**Simulation of Oleuropein Structural Conformation in Vacuum, Water and
Triolein-water systems using Molecular Dynamics**

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21 **Abstract**

22 Oleuropein, the main phenolic compound of olive leaves, exhibits a unique blend of
23 biological activities and has been shown to locate itself at the oil-water (O/W) interface.
24 This behavior could influence on the physico-chemical properties of dispersed systems
25 such as emulsions. In this work, we study the effect of the microenvironment (vacuum,
26 water, and triolein-water) on the conformational preferences of oleuropein using
27 molecular dynamics (MD) simulations at 300 K for at least 30 ns. The seven torsions
28 that describe the flexible skeleton of oleuropein were monitored together with the
29 distance between the glucose (Glu) and hydroxytyrosol (Hyd) moieties ($d_{\text{glu-hyd}}$) of the
30 molecule. The obtained trajectories demonstrated that oleuropein adopts different
31 conformations that depend on the environment. The preferential conformers in each
32 system were analyzed for their molecular geometry and internal energy. In vacuum, the
33 oleuropein preferential conformation is tight with the glucose moiety in close proximity
34 with the hydroxytyrosol moiety. In water, oleuropein preferential conformers presented
35 large differences in their structural properties, varying from a close like U form, and a
36 semi-opened form, to an opened form characterized by high fluctuations in $d_{\text{glu-hyd}}$
37 values. In a triolein-water system; oleuropein tends to adopt a more open form where
38 the glucose moiety could be approximately aligned with the hydroxytyrosol and elenolic
39 acid moieties. Based on a calculation at the HF/6-31G* level, these flexibilities of
40 oleuropein required energy of 19.14 kcal/mol in order to adapt the conformation
41 between water and triolein-water system. A radial distribution function (RDF) analysis
42 showed that specific hydroxyl groups of Hyd and Glu interact with water molecules,
43 enabling us to understand the amphiphilic character of oleuropein at the triolein-water

44 interface. MD calculations together with interfacial tension measurements revealed the
45 oleuropein binding at O/W interface is an enthalpy driven mechanism.

46

47 1. Introduction

48 Major and minor biomolecules from olive plants and its processing byproducts have
49 been consumed for a long time in the Mediterranean diet without an understanding of
50 their structure, function, and interactions within the olive fruit or in the human body
51 (Uccella, 2000). Advanced scientific research has revealed that a Mediterranean diet
52 rich in olive oil is associated with a decrease in risk for cardiovascular disease, obesity,
53 and diabetes (López-Miranda et al., 2010). These protective functions may originate
54 from the lipid components of such a diet as well as phytochemicals including
55 polyphenols, which occur in olive oil and olive plants. Among protective polyphenols,
56 oleuropein, which is the main component of bitterness in olives, is well known to
57 exhibit a unique blend of biological activities (Bulotta et al., 2013; Cicerale, Lucas, &
58 Keast, 2010). Oleuropein is a secondary metabolite synthesized via a branching in the
59 melalonic acid during the formation of oleosides (Omar, 2010). Oleuropein can be
60 extracted from the leaves and drupes of the olive tree (*Olea europaea* L.), especially
61 present in the leaves (60–90 mg/g dry weight) (Ghanbari, Anwar, Alkharfy, Gilani, &
62 Saari, 2012). Purified oleuropein and its aglycon forms were characterized
63 spectroscopically using the NMR and FABS-MS methods (Capasso et al., 1997).

64 The oleuropein structure depicted in Fig.1, shows that this polar glycoside includes
65 three compartments, a glucose molecule, elenolic acid and hydroxytyrosol. During fruit
66 maturation or industrial processing of extra virgin olive oil, enzymatic hydrolysis by

67 β -glucosidase results in cleavage of the glucose moiety from the non-polar oleuropein
68 aglycone, and the hydrolysis action of esterase engenders the liberation of
69 hydroxytyrosol and elenolic acid (Gutierrez-Rosales, Romero, Casanovas, Motilva, &
70 Mínguez-Mosquera, 2010). Oleuropein provides a natural defense against pathogenic
71 attack and plays a role in structuring the cell-wall skeleton by acting as a cross- linker
72 between the different polysaccharide moieties in the plant cells (Uccella, 2000).

73 The activity of polyphenolic compounds depends on their structural characteristics
74 (Leopoldini, Russo, & Toscano, 2011). Molecular conformation can be investigated
75 using a multitude of scientific techniques including X-ray crystallography, NMR
76 spectroscopy, and computational modeling. A structural clarification of oleuropein in a
77 human biological environment would be of great interest since it helps us to understand
78 the mechanisms of its biological activities as correlated with its physico-chemical
79 properties. In this field, numerous works have focused on the conformation of the
80 oleuropein binding to some key proteins involved in the pathway of oleuropein
81 mediated diseases using molecular modeling (MD). MD studies indicate that oleuropein
82 binds to the peroxisome proliferator activating receptors involved in atherosclerosis
83 expression (Andreadou et al., 2006; Huang, Huang, & Lee-Huang, 2010). Oleuropein
84 conformation was strongly dependent on the receptor type α , β , or γ (Huang et al.,
85 2010). Lee-Huang et al. (2007) investigated conformation of the oleuropein binding to
86 the retrovirus (HIV-1) envelop glycoprotein (gp41) using molecular docking and MD
87 simulations. The conformation of isolated oleuropein in vacuum has shown a closed
88 structure in which its phenolic hydrogen forms a hydrogen-bond network with the
89 hydroxyl groups of the glucose moiety(Gikas, Bazoti, & Tsarbopoulos, 2007). However,
90 the MD simulations of oleuropein in vacuum did not consider the chemical reactions

91 that are carried out in solution, where this compound would really exist. Thus, a study
92 of a possible solvent effect on oleuropein conformation should be considered. For
93 instance, an understanding of oleuropein conformation within the olive cellular
94 environments will be very important for interpreting its histological distribution, and
95 investigating its reactivity with enzymes and other physiological partners.

