taylorgrams obtained in the case of leuprorelin HIP in SEDDS diluted with saline solutions

257 having different ionic strengths, the detection wavelength was 193 nm which is specific for the
258 peptides in this work. When the HIP in SEDDS was dispersed in water, the obtained R_h ($11.87 \pm$
259 0.46 nm; $n=5$) was the one corresponding to the microdroplets size obtained with 1-
260 methylnaphthalene, suggesting that the peptide remained inside the microdroplets. This can be
261 explained by the fact that the $\log P$, defined as the ratio of peptide-docusate complex between
262 octanol and demineralized water, of Leuprorelin-docusate HIP is 3 (Griesser et al., 2017), which
263 is close to the value of 1-methylnaphthalene ($\log P = 3.87$, (Haynes, 2015)). However, when the
264 salt concentration increased, a decrease in the hydrodynamic radius was observed (Figure 2B and
265 Figure 3) suggesting the release of the drug from the droplet. It is noteworthy that the peak shape
266 was always Gaussian, showing a fast exchange kinetics of the peptide between the droplet (under
267 its HIP form) and the continuous phase (under its free form). The R_h decreased exponentially
268 from 11.87 ± 0.46 nm in water to 2.81 ± 0.07 nm, at 150 mM ionic strength. The latter value
269 suggests the release of a large amount of peptide, but not the totality.

270 In the case of Desmopressin-docusate HIP a relatively narrow peak was observed, even at low
271 ionic strength (in water), as displayed in Figure 2C. The R_h decreased from 4.13 ± 0.18 nm in
272 water to 0.70 ± 0.01 nm at 150 mM ionic strength, showing a total release of the peptide from the
273 droplets at physiological ionic strength. In fact, the complex between desmopressin and docusate
274 is much less hydrophobic ($\log P = 0.5$; (Griesser et al., 2017)) than the complex between
275 leuprorelin and docusate. These results are in good agreement with what was stated by Bernkop-
276 Schnürch and Jalil (Bernkop-Schnürch and Jalil, 2018) concerning the impact of $\log P$ on drug
277 release from SEDDS.



278
 279 **Figure 3:** Average hydrodynamic radii obtained for 1-methylnaphtalene marked SEDDS,
 280 Leuporelin-docusate HIP in SEDDS and Desmopressin-docusate HIP in SEDDS as a function
 281 of the ionic strength. Closed symbols are for samples diluted in water or NaCl solutions. Open
 282 symbols are for samples diluted in PBS buffer (phosphate buffer 10 mM + 154 mM salt, pH 6.8,
 283 ionic strength 159 mM). Dotted lines are guide for the eyes. Error bars are standard deviations
 284 obtained for 5 repetitions. When not visible the error bar is smaller than the size of the symbol.
 285 For detailed compositions of the TDA samples and mobile phase see experimental section or
 286 Table S1.

