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1 **Encapsulation of flavonoid in multiple emulsion using spinning disc reactor technology**

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6 **Abstract**

7 Rutin (quercetin-3-rutinoside) and anthocyanin flavonoids have numerous biological
8 activities which are beneficial to human health such as antioxidant and anti-inflammatory
9 effects. In order to aid delivery of their health benefits, an attempt has been made to
10 encapsulate rutin and Hibiscus anthocyanins in multiple emulsions using a spinning disc
11 reactor (SDR) as a novel processing aid. The encapsulation of flavonoids may prolong their
12 shelf-life and increase their bioavailability for absorption by the body (Munin & Edwards-
13 Lévy, 2011).

14 The advantage of using SDR technology in the second stage of emulsification is that it does
15 not break the droplets of the primary emulsion. The time-dependent stability of the multiple
16 emulsions was investigated using particle size, microscopy, visual assessment and stability
17 index measurements. At 2 wt. % emulsifier, Brij 78 was found to be capable of producing
18 uniform droplets of the final W/O/W emulsion in the size range of 13-15 μm . The results
19 show that the SDR technology can be used as an alternative process for making stable
20 W/O/W multiple emulsions with a fairly narrow droplet size distribution.

21 Rutin and anthocyanins were successfully encapsulated within the internal aqueous phase of
22 W/O/W multiple emulsions, giving an encapsulation efficiency of >80%. In the presence of
23 flavonoids, a reduction in the average particle size has also been observed, possibly due to its
24 surface active properties. Confocal laser microscopy confirmed the successful formation of
25 SDR-processed multiple emulsions.

26 *Keywords:* Spinning disc reactor; W/O/W emulsion; Rutin; Anthocyanins; Encapsulation;
27 Antioxidant

28 **1. Introduction**

29 Multiple emulsions have a number of potential benefits over the conventional oil-in-water
30 (O/W) emulsions for certain applications such as reducing fat content (Gaonkar, 1994;
31 Lobato-Calleros, Rodriguez, Sandoval-Castilla, Vernon-Carter & Alvarez-Ramirez, 2006) or
32 encapsulation of the functional food components (Benichou, Aserin & Garti, 2004) and active
33 molecules (Kanouni, Rosano & Naouli, 2002; Laugel, Chaminade, Baillet, Seiller & Ferrier,
34 1996; Tokimitsu, Kobayashi, Uzu & Arisawa, 1990) in the inner aqueous phase. Thus,
35 multiple emulsions have potential as micro carriers of hydrophilic or lipophilic ingredients
36 entrapped in their internal droplets which are subsequently released. Encapsulation within the
37 inner emulsion can allow the masking of odour or taste and protection against oxidation by
38 light or enzymatic degradation, to prolong shelf-life. Controlled release of the active
39 ingredients can be produced by dilution, shear, or other agitation (Kanouni, Rosano &
40 Naouli, 2002; Muschiolik, 2007).

41 Generally, multiple emulsions are prepared by a two stage emulsification process: firstly, a
42 simple W/O emulsion is made using a low HLB (hydrophilic-lipophilic balance) emulsifier
43 under intense homogenization conditions. In the second stage, the primary water-in-oil (W/O)
44 emulsion is dispersed in an aqueous phase containing high HLB emulsifier under lower shear
45 conditions, preventing rupture of the internal droplets as far as possible, to produce a W/O/W
46 multiple emulsion (Pal, 2008).

47 The loss of the internal phase due to the excessive shear stress during the production of the
48 secondary emulsion is a major problem and much research has been carried out to try to
49 overcome this difficulty (Liu, Ma, Meng & Su, 2005). The release rate of the internal droplets

50 is directly proportional to the applied shear stress and only moderate shear can be applied in
51 order to produce multiple emulsions that retain a significantly high percentage of the internal
52 phase (van der Graaf, Schroen & Boom, 2005).

53 Hence it is desirable to use low shear device to prevent expulsion of the internal droplets to
54 the external continuous phase in order to produce highly stable multiple emulsions (Pal,
55 2008). However, low-shear conditions cannot be used with most conventional emulsification
56 equipment without yielding droplets that are unacceptably large or have an unacceptably
57 wide droplet distribution, which eventually leads to unstable products (van der Graaf,
58 Schroen & Boom, 2005).