96 Recently, several biological activities of oleuropein have been correlated to its
97 surface activity (Di Mattia, Sacchetti, & Pittia, 2011). For instance, the antimicrobial
98 activity of oleuropein can be attributed to its ability to damage the bacterial membrane
99 (Bisignano et al. 1999). A few research groups suggested that the effectiveness of
100 oleuropein, as an antioxidant in a membrane of phospholipid bilayer, is associated with
101 the membrane surface rather than penetration of oleuropein into the membrane
102 (López-Miranda et al. 2010; Caturla, Pérez-Fons, Estepa, & Micol, 2005).

103 From a physical point of view, the surface activity of oleuropein would be important
104 during extraction of the olive oil, which involves several unit operations of biphasic
105 systems: mainly malaxation and centrifugation (Di Giovacchino, Costantini, Ferrante, &
106 Serraiocco, 2002). Our previous study reveals that oleuropein was able to significantly
107 reduce the interfacial tension of triglyceride oil-water interfaces (Souilem et al. 2014),
108 which confirms its high surface activity. We also proved that oleuropein is the major
109 surface active compound in olive leaf extract (OLE). Ghahfarokhi et al. (2015)
110 suggested the use of OLE as a naturally occurring surfactant since it was able to reduce
111 the interfacial tension between kerosene and water from 36.5 to 14 mN/m. Souilem et al.
112 (2014) used microchannel emulsification to demonstrate the ability of oleuropein to
113 stabilize oil-in-water (O/W) emulsions. Although this experimental evidence about the

114 O/W interfacial activity of oleuropein, there is no available explanation at molecular
115 scale, on the behavior of oleuropein at O/W interface. An investigation on the change
116 of oleuropein conformation upon adsorption to the O/W interface could lead to a better
117 understanding of its amphiphilic character.

118 In this paper we extend our earlier study by focusing on the active conformation of
119 oleuropein at the O/W interface and its conformational change according to its nature
120 and the media (vacuum, water, and triolein-water systems) using MD simulations.

121

122 **2. Computational methods**

123 *2.1. Setup model and simulation details*

124 The initial structures of oleuropein and triolein were generated using molecular
125 modeling software (ChemDraw). To overcome the high flexibility of the molecule, a
126 conformational search had been done to obtain the most stable torsional angle of the
127 molecule. The configuration at the lowest energy was selected to be further subjected to
128 a full geometry optimization at the force field B3LYP/6-31G (d,p) by the application of
129 Gaussian 09.

130 The topology of oleuropein was generated through the Dundee PRODRG2 Server. The
131 atomic charge of oleuropein was obtained from the optimized structure at the
132 B3LYP/6-31G (d,p) level of theory. Bond interactions were calculated based on the
133 GROMOS96 force field. A united atom force field (<http://wcm.ucalgary.ca/tieleman>)
134 based on lipid parameters (Tieleman and al. 2006) were applied to triolein. The
135 extended simple point charge (SPC/E) model of water was used to describe the solvent

136 water. MD simulations were carried out in triplicate using the GROMACS 4.6.1
137 software package. The systems for oleuropein in vacuum, water and triolein-water were
138 setup as follows.

139 2.1.1. *Vacuum system*

140 Oleuropein was minimized without any constraints before 50ns. MD simulations at 300
141 K. The non-bonded interactions were settled with a cutoff of 0.7 nm.

142 2.1.2. *Water system*

143 A single oleuropein molecule was placed in the center of a cubic box 5 nm inlength.
144 The total number of water molecules was 4115. The system was first subjected to an
145 initial minimization for 100 ps, followed by equilibration in a canonical ensemble
146 (NVT) for 200 ps and an isothermal-isobaric ensemble (NPT) for 200 ps and 400 ps,
147 respectively, under periodic boundary conditions and with a time step of 2 fs. The
148 temperature was set to 300 K using a Parrinello-Rahman thermostat with a coupling
149 constant of 0.1 ps. The total pressure was isotropically kept at 1 atm using a
150 Parrinello-Rahman pressures coupling of 0.5 ps. Electrostatic interactions were modeled
151 with the particle mesh Ewald (PME) approach using a grid spacing of 0.16 nm. The MD
152 simulation was subsequently conducted for 50 ns at constant temperature of 300 K
153 under the NPT ensemble as previously set conditions.

154 2.1.3. *Triolein-water system*

155 A box of triglyceride phase composed of 199 triolein molecules was generated,
156 minimized and equilibrated by GROMACS. A oleuropein-triolein-water system was
157 then built from three layers of water, triolein and water as illustrated in Fig. 2.b. The

158 dimensions of the resulting box were 6.82 nm X 6.82 nm X 20.62 nm. The oleuropein
159 phase having a thickness of 6.92 nm, was intercalated between two opposing cubic
160 water boxes aligned along the z-axis. Each water box has a thickness of 6.85 nm and
161 21271 molecules. Subsequently, one oleuropein molecule was added at each
162 triolein/water interface. Three sets of simulations with different starting orientation of
163 oleuropein were conducted (Fig.2.b). During the first simulation (S1), oleuropein was
164 placed where its glucose moiety was orientated into the triolein phase. In the second
165 simulation (S2), oleuropein was placed such that its glucose moiety was oriented into
166 the water phase. The final simulation (S3) was carried with oleuropein in which the
167 glucose and hydroxytyrosol moieties had been aligned parallel to the interface.