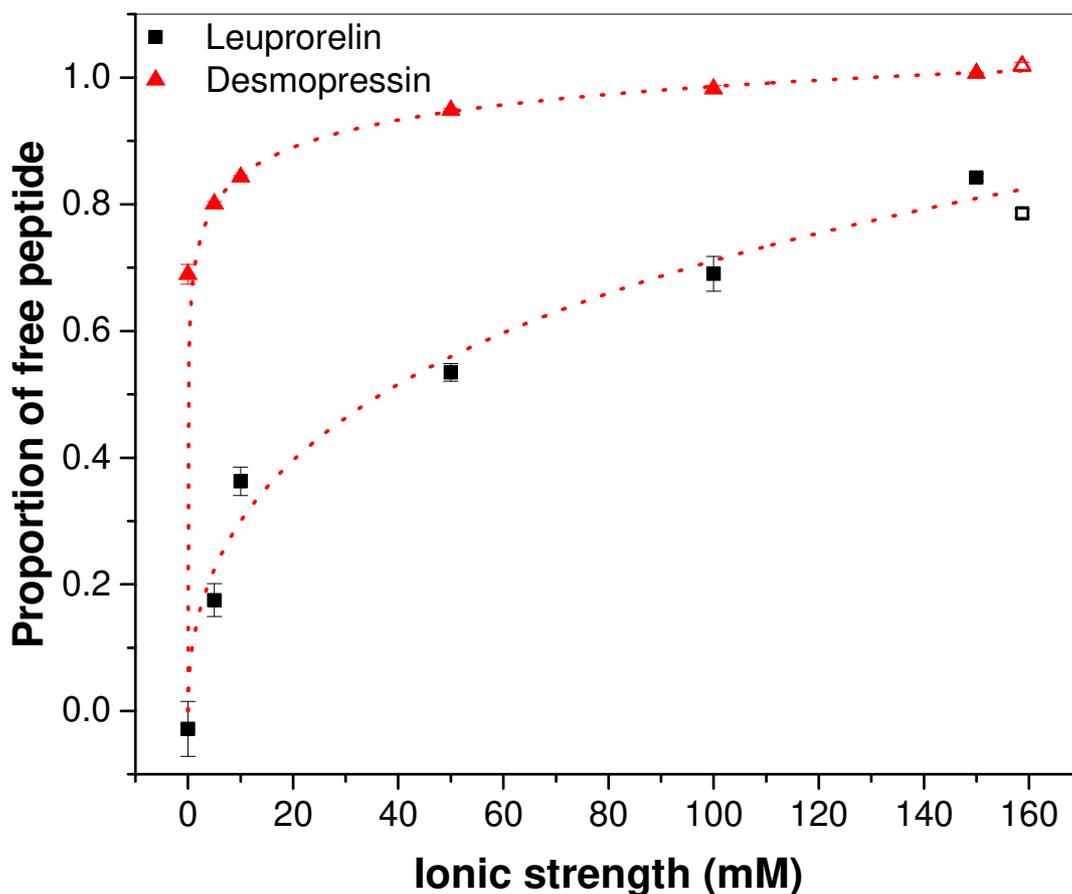
287 Furthermore, in order to check that the decrease in size was due to the release of the drug and not
 288 a change in the shape of the microdroplets, 1-methylnaphthalene was added to the HIP solution
 289 in SEDDS. Since 1-methylnaphthalene is always located in the microdroplets, if there is a
 290 change in size it can be quantified. The results showed that at the specific wavelength of 1-
 291 methylnaphthalene (226 nm), the peak was broader than at the specific wavelength of the HIP
 292 (193 nm) (see supporting information Figure S6 and Table S3), suggesting the presence of the

293 microdroplets and confirming that the peptide was released with fast kinetics compared to the
294 analysis time (a few min).

295 From these observations, knowing the size of the peptide alone and of the microdroplet at the
296 different ionic strengths, the proportion of free peptide can be calculated using the following
297 equation:

$$298 \quad R_{h,average} = xR_{h,peptide} + (1-x)R_{h,microdroplet} \quad (8)$$

299 where x is the molar fraction of free peptide, $R_{h,peptide}$ the hydrodynamic radius of free peptide
300 obtained by TDA by injecting the peptide in its free form, $R_{h,microdroplet}$ the hydrodynamic radius
301 of the microdroplets determined using 1-methylnaphthalene as a marker, and $R_{h,average}$ the
302 hydrodynamic radius obtained for the peptide-docusate HIP formulation at given ionic strength.
303 $R_{h,average}$ is the weight average hydrodynamic radius obtained by TDA with a mass concentration
304 sensitive detector (UV detector in this work). Solving equation (8) for the quantification of free
305 peptide proportion in SEDDS solutions diluted 36 times in pure water showed that 100 % of
306 leuprorelin were inside of the microdroplets, while only 30 % of the peptide remained in the
307 microdroplet in the case of desmopressin.