59 In the recent years, there has been growing interest in the role of flavonoids in maintaining
60 human health. Flavonoids have become a regular part of the human diet (Havsteen, 1983;
61 Pierpoint, 1986) and are of importance as antiscorbutic (anti-scurvy) agents added to food
62 (Roger, 1988).

63 Rutin (quercetin-3-rutinoside) is one of the primary flavonoids in a number of plants (Kim,
64 Lee, Kim, Park, Kwon & Lee, 2005) such as buckwheat. It has numerous biological activities
65 which are beneficial to human health such as antioxidant effect (Gao, Xu, & Chen, 2003;
66 Kozlov, Ostrachovitch & Afanas, 1994), protective effect against hepatotoxicity (Janbaz,
67 Saeed & Gilani, 2002), and anti-inflammatory effect (Cruz, Galvez, Ocete, Crespo, Sanchez
68 de Medina & Zarzuelo, 1998; Guardia, Rotelli, Juarez & Pelzer, 2001). Reynolds (1996)
69 suggested that rutin can be used to improve capillary function by reducing abnormal leakage
70 and it has been administered to reduce capillary impairment and venous insufficiency of the
71 lower limb. However, the solubility of rutin and many other flavonoids in water (or oil) is
72 low (Luo, Murray, Yusoff, Morgan, Povey & Day, 2011; Luo, Murray, Ross, Yusoff,
73 Morgan, Povey & Day, 2012).

74 The only group of flavonoids that has reasonable solubility in water is the anthocyanins.
75 Anthocyanins have a high potential for use as natural colorants due to their attractive orange,
76 red, purple, and blue colours. However, they can be quite unstable chemically (Fennema,
77 2008) depending on the flavonoid concentration, pH, temperature, light intensity, the
78 presence of metallic ions, enzymes, oxygen, ascorbic acid, sugars and their degradation
79 products and sulphur dioxide, among others (Cevallos, Bolyvar & Cisneros-Zevallos, 2004).
80 The colour stability is generally more stable at low pH, e.g., pH 2 (Selim, Khalil, Abdel-Bary
81 & Azein, 2004).

82 Anthocyanins are also good natural antioxidants which may provide an array of health
83 promoting benefits (Tsuda, Kato & Osawa, 2000). Almajano, et al., (2008) reported that W/O
84 emulsions containing tea extracts have shown strong antioxidant activity against oil
85 oxidation. However, anthocyanins have received less attention than other flavonoids; despite
86 their widespread occurrence, possibly due to their instability. Multiple emulsions are a way of
87 possibly protecting anthocyanins in foods.

88 Extracts of *Hibiscus sabdariffa* are known to contain a significantly high amount of
89 anthocyanins and have been reported to decrease blood pressure (Haji Faraji & Haji
90 Tarkhani, 1999; Onyenekwe, Ajani, Ameli & Garnamel, 1999) and have anti-tumor, immune-
91 modulating and anti-leukemic effects (Muller & Franz, 1992; Tseng, Kao, Chu, Chou, Lin &
92 Wang, 2000). Wang et al., (2000) have reported protective effects against oxidative stress in
93 rats.

94 In previous work (Akhtar & Dickinson, 2000) water-in-oil-in-water multiple emulsions
95 were prepared via a two stage emulsification process using a jet homogeniser alone. The
96 jet homogenisation produced multiple emulsions with a wide range of droplet sizes (0.5 –
97 16 µm), a highly poly dispersed system which had lower encapsulation efficiency (40 –
98 60%) due to high shear mixing. The aims of this study were to test the advantages of

99 combining SDR technology with a jet homogenizer for producing multiple emulsions for
100 effective encapsulation and protection of some of these flavonoids. The jet homogenizer
101 is capable of reproducibly fine aqueous (or oil) droplets of a narrow size distribution,
102 whilst the SDR can provide very controllable and low shear conditions for producing the
103 secondary emulsion. The SDR equipment used for processing multiple emulsions is
104 shown elsewhere (Akhtar, Blakemore, Clayton & Knapper, 2009). The SDR is essentially
105 a 20 cm diameter rotating disc heated up to 250 °C with a speed range of 200–3000 rpm.
106 In the SDR, the emulsion phases are fed into the center of the disc and the centrifugal
107 force drives the emulsion phases towards the edge of the disc as a thin film. When the
108 film breaks at the edge of the disc, it creates uniform emulsion droplets with a narrow
109 droplet size distribution. The multiple emulsions formed were characterized and tested
110 for their stability via particle size analysis, creaming, confocal microscopy and
111 spectrophotometry.