168 Initially, the system was subjected to energy minimization using the Steepest Descent
169 and Conjugated gradient methods to remove the initial strain. Equilibration was carried
170 out under canonical ensemble for 100ps. The temperature was raised to 300 K and held
171 constant using a Parrinello-Rahman thermostat with a coupling constant of 0.1 ps.
172 Electrostatic interactions were modeled using the particle mesh Ewald (PME) approach
173 with a grid spacing of 0.16 nm and an interpolation order (PME order of 4). Van der
174 Waals interactions were computed with a cut-off of 1.4 nm. All bonds involving
175 hydrogen atoms were constrained using the LINCS algorithm. The integration time step
176 was 2 fs. The density of the system was equilibrated in the isothermal-isobaric (NPT)
177 ensemble for 200 ps. The total pressure was held at 1 atm using a Parrinello-Rahman
178 pressure coupling of 0.5 ps. Production simulations were carried out for 30 ns at a
179 constant temperature of 300 K under the NPT ensemble as previously set conditions. To
180 maintain a stable interface, semi-isotropically pressure coupling was applied allowing
181 the length of the box in z direction to change but holding the box lengths in the x and y

182 directions fixed to their initial values. Such an MD setup has previously been
183 demonstrated to be optimal for simulating the equilibrium properties of a
184 triglyceride-water interface (Henneré et al. 2009).

185 2.2. Analysis of MD trajectories

186 2.2.1. Torsions analyses

187 The conformation of oleuropein was analyzed by selecting of the seven important
188 torsions (T_1 , T_2 , T_3 , T_4 , T_5 , T_6 and T_7) defined in Fig.2.a. These torsions belong to the
189 backbone skeleton of oleuropein and describe the possible rotational degrees of freedom
190 along the three successive chemical-bond vectors between elenolic acid and glucose.
191 The torsions were preset to vary within the range -180° to $+180^\circ$. Their distributions
192 were analyzed from the frames, after reaching thermodynamic equilibrium.
193 Representative conformers of oleuropein in each environment were chosen according to
194 the highest population of the determined torsional angles.

195 2.2.2. Energy calculation

196 To complement the structural analysis, potential energy calculations were
197 performed for the selected preferable snapshots using the Gaussian09 program
198 package. Hydrogen and heavy atoms in each oleuropein conformer were optimized at
199 HF/3-21 G and the energy was obtained at HF/6-31G* levels.

200 2.2.3. Distance analysis

201 To approximate the spatial flexibility of oleuropein in the different studied systems; the
202 end-to-end distance of the molecule ($d_{\text{glu-hyd}}$) was measured during the simulations.

203 $d_{\text{glu-hyd}}$ represents the distance between the centers of the hydroxytyrosol and glucose
204 moieties.

205 2.2.4. Radial distribution function (RDF) analyses

206 The RDF analysis aimed to investigate the hydrogen bonding between functional groups
207 of oleuropein (hydroxyl and ester) and environmental water molecules. Therefore, pair
208 radial distributions were analyzed between the oxygen atoms in the oleuropein molecule
209 (O2, O3, O4, O5, O6, OAR, OAV, OAW, OBE and OBA) and the oxygen atoms of
210 water molecules. The distance of the 1st hydration shell and the numbers of water
211 molecules were obtained for both the water and the triolein/water system. The
212 heteroatomic distances within 3.00 Å were considered for the hydrogen bonding
213 interactions.

214 2.2.5. Determination of the standard enthalpy of oleuropein adsorption

215 2.2.5.1. MD calculations

216 Adsorption enthalpy of oleuropein in triolein-water system was obtained from the last
217 15 ns of production trajectories using molecular mechanics Poisson-Boltzmann surface
218 area (MM-PBSA) approach implemented in g_mmpbsa (Rashmi et al. 2014). Briefly,
219 binding enthalpies between oleuropein and triolein in gas phase were combined with the
220 polar solvation and nonpolar solvation energy according to the thermodynamic cycle.
221 Implicit solvation model was applied using dielectric constant of 80 with grid space of
222 1.5 Å. Solvent probe radius was 1.4 Å. In total, 1000 snapshot structures were used in
223 the MM-PBSA calculations and analysis.

224 2.2.5.2. Empirical calculations

225 The standard enthalpy of oleuropein adsorption at soybean oil/water interface was
226 determined based on interfacial tension measurements following the method described
227 by (Krassimir and Kralchevsky. 2012).

228 Interfacial tension measurements were carried out using a pendant drop tensiometer
229 analyzer (PD-W, Kyowa Interface Science Co., Ltd., Saitama, Japan); following the
230 method by (Souilem et al. 2014). Measurements were carried out using aqueous
231 solutions of highly purified oleuropein (purity 93%) (Extrasynthèse, France) and refined
232 soybean oil (Wako Pure Chemical Industries, Ltd. (Osaka, Japan)).

233 First, the standard free energy of oleuropein adsorption ΔG was determined by linear
234 fits of interfacial tension isotherms at different temperatures (15°C, 25°C, 35°C and
235 45°C) with the help of the theoretical model of adsorption defined by Eq. (1):

$$236 \quad KT \ln \left(\frac{C}{W} \right) = \frac{1}{\Gamma_d} \pi_s + \Delta G \quad (1)$$

237 Where Γ_d (const) is the adsorption layer of oleuropein, $\omega = \rho_w/18$ is the molar
238 concentration of water; ρ_w (g/L) is the mass density of water and π_s (mN/m) is the
239 surface pressure. ΔG is determined from the intercept of the linear regression of plots
240 from experimental data for $\pi_s(C)$ as $KT \ln \left(\frac{C}{W} \right)$ in accordance with Eq. (1). The Gibbs
241 free energy (ΔG) of an isothermal transfer of surfactant molecules from bulk solution to
242 interface is defined by Eq. (2):

$$243 \quad \Delta G = \Delta H - T\Delta S \quad (2)$$

244 Where ΔH (KJ/mol) is the free enthalpy of adsorption and ΔS (KJ/mol) is the free
245 entropy of adsorption. ΔH is determined from the intercept of the linear regression of
246 plots from the obtained experimental ΔG as T° in accordance with Eq. (2).