308
 309 **Figure 4:** Proportion of free peptide (x) as a function of the ionic strength for Leuprorelin-
 310 docusate HIP in SEDDS and Desmopressin-docusate HIP in SEDDS. x was determined by
 311 solving equation (8) at each ionic strength. Closed symbols are for samples diluted in water or
 312 NaCl solutions. Open symbols are for samples diluted in PBS buffer (phosphate buffer 10 mM +
 313 154 mM salt, pH 6.8, ionic strength 159 mM). Dotted lines are guide for the eyes. Error bars are
 314 standard deviations obtained for 5 repetitions, when not visible the error bar is smaller than the
 315 size of the symbol. **For detailed compositions of the TDA samples and mobile phase see**
 316 **experimental section or Table S1.**

317

318 **3.3 Ionic strength effect on the release of peptides from SEDDS**

319 Figure 4 represents the evolution of the proportion of free peptide (x) as a function of ionic
 320 strength in the case of leuprorelin and desmopressin, as calculated by equation (8) using TDA
 321 results. As can be seen from this figure, in the case of leuprorelin in water the totality of the

322 peptide was inside the SEDDS nanodroplets, while the amount of free peptide increased up to 80
 323 % at the highest studied ionic strength (150 mM and PBS buffer pH 6.81, ionic strength 158
 324 mM). In the case of desmopressin, 70% of peptide were already released in water, and more than
 325 95 % was released for ionic strengths higher than 50 mM. It is noteworthy that 100% of peptide
 326 were released above 150 mM. This study clearly demonstrates that TDA can be used to
 327 determine the released amount of peptide in complex HIP/SEDDS formulations. In fact, the
 328 formation of the complex between the peptides and sodium docusate is based on electrostatic
 329 attraction which is sensitive to the ionic strength. The higher the ionic strength in the release
 330 medium is, the more HIPs will fall apart. Raising the salt concentration results in a partial
 331 dissociation of HIPs and, consequently, an increase in peptide solubility in the release medium.
 332 However the more hydrophobic (higher log*P*) the complex is, the more it is protected by the
 333 microdroplet, and thus less subject to dissolution i.e. release in the continuous medium.

334 If we consider the following equilibrium:



$$336 \quad D_{SEDDS/w} = \frac{[P_{SEDDS}]}{[P_w]}$$

337 (10)where P_w is the peptide in the release medium composed of water, different salt
 338 concentrations ranging from 0 to 150 mM, and 1.98 mM of docusate (see section 3.1), P_{SEDDS} is
 339 the peptide in its peptide-docusate HIP form located inside of the SEDDS droplets under the
 340 dilution and composition conditions of this work, and $D_{SEDDS/w}$ is the partitioning coefficient
 341 defined as the ratio of the peptide concentrations.

342 If we consider the fact that in TDA the measured average hydrodynamic radius is a weight
 343 average hydrodynamic radius, equation (8) can be used and can be rewritten as:

$$344 \quad R_{h,TDA} = \frac{[P_w]}{[P_w] + [P_{SEDSS}]} R_{h,peptide} + \left(1 - \frac{[P_w]}{[P_w] + [P_{SEDSS}]} \right) R_{h,microdroplet} \quad (11)$$

345 It is important to note that $R_{h,peptide}$ and $R_{h,microdroplet}$ are measured independently by TDA.
 346 Combining equations (10) and (11), one can express the partitioning coefficient $D_{SEDSS/w}$ as a
 347 function of the experimentally measured hydrodynamic radii:

$$348 \quad D_{SEDSS/w} = \frac{R_{h,peptide} - R_{h,TDA}}{R_{h,TDA} - R_{h,microdroplet}}$$

349 (12)