112 **2. Materials and methods**

113 *2.1. Materials*

114 The low HLB lipophilic polymeric emulsifiers Arlacel P135 (polyethylene-30
115 dipolyhydroxystearate), HLB = 4 – 5, and Cithrol PG3PR (polyglycerol-3
116 polyincinoleate), HLB = 2 – 2, were purchased from ICI (Middlesbrough, England) and
117 Croda (Hull, England), respectively. The high HLB hydrophilic emulsifiers, Brij 78
118 (polyoxyethylene (20) stearyl ether), HLB = 15.3, and Synperonic PE/F127, HLB = 16,
119 were purchased from Croda Ltd (Hull, England). A pH 7 buffer was prepared from
120 sodium dihydrogen orthophosphate dihydrate and di-sodium hydrogen orthophosphate,
121 purchased from Fisher Chemicals (UK). Potassium chloride (>99%, reagentplus) was
122 purchased from Sigma Aldrich and hydrochloric acid (37%, general purpose grade) was

123 obtained from Riedel-de Haen, Germany.
124 Rutin trihydrate (Quercetin-3-rutinoside) (95%) was purchased from Sigma Aldrich (St
125 Louis, MO, USA). Sunflower oil (refractive index 1.463) was purchased from a local
126 supermarket (Morrison's, Leeds). *Hibiscus sabdariffa* (Rosella) plants were purchased
127 from a local market in Nigeria and their species verified by the Agricultural Development
128 Programme (ADP), Benin City, Nigeria. All solutions were prepared using double
129 distilled water.

130

131 *2.2 Preparation of rosella extract*

132 Rosella extract was made by boiling 40 g of freshly ground dried calyx in 1560 g of water for
133 15 minutes. The solution was filtered through a 0.5 µm filter paper Whatman grade 1, then
134 concentrated in a rotary evaporator (under vacuum) at 40 °C for 2 hours. The concentrated
135 extract was stored in a volumetric flask covered with aluminium foil and stored at 4 °C. The
136 UV absorbance spectrum of Rosella was obtained by measuring the absorbance in the
137 wavelength range of 250-550 nm using a spectrophotometer (CECIL CE3021, Tabot
138 Scientific Ltd UK).

139 Figure 1(a) shows a full spectrum of Rosella with maximum absorbance of 518.6 nm.
140 Dilutions of the extract with pH 2 buffer were made in order to obtain a calibration curve, as
141 shown in Figure 1(b), so that the concentration of Rosella anthocyanins in the emulsions
142 could be determined by measuring the absorbance of the serum layer. Absorbance
143 measurements at 519 nm at each concentration were taken in triplicate.

144 *2.3 Preparation of primary W/O emulsions*

145 For encapsulating rutin, the aqueous phase was a pH 7 buffer prepared by combining 195 mL
146 of 0.2 M NaH₂PO₄ with 305 mL of 0.2 M Na₂HPO₄. The oil phase was prepared by

147 dissolving 4 wt% Arlacel P135 into sunflower oil with gentle stirring and heating at 50°C.
148 The water-in-oil emulsions (20 vol% water) were prepared at ambient temperature using a
149 laboratory-scale jet homogenizer (Burgaud, Dickinson & Nelson, 1990) working at the
150 operational pressure of 300 bar For encapsulating the Rosella anthocyanin extract, a mixture
151 of 50 mL of 0.2 M KCL plus 13 mL of 0.2M HCl was used to make the aqueous phase of pH
152 2. Cithrol PG3PR emulsifier 1.6 – 4.5 wt% was dissolved in sunflower. The primary W/O
153 emulsion (20 vol % aqueous phase) was prepared as above for the rutin system.