247

248 **3. Results and discussion**

249 *3.1. Equilibrium profile of the system*

250 Analyses were performed after the system shifted towards equilibrium. Equilibrium of
251 the system was monitored through the root mean squared deviation (RMSD) and
252 density. RMSD values of oleuropein reached equilibrium after 200 ps for vacuum, 2 ns
253 for water and 15 ns for triolein-water systems.

254 The density profiles of water and triolein in both water and triolein-water system along
255 the z-axis was monitored as seen in Fig. 4. At equilibrium, the obtained water and
256 triolein densities were 1000 and 926 kg/m³, which represents the experiment data of 998
257 kg/m³ and 890 kg/m³, respectively at 300 K.

258 *3.2. Location of oleuropein in triolein-water system*

259 Figure3.b plots the density of the components in the simulation system along the z-axis.
260 Oleuropein exhibits two peaks (6.99 nm and 13.58 nm), indicating that oleuropein is
261 located on both sides of the triolein-water interfaces. Our observations are in line with
262 the corresponding experimental conclusions, that oleuropein exhibits interfacial activity
263 at the triglyceride-water interface (Souilem et al., 2014). Oleuropein caused a significant
264 decrease of the interfacial tension at the triglyceride-water interface and showed an
265 emulsifying ability to stabilize O/W droplets using microchannel emulsification
266 (Souilem et al., 2014).

267 *3.3. Conformational analysis*

268 To gain further insight into the details of the environmental effects, the torsions
269 distribution in each environment was analyzed. Considering the large number of

conformers generated from the MD simulations, the distribution of each torsion that was
 previously defined in Fig.2.a, was determined. It can be easily seen from Fig.4 that the
 resulting distribution plots for the major torsions are different in vacuum, water or
 triolein-water systems except torsion 7, reflecting the flexibility of oleuropein structure
 in different surrounding microenvironments. The highest preferential locations of the
 studied torsions are identified in Fig.4 as T_{nt}^{Sys} (nt: number of torsion; Sys (system
 (Vacuum (VAC), Water (WAT), Triolein-water (TRI-WAT)). Analyzing torsion1, two
 dominant populations with maxima $(T_1^{VAC1}, T_1^{VAC2}) = (T_1^{WAT1}, T_1^{WAT2}) = (T_1^{TRI-WAT1},$
 $T_1^{TRI-WAT2}) = (-90^\circ, 92^\circ)$ were observed in all vacuum, water and triolein-water systems.
 The similar observed distribution plots of T_1 were characterized by a similar amplitude
 of the maxima (-90°) at around 18% indicating a low solvent effect on the torsion 1.
 Torsion 2 exhibited different distribution plots in the studied systems. In vacuum, T_2
 was more frequently located at $T_2^{VAC1}=176^\circ$ with an amplitude of 13.5% but it could
 also be located at $T_2^{VAC2}=-65^\circ$ and $T_2^{VAC3}=71^\circ$ with lower amplitudes of 3.7% and
 7.5%, respectively. Conversely, in water, T_2 is located at two symmetric maxima
 $(T_2^{WAT1}, T_2^{WAT2}) = (-65^\circ, +65^\circ)$ having an amplitude of 12.9%. We e also notice a
 possible location T_2^{WAT3} at 176° but with lower observed amplitude at around 5.8%.
 In triolein-water, torsion 2 has the same preferential location as in vacuum. $T_2^{VAC1}=$
 $T_2^{TRI-WAT1}=176^\circ$, but presents a higher amplitude of 22%. The different preferential
 angle of torsion 2 between the water and triolein-water systems suggests a staggered
 conformation of the bond (CBD-CBC) and (CBB-OBA) might contribute to oleuropein
 partitioning into the hydrophobic triolein phase. Analyzing torsion 3, two symmetric
 dominant states were observed. In vacuum, the states have ranges of $[-138^\circ, -34^\circ]$ and
 $[27^\circ, 143^\circ]$, with maxima T_3^{VAC1} and T_3^{VAC2} of -75° and 82° . In water and in