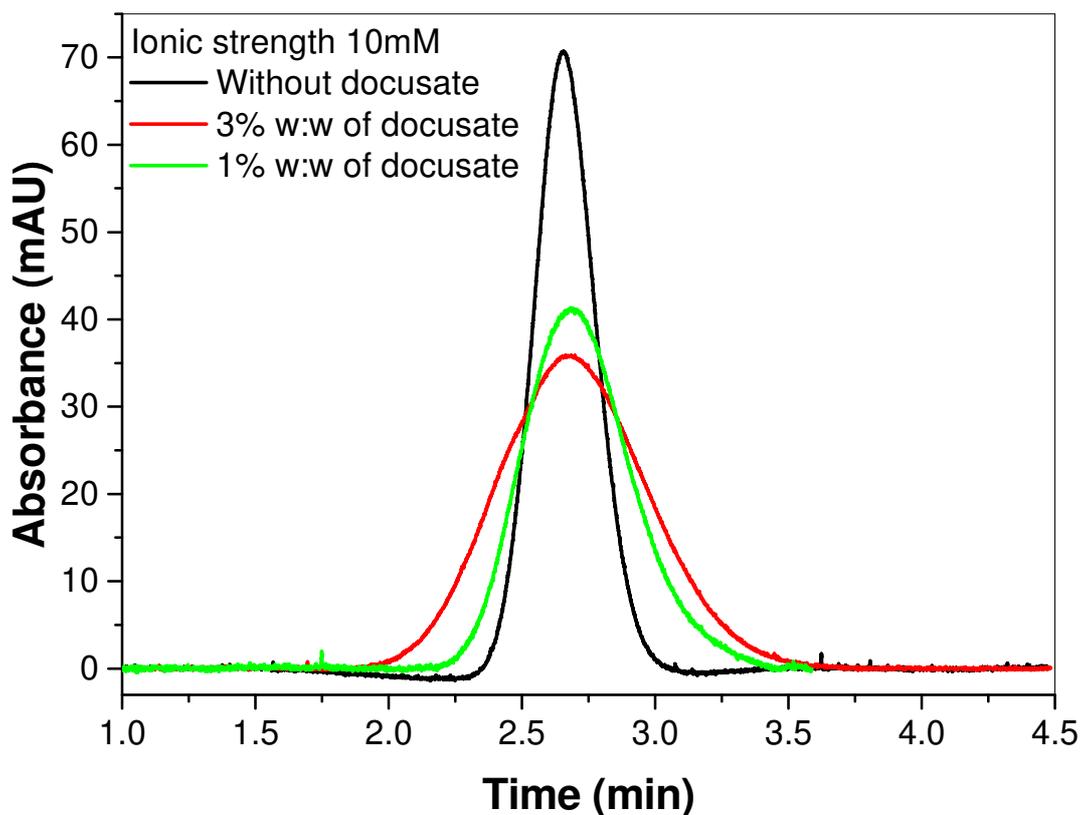
350 By using equation (12) the partitioning coefficient of the peptide-docusate complex between the
 351 SEDDS and the aqueous phase containing 1.98 mM of docusate can be calculated. Experimental
 352 results are gathered in Table 1. As can be seen from this table, $D_{SEDSS/w}$ for leuprorelin in pure
 353 water could not be calculated as $R_{h,TDA}$ was equal to $R_{h,microdroplet}$ because of the presence of the
 354 totality of the peptide in the SEDDS droplets. When the ionic strength increased the $\log D_{SEDSS/w}$
 355 decreased from 0.67 (at 5 mM) and -0.68 (at 150 mM) for Leuprorelin and from -0.63 (at 5 mM)
 356 and -2.32 (at 150 mM) for Desmopressin showing that the presence of salt dissociated the
 357 complex and partially (or totally) inhibited its entry into the SEDDS droplets.

358 Table 1: Calculated partitioning coefficients $D_{SEDSS/w}$ using equation (12) and $\log D_{SEDSS/w}$
 359 between the SEDDS droplets under this work experimental conditions (36 fold dilution) and the
 360 aqueous phase containing 1.98 mM of docusate, for leuprorelin-docusate and desmopressin-
 361 docusate ion pairs (10% w:w in the SEDDS) at different ionic strength ranging from 0 to 150
 362 mM by the addition of NaCl.