triolein-water systems, the ranges of the states are shifted to $[-176^{\circ}, -74^{\circ}]$ and $[67^{\circ}, 178^{\circ}]$. Two symmetric preferential torsional angle $T_3^{\text{WAT1}} = -139^{\circ}$ and $T_3^{\text{WAT2}} = 153^{\circ}$ with a similar occurrence of 10.2% were observed in water. In triolein-water, a larger expansion of torsion 3 was observed leading to the staggered conformation of bond (CBC-CBB) and (OBA-CAZ) at $T_1^{\text{TRI-WAT1}} = -179^{\circ}$. The distribution plots of torsion 4 showed a pronounced solvent effect on its motion. In fact, in vacuum, torsion 4 is more frequently at a staggered conformation T_4^{VAC1} at -179° with high amplitude of 26%. However, in water or triolein-water, T_4 varies among $-177^{\circ}, -74^{\circ}$ and 82° . The maximum T_4 observed in water was $T_4^{\text{WAT1}} = -74^{\circ}$ and the highest observed location in triolein-water was $T_4^{\text{TRI-WAT1}}$ at -179° . Analyzing torsion 5, two parabolic dominant states having the ranges of $[-152^{\circ}, -65^{\circ}]$ and $[15^{\circ}, 126^{\circ}]$ described the populations of torsion 5 in vacuum with a preference for $T_5^{\text{VAC1}} = 80^{\circ}$ having an amplitude of 9.5%. In water and triolein-water systems, torsion 5 has more flexibility, explained by the border distribution plots while keeping the same preferential angle at $T_5^{\text{WAT1}} = T_5^{\text{TRI-WAT1}} = 80^{\circ}$. Torsion T_6 exhibits two dominant states in vacuum, with the highest preference at $T_6^{\text{VAC1}} = -67^{\circ}$. In water and triolein-water systems, different distribution plots of T_6 were observed, indicating the presence of intermolecular interactions in the presence of water or triolein. In water, T_6 has the highest population at $T_6^{\text{WAT1}} = 74^{\circ}$ but in the triolein-water system, it was observed to be distributed at two angles $T_6^{\text{TRI-WAT1}} = 74^{\circ}$ and $T_6^{\text{TRI-WAT2}} = 164^{\circ}$ having a similar amplitude of around 17%.

Torsion angle T_7 linking the glucoside moiety to the oleuropein aglycone showed a single and sharp preferential peak at around -80° in all systems, indicating its high intramolecular rigidity through the simulation periods. In the triolein-water system with different starting orientations of the oleuropein, the distribution plots were reproduced

318 at the two interfaces for torsions T_2 , T_3 , T_4 and T_7 . However, small deviations were
319 obtained for torsions T_1 and T_5 which could explain the high flexibility of these torsions
320 in a biphasic system.

321 Summarizing the above results, we noticed that torsions T_2 , T_3 and T_4 showed the
322 highest microenvironmental dependence since they exhibited different distribution plots
323 and preferential angles depending on the system. In addition, the carboxyl ester group
324 linking hydroxytyrosol and elenolic acid is considered to be the key flexible region in
325 the oleuropein molecule.

326

327 3.4. Flexibility of oleuropein

328 3.4.1. Conformation of oleuropein in vacuum

329 The distribution of torsional angles in vacuum (Fig.4) showed that the preferential
330 torsions (T_5^{VAC1} and T_6^{VAC1}) are acute (81° and 69°), suggesting that oleuropein folds
331 into a closed molecule. The distance ($d_{\text{glu-hyd}}$) during simulation was monitored for
332 further investigation and distributions of $d_{\text{glu-hyd}}$ were illustrated in Fig. 5. The average
333 distance $d_{\text{glu-hyd}}$ was relatively stable at 0.6 nm with small fluctuations during the
334 simulations. This confirms the close proximity of hydroxytyrosol to glucose and is
335 consistent with the fact that all conformers have a tight structure (Fig6.a). Nevertheless,
336 $d_{\text{glu-hyd}}$ exceeds 0.32 nm, indicating that there is no intramolecular interaction between
337 the atoms forming the hydroxytyrosol and glucose moieties. The corresponding $d_{\text{glu-hyd}}$
338 values of the highest observed conformers C_{vac}^1 , C_{vac}^2 , C_{vac}^3 and C_{vac}^4 were 0.589 nm,
339 0.641 nm, 0.594nm and 0.645 nm, respectively.

340

341 *3.4.2. Conformation of oleuropein in water*

342 Water highly influences on the torsion angle distribution (Fig.4), which would be
343 reflected in the conformational preference of oleuropein. The shift of T_3 (T_3^{VAC1} ; T_3^{VAC2})
344 from $(-79^\circ, +79^\circ)$ to the maxima (T_3^{WAT1} ; T_3^{WAT2}) $(-139^\circ, 154^\circ)$ in water may cause the
345 glucose moiety to be located further from the phenolic group of hydroxytyrosol in water.
346 Figure5.b depicts the distributions of $d_{glu-hyd}$ in the water system. The $d_{glu-hyd}$ distance
347 varied from 0.4 to 1.4 nm with an average distance of 0.8 nm. The high frequency of the
348 distance variation might reflect a fast dynamics of oleuropein between the tight and
349 open forms. The torsions analyses were in agreement with the distance analysis since
350 oleuropein preferential conformers (Fig.6) presented a large difference in their structural
351 properties, varying from a close like U form (C_{wat}^1) having a d_{glu_hyd} distance of 0.570
352 nm, and a semi-open form (C_{wat}^3) having a $d_{glu-hyd}$ of 1.074 nm to an opened form like
353 the conformer, C_{wat}^4 with a $d_{glu-hyd}$ of 1.214 nm. The high flexibility of oleuropein is
354 expected to be potentially influenced by the presence of water, which may interact with
355 a number of OH groups throughout the oleuropein molecule by forming hydrogen
356 bonds.

357

358 *3.4.3. Conformation of oleuropein in the triolein-water system*

359 The preferential torsions ($T_2^{TRI-WAT}$, $T_3^{TRI-WAT}$ and $T_4^{TRI-WAT}$) linking the hydroxyphenyl
360 and elenolic acid moieties have the same rotation around $(-180^\circ, 180^\circ)$ (Fig.4)
361 suggesting that oleuropein folds into an open molecule. Similarly to the water system,
362 $d_{glu-hyd}$ ranged from 0.4 to 1.4 nm with an average distance of 0.84 nm. However, less
363 fluctuation of the distance was observed as compared to its motion in the water system.
364 The structures of the highest probable conformers in the triolein-water system are

represented in Fig. 6c. The top 10 highest probable conformers for oleuropein followed an opened conformation. However, there are several notable differences in the orientations of the glucose and hydroxyphenyl moieties in a set of opened conformations. $d_{\text{glu-hyd}}$ of the different selected conformers varies between 1.008 nm for $C_{\text{tri_wat}}^8$ and 1.377 nm for $C_{\text{tri_wat}}^6$. The greatest $d_{\text{glu-hyd}}$ distance forces the glucose, elenolic acid and hydroxytyrosol to be approximately aligned and indicates high repulsive forces inside the molecule.

3.5. Energy determination of preferential conformers

Since the major conformations of oleuropein were different for each system, the required energy to adopt each conformation was predicted (Table 1). In vacuum, S_{vac}^1 was the most stable conformation since it has the lowest energy. The energy difference between the lowest energy conformer and the highest energy conformer in vacuum is about 9.73 kcal/mol, which strongly indicates that oleuropein can easily adopt these different conformations. Similarly, based on the energy values of the highest probable conformers, the stability of oleuropein in water appears in this order: $C_{\text{wat}}^4 > C_{\text{wat}}^2 > C_{\text{wat}}^1 > C_{\text{wat}}^3$, which means that the most stable conformation in water has an open form where the glucose, elenolic acid and hydroxytyrosol are approximately aligned. However, the energy barrier between the closed conformer and the open form is small, about 1.92 kcal/mol, confirming that oleuropein can easily convert between the two forms.

In the triolein-water system, oleuropein showed flexibility lower than that in the water system since all of the conformational states have a relatively open form (Fig.6.c). All of the conformers are geometrically symmetric; they differ in the orientation of their

389 glucose and hydroxyphenyl moieties. The conformer ($C_{\text{tri-wat}}^9$) presented the lowest
390 energy, which reflects its higher stability in the triolein-water system when adopting this
391 conformation state. $C_{\text{tri-wat}}^9$ is characterized by a $d_{\text{glu-hyd}}$ of 1.142 nm, which is lower than
392 the distance of $S_{\text{tri-wat}}^6$, so repulsive forces are minimized in this structure. The energy
393 barrier of 14.39 kcal/mol makes the difficult to change from the conformer $C_{\text{tri-wat}}^9$ to
394 $C_{\text{tri-wat}}^2$.

395 When comparing the energy difference between all the systems, we found that the
396 energy barrier of oleuropein to convert from the tight form representing the lowest
397 energy in vacuum to the lowest energy form in a water system is around 5.24 kcal/mol.
398 However, the energy barrier between the lowest energy conformer in water and
399 triolein-water systems is around 19.14 kcal/mol. This energy barrier is required for
400 oleuropein to cross the system. The O/W interfacial activity of oleuropein might
401 originate from specific hydrogen bonding and hydrophobic interactions at the
402 triolein-water interface that is expected to greatly contribute to its stabilization.

403 3.6. Radial distribution function analysis: Interaction of oleuropein with the 404 surrounding environment

405 Radial distribution function analyses (RDF) for the water solution in water and in
406 triolein-water systems were calculated based on the MD simulations. We focus on the
407 oxygen atom, *i.e* O (oleuropein)- O (water), where O (oleuropein) refers to an oxygen
408 atom belonging to oleuropein and O (water) refers to the surrounding water. Based on
409 these results, hydrogen bonding is expected when the cut-off of the well-defined peak at
410 a distance below 3.5 Å. Examples of radial distribution from different oxygen atoms in
411 the oleuropein molecule are presented in Fig.7. The oxygen atoms present in the glucose

moiety reveal the existence of hydrogen bonding between the hydroxyl groups of oleuropein (O2, O3, O4 and O6), and water molecules both in bulk water and triolein/water systems. For instance, for O6, there were sharp peaks at around 3.2 Å in water and 3Å in triolein/water. However, the presence of water molecules was steadier in water (9.97 water molecules) than in the triolein/water system (2.52 water molecules). A similar solvation effect was caused by the triolein/water system when analyzing the oxygen atoms OBK and OBL belonging to the hydroxytyrosol moiety of oleuropein (Fig.7.b). These atoms have stronger hydrogen bonding in a water system.

Analyzing the RDF plots for oxygen atoms(OAR, OAW, OAV, OBE, and OBA), belonging to the elenolic acid moiety, one or more water molecules was found around VCDB HY67UJP/MQWSOAR, OAV and OBE in the water system (Table 2), confirming the existence of hydrogen bonding in these regions. However, these curves were significantly less intense in the triolein/water system, suggesting that an effective desolvation happened beyond the first shell of the corresponding oxygen atoms (Table 2).For OAR, the shell completely disappears, which leads to the inaccessibility of water molecules to the elenolic acid moiety.

Summarizing the above results, when we compare the RDF plots in water and in triolein/water systems, the main differences are found for the O (elenolic acid)-O water interactions and to lower extents in the glucose and hydroxytyrosol moieties. These results confirm the O/W interfacial activity of oleuropein which was assumed based on the lowering of solvation of the main oxygen atoms in oleuropein. The absence of hydrogen bonding in the elenolic acid region suggests its partitioning into the triolein phase due to hydrophobic interactions.