$D_{SEDSS/w}$	$\log D_{SEDSS/w}$
---------------	--------------------

Ionic strength (mM)	Leuprorelin	Desmopressin	Leuprorelin	Desmopressin
0	NA	0.450	NA	-0.35
5	4.625	0.236	0.67	-0.63
10	1.716	0.173	0.23	-0.76
50	0.847	0.044	-0.07	-1.35
100	0.465	0.026	-0.33	-1.59
150	0.207	0.005	-0.68	-2.32

363
364 It is important to note that the release medium contained 1.98 mM of docusate, otherwise a
365 signal mismatch was obtained when SEDDS were diluted in water and was difficult to interpret
366 (supporting information, Figure S2). However, when the ionic strength was increased, and in the
367 absence of sodium docusate, a complete release of the peptide was observed, even at low ionic
368 strength (see black trace in Figure 5). Changing the docusate concentration changed the average
369 R_h , which was 1.22 nm (98 % of free peptide), 1.87 nm (92 % of free peptide), 3.24 nm (78% of
370 free peptide) and 7.23 nm (37% of free peptide) for 0, 0.33, 0.66 and 1.98 mM of docusate,
371 respectively, at 10 mM ionic strength. This can be due to the fact that the presence of docusate in
372 the release media allows the HIP dissociation equilibrium to be displaced toward the formation
373 of HIP and the formed complex is rapidly incorporated into the droplets. In conclusion, it was
374 shown that the composition of the release medium drastically influences the amount of released
375 peptide, and thus, the partitioning coefficient of the HIP.



376

377 **Figure 5:** TDA of leuprorelin-docusate HIP in SEDDS diluted 36 times in 10 mM NaCl solution
 378 in the absence of sodium docusate from the mobile phase and its presence in the mobile phase (at
 379 two different final concentrations 0.66 and 1.98 mM). Experimental conditions: μ -SIL-DNA
 380 neutral coated fused silica capillary, 45 cm (36.5 cm to detector) \times 75 μ m i.d.; Mobile phase:
 381 SEDDS + x % w:w of docusate ($x = 0, 1$ or 3 % w:w) diluted 36 times in 10 mM NaCl solution.
 382 Mobilization pressure: 50 mbar. Hydrodynamic injection: 50 mbar for 2 s. Injected samples:
 383 leuprorelin-docusate HIP (7% w:w of peptide and 3% w:w sodium docusate) in SEDDS diluted
 384 by a factor of 36 in 10 mM NaCl without the addition of docusate. Temperature: 37°C; UV
 385 detection at 193 nm. For detailed compositions of the TDA samples and mobile phase see
 386 experimental section or Table S1.

387 Furthermore, if we consider the following equilibrium:



389 Having an ion pair equilibrium constant, K_{IP} , defined as:

$$390 \quad K_{IP} = \frac{[PL_n]}{[P_w][L]^n} \quad (14)$$

391 where P_w is the peptide, L is the docusate, PL_n is the complex and n is the stoichiometry
 392 coefficient for the docusate ($n = 2$ in the case of leuporelin-docusate complex (Griesser et al.,
 393 2017)). K_{IP} is dependent on the temperature, on the ionic strength of the medium and on the
 394 composition of SEDDS and continuous medium. If the low solubility of the complex in the
 395 aqueous phase is considered, it can be stated that the complex is quantitatively inside of the
 396 droplets *i.e.* $PL_n = P_{SEDDS}$; equations (10) and (14) can thus be combined to obtain:

$$397 \quad D_{SEDDS/w} = K_{IP} [L]^n \quad (15)$$

398 Thus, $\log K_{IP}$ can be calculated for different docusate concentrations using the following
 399 equation:

$$400 \quad \log K_{IP} = \log D_{SEDDS/w} - n \log [L] \quad (16)$$

401 In the case of figure 5, when the SEDDS were diluted in 10 mM NaCl solution containing
 402 various concentrations of sodium docusate, a roughly constant value of $\log K_{IP}$ was obtained (see
 403 Table 2), showing the validity of the proposed calculations.