435

436 *3.7. Link between conformation and O/W interfacial activity of oleuropein*

437 We have confirmed the O/W interfacial location of oleuropein by the use of three sets of
438 simulations S1, S2 and S3 starting either from a parallel or a perpendicular initial
439 orientation of oleuropein to the triolein-water interface (Fig.2b). The energy barrier
440 between the lowest energy conformers in vacuum and water is around 5.24 kcal/mol.
441 However, the energy barrier between the lowest energy conformers in vacuum and
442 triolein-water is around 13.90 Kcal/mol. This high energy barrier deems more stability
443 of oleuropein in the triolein-water system and leads to a specific adsorption
444 conformation adopted at the interface. RDF analysis suggested that the conformational
445 change of oleuropein significantly affected its hydrophilic and hydrophobic properties.
446 The semi-open conformation of oleuropein in water had total water coordination as high
447 as 42 molecules while the open conformation of oleuropein at the triolein-water
448 interface had a total water coordination 11 molecules. The oleuropein at the
449 triolein-water interface had the adsorption enthalpy of -36.21 to -34.83 kcal/mol. This
450 suggests that the open conformation of oleuropein greatly increases its hydrophobicity,
451 and facilitates its binding at the triolein-water interface. Torsional analysis together with
452 the $d_{\text{glu-hyd}}$ variation during equilibrium revealed an open conformation of oleuropein at
453 the triolein-water interface but did not inform about the orientation of the molecule
454 toward the interface. The interfacial activity of oleuropein is assumed to be due the
455 presence of hydrophilic and hydrophobic moieties. Our previous assumption
456 approached oleuropein to the glucose ester surfactant where the hydrophilic moiety is
457 formed by glucose while the hydrophobic tail is formed by oleuropein aglycone
458 (Souilem et al. 2014). However, RDF analysis showed the presence of hydrogen

459 bonding related to OBK and OBL oxygens, which implies the partitioning of some
460 branches of hydroxytyrosol into the water phase. Based on the above results, we
461 propose that oleuropein adopted a horizontal open conformation parallel to the
462 triolein-water interface following the illustration in Fig. 9, in which the hydrophobic
463 part is formed by elenolic acid whereas the hydrophilic part is formed by the glucose
464 moiety and some branches of hydroxytyrosol. The strong hydrophobic intermolecular
465 interactions explained the lower conformational dynamics of oleuropein at the
466 triolein-water interface as compared to the water system, where the molecule could
467 easily convert between the open and close forms (Fig.7b). The proposed orientation of
468 oleuropein at the triolein-water interface may explain the large areas of occupied
469 oleuropein per interface determined experimentally using the Gibbs theory (Souilem et
470 al. 2014). To gain information about the nature of adsorption of oleuropein at
471 triolein-water interface, we have calculated the enthalpy of its adsorption for the three
472 sets of simulations S1, S2 and S3 mentioned above. The obtained enthalpies values
473 converged to similar ranges of values, -36.21 ± 4.26 , -34.83 ± 3.66 and -34.99 ± 6.47
474 kcal/mol for the configurations in S1, S2 and S3, respectively. $\Delta H < 0$ implies that
475 adsorption occurs spontaneously and it is exothermic, a result in agreement with the
476 higher obtained energies of conformers in triolein-water system (Table 1). Although the
477 enthalpy of adsorption is a fundamental reflection of the interaction between the
478 adsorbate and an interface, it could be determined experimentally based on interfacial
479 tension measurements (Krassimir and Kralchevsky.2012). The interfacial tension of the
480 pure triolein-water system has been measured by Meyers et al. (2012) using a pendent
481 drop tensiometer to be 32.1 mN/m, a value which is close to the interfacial tension of the
482 pure soybean oil-water system (31.5 mN/m) (Goankar, 1989). Therefore, we roughly

483 estimated the free enthalpy of adsorption of oleuropein based on interfacial tension
484 results of oleuropein containing soybean oil-water systems (Fig. 9). Interfacial tension
485 data at different temperatures were plotted in accordance with Eq (1) (Fig. 9a). The
486 internal free energy of oleuropein adsorption (ΔG) was derived from the intercepts of
487 linear regressions of these plots (Fig. 9b). ΔG was around -35.81 and -41.73 KJ/mol for
488 25°C and 45°C, respectively. The obtained experimental ΔG values were plotted against
489 temperature in order to extract ΔH according to the fit with Eq (2) (Fig. 9c). The obtained
490 experimental enthalpy was around -31.86 KJ/mol (-7.61 Kcal/mol). Experimental
491 calculations confirmed that oleuropein adsorption at O/W interface is an enthalpy driven
492 mechanism. The higher enthalpy of adsorption obtained experimentally is expected since
493 many oleuropein molecules are aggregated at the interface and the enthalpy of adsorption
494 is dependent on the adsorbate concentration (Anwar-ul-Haq et al. 2013).
495 Complementary MD simulations of the adsorption of many oleuropein molecules at
496 triolein-water interface would offer a better comparison with the experimental
497 adsorption kinetics of oleuropein. Moreover, we aim to extend our studies to look at the
498 effect of pH, ionic strength and temperature on the interfacial activity of oleuropein, and
499 ultimately hope to lead a better understanding of the factors that control its stability and
500 delivery when incorporated in multiphasic systems

501

502 **4. Conclusions**

503 The present study provides accessible conformational models for oleuropein in different
504 environments (vacuum, water and triolein-water systems). The data indicated that
505 oleuropein undergoes extensive conformational changes depending on the
506 microenvironment. The nature of the molecule dynamics whether it is in open,

507 semi-open or tight form could be easily identified from torsion analyses and from the
508 distance between the glucose and hydroxyphenyl moieties of oleuropein. Water
509 mediated hydrogen bonding was greatly involved in stabilizing the three-dimensional
510 conformation of oleuropein. The absence of hydrogen bonding with water molecule in
511 the elenolic acid moiety of oleuropein might be the key towards its stabilization at the
512 triolein-water interface, mainly due to hydrophobic interactions. This MD study
513 confirmed our previous experiment results regarding the surface activity of oleuropein
514 at the triglyceride-water interface (Souilem et al. 2014) and provides us with a full
515 understanding of the amphiphilic character of oleuropein at the molecular level. The
516 surface activity of oleuropein might influence the preparation, stability and delivery of
517 food emulsions.

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522

523 **Figures and tables captions**

524 **Fig. 1.** Chemical structure of (a) oleuropein and (b) triolein.

525 **Fig.2. (a)** Atom numbering of oleuropein and definition of the seven torsional angles.(b)
526 Sets of simulations of oleuropein in triolein-water system.

527 **Fig.3.** Density profiles of the different systems. (a) water; (b) oleuropein-water-triolein
528 system.

529 **Fig.4.** Dihedral angles distributions in vacuum (left side), water (center) and
530 triolein-water (right side) systems.

531 **Fig.5.** Distributions of distance between hydroxytyrosol and glucose moieties ($d_{\text{glu-hyd}}$)
532 throughout the trajectories during equilibrium: (a) vacuum; (b) water; (c) triolein-water
533 system.

534 **Fig.6.** Highest probable conformers of oleuropein in (a) vacuum, (b) water and (c)
535 triolein-water systems. (d) Alignment of oleuropein in vacuum (left side), water (center), and
536 triolein-water (right side) system

537 **Fig.7.** Oleuropein-water radial distribution functions:(a) water ; (b) triolein-water

538 **Fig.8.** Partitioning of oleuropein at the triolein-water interface

539 **Fig.9.** Interfacial tension data and related adsorption enthalpies of oleuropein at soybean
540 oil-water interface.

541 **Table 1.**Dihedral angles and energy information for the highest probable conformations
542 of oleuropein in vacuum, water and triolein-water systems.

543 **Table 2.**Radial Distribution Function (RDF) analyses results.(a) water system.(b)
544 triolein-water system.

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643

644

Fig 1.

(a)

(b)

Fig 2.

(a)

(b)

Fig 3.

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Fig 4.

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Fig 5.

(a)

(b)

(c)

(d) Alignment of oleuropein in vacuum (left side), water (center), and triolein-water (right side) system

Fig6.

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Table 1. Dihedral angles and energy information of the highest probable conformations of oleuropein in vacuum, water and triolein-water systems

Torsions in vacuum system								
Conformer	Energy-difference (kcal/mol)	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇
S _{vac} ¹	0	-83.33	-170.08	-74.83	-179.85	85.61	-60.78	-67.77
S _{vac} ²	6.48	-69.01	162.38	-60.66	-173.69	97.89	-60.70	-75.44
S _{vac} ³	9.73	-83.45	-173.96	-86.80	179.41	76.96	-57.70	-98.32
S _{vac} ⁴	5.95	-89.29	175.23	-89.24	177.89	85.34	-50.49	-101.83
Torsions in water system								
Conformer	Energy	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇
S _{wat} ¹	1.92	-99.16	-72.81	152.04	-79.03	64.74	57.75	-64.69
S _{wat} ²	0.02	-86.88	73.17	155.35	-89.63	59.19	84.18	-69.19
S _{wat} ³	6.13	-89.02	-67.98	-148.36	-81.94	72.02	64.77	-81.47
S _{wat} ⁴	0	-95.11	71.28	-146.72	-116.80	93.95	79.03	-96.61
Torsions in triolein-water system								
Conformer	Energy	T ₁	T ₂	T ₃	T ₄	T ₅	T ₆	T ₇
S _{tri-wat} ¹	19.83	-98.44	-177.94	159.70	-175.13	81.61	101.80	-77.89
S _{tri-wat} ²	14.39	-82.90	-155.65	-170.59	-174.99	107.64	154.81	-90.02
S _{tri-wat} ³	21.37	93.88	-148.72	-149.98	151.00	84.64	146.54	-74.49
S _{tri-wat} ⁴	18.99	-81.45	174.15	-159.95	166.91	81.74	84.05	-95.73
S _{tri-wat} ⁵	18.99	93.38	169.62	-171.04	159.61	75.92	138.24	-74.41
S _{tri-wat} ⁶	17.26	97.56	163.46	170.30	-179.87	71.55	80.82	-116.54
S _{tri-wat} ⁷	02.27	-92.41	164.83	-172.70	-167.37	75.23	74.54	-84.58
S _{tri-wat} ⁸	06.07	-84.98	-179.34	-175.61	164.82	74.61	74.33	-94.41
S _{tri-wat} ⁹	0	-86.31	164.99	-169.93	166.32	100.99	162.19	-125.13
S _{tri-wat} ¹⁰	04.12	-83.57	170.95	178.64	177.44	94.82	162.84	-125.10

Table 2: RDF results in water and in triolein-water systems

Moiety	Oxygen label	Water		Triolein/water	
		r (Å)	ⁿ (Owater)	r (Å)	ⁿ (Owater)
Glucose	O2	3.04	2.11	3.00	0
	O3	2.96	3.90	2.98	0.83
	O4	3.10	2.93	3.16	1.88
	O5	3.02	0.88	**	**
	O6	3.20	9.97	3.04	2.52
Elenolic acid	OAR	3.46	5.55	*	*
	OAV	2.78	2.11	2.82	0.83
	OAW	**	**	**	**
	OBE	2.80	2.44	2.84	0.97
	OBA	**	**	**	**
Hydroxytyrosol	OBK	3.04	5.61	2.94	2.17
	OBL	2.96	6.12	2.92	2.04

*: no shell **: r> cut-off distance

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Fig. 7

Fig. 8

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Fig. 9