404 **Table 2:** Influence of docusate concentration in the release media on the partitioning coefficient
 405 $\log D_{SEDDS/w}$, the ion pair formation constant $\log K_{IP}$ and the fraction of the released peptide. Ionic
 406 strength in the release medium is of 10 mM.

Docusate Concentration (mM)	$\log D_{SEDDS/w}$	$\log K_{IP}$	$R_{h,TDA}$ (nm)	%free peptide
0	-2.57	NA	1.22	98%
0.33	-1.12	5.84	1.87	92%
0.66	-0.57	5.79	3.24	78%
1.98	0.23	5.63	7.23	37%

407

408 It is important to note that, for all the studied ionic strengths, the obtained elution profile did not
 409 evolve with time (at least within a time range of several hours), showing that a rapid equilibrium

410 is reached between the HIP concentration in the microdroplets and the continuous medium. This
411 observation confirms the hypothesis postulated by Bernkop-Schnürch and Jalil (Bernkop-
412 Schnürch and Jalil, 2018). Furthermore, it showed, for the first time, that the release of the
413 peptides from the microdroplets is related to the $\log P$ value of the HIP, to the ionic strength of
414 the medium and to the composition of the continuous medium (i.e. presence of free docusate).

415 **4. Conclusion**

416 The aim of this work was to show that the hydrophobicity of the peptide-surfactant complex is an
417 important parameter allowing the peptide to be hosted by the SEDDS nanodroplets, and to study
418 the influence of the ionic strength on the release of the peptide from the formulation. The use of
419 the TDA method was found to be an excellent way to quantify the proportion of released peptide
420 in SEDDS + HIP formulation. For that, it was demonstrated experimentally that the addition of
421 surfactant to the mobile phase was required to avoid a mismatch between the sample zone
422 containing the SEDDS + HIP and the SEDDS mobile phase. It was also experimentally
423 confirmed that the $\log P$ value of the HIP can be used as a good indicator of the peptide load in
424 the SEDDS nanodroplets. In the case of the leuprorelin-docusate complex ($\log P = 3$), the totality
425 of the complex was located inside the SEDDS microdroplets. The increase of the ionic strength
426 lead to the release of the peptide from the nanodroplets (up to 80% of free peptides at
427 physiological ionic strength (150 mM)). As for desmopressin-docusate complex ($\log P = 0.5$) a
428 fraction of 30 % of peptide was located in the nanodroplets, and the increase of the ionic strength
429 to values around 50 mM leads to the release of over 95 % of the peptides. **Further, the**
430 **concentration of added surfactant to the release medium allowed the tuning of the peptide release**
431 **from the SEDDS droplets. It was also shown in this work, that one can calculate the $\log D_{SEDDS/w}$**
432 **values for each experiment under the operating experimental conditions.** Due to its simplicity,

433 low sample consumption, absolute method (no calibration), TDA might become a
434 characterization technique of importance for formulators developing drug release technologies
435 based on SEDDS.

436 **5. Supporting information**

437 Figures S1, S2, S3, S4, S5, S6, S7 and tables S1, S2 and S3

438 **6. Acknowledgments**

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440 **7. Notes**

441 Vincent Jannin and Frédéric Demarne were employed by Gattefossé SAS manufacturing and
442 selling Capryol 90 and Transcutol HP at the time this study was performed.

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508

509

Graphical Abstract

Peptide release from SEDDS nanodroplets followed by TDA

*HIP (peptide-surfactant hydrophobic ion pair)
in SEDDS*