154

155 *2.4 Preparation of W/O/W multiple emulsions*

156 The primary W/O emulsions (20 vol% oil) were dispersed into a secondary water phase
157 (80 vol% pH 2 buffer) containing 1 wt% of Synperonic PE/F127 or Brij 78. The mixture
158 was gently stirred for 5 minutes and then passed over the SDR disc rotating at 2000 rpm
159 at ambient temperature at a flow rate of 7 ml s⁻¹ to produce W/O/W emulsion.

160 The SDR has an excellent heating and cooling facility, in the range of +200 to -20 °C, by
161 using heat transfer fluids in a water bath. The spinning disc has a speed range of 200 to
162 3000 rpm with a flow rate in the range of 0.5 to 8 ml s⁻¹. The main vessel has been
163 designed to mechanically withstand pressures of up to 15 bar. Two standard gear pumps
164 (Micropump Inc., Vancouver, WA, USA) have been incorporated into the main controller
165 unit. Which is used depends on the viscosity of the material being spread onto the
166 spinning disc.

167

168 *2.5 Particle size measurement*

169 Primary W/O emulsion droplet size distributions were measured using a Zetasizer Nano-ZS
170 (Malvern Instruments, Malvern, UK), whilst the droplet size distributions of the W/O/W
171 multiple emulsions were measured using a Mastersizer Hydro 2000 (Malvern Instruments,

172 Malvern, UK). The refractive indices of water and sunflower oil were set at 1.330 and 1.463,
173 respectively, with the optical absorption parameter was set at 0.001. The mean droplet size
174 was characterised by surface weighted mean diameter (d_{32}) and volume weighted mean
175 diameter (d_{43}) defined by:

$$176 \quad d_{32} = \frac{\sum_i d_i^3}{\sum_i n_i d_i^2}, \quad d_{43} = \frac{\sum_i n_i d_i^4}{\sum_i n_i d_i^3}$$

177 where n_i is the number of droplets of diameter d_i .

178 *2.4 Confocal laser scanning microscopy*

179 The microstructure of the W/O/W emulsions was observed using a confocal scanning
180 laser microscope (CLSM). The observations were made at ambient temperature and
181 immediately after the preparation of the emulsions. Nile Red (25 μ l of 0.01% w/v dye in
182 polyethylene glycol per 2.5 g of emulsion sample) was used to highlight the oil phase,
183 using an excitation wavelength of 488 nm and collecting wavelengths 523–650 nm.

184

185 *2.5 Visual assessment of emulsion stability*

186 The instability of emulsions due to creaming was determined visually by measuring the
187 serum layer separation at room temperature over the storage period. W/O/W emulsion were
188 poured into glass tubes (100 mm height, 13 mm diameter) and sealed to prevent evaporation
189 and stored at room temperature for a period of 21 days. The creaming stability was assessed
190 visually by measuring the thickness of the cream layer and was calculated as follows:

$$\% \text{ serum separation} = \frac{\text{height of cream layer} \times 100}{\text{total height of emulsion}}$$

191

192 *2.6 Encapsulation efficiency*

193 Multiple emulsions were poured into centrifuge tubes (diameter 20 mm, 100 mm length; 16
194 ml) and centrifuged (Beckman Coulter; AllergaTM X-22 Centrifuge) at 12500 rpm for 30

195 min. Samples of the lower aqueous phase (serum layer) were carefully removed via a syringe
196 and their absorbance at 519 nm measured using the spectrophotometer. Absorbance of each
197 sample was measured in triplicate and the concentration of flavonoid was determined from
198 the calibration standard curve presented in Figure 1(b).

199

200 **3 Results and discussion**

201 *3.1 Particle-size distribution of emulsions with and without flavonoids*

202 The particle-size distributions of the primary 20 vol% W/O emulsions stabilised by 1.6 wt%
203 polymeric emulsifier with and without rutin are shown in Figure 2. Both the primary
204 emulsions showed very similar monomodal distributions with z-average of 128 nm and
205 polydispersity index of 0.034. Thus, including 90 μ M rutin in the aqueous phase did not
206 change the water droplet size significantly. The particle-size distributions of the W/O/W
207 multiple emulsion with and without rutin are compared in Figure 3. The distributions are
208 almost identical, with a slightly higher proportion of smaller droplets when rutin is present.
209 Luo et al., (2012) recently showed that rutin is weakly surface active, so that some leakage of
210 rutin from the primary emulsion and its acting as an emulsifier of the W/O/W emulsions may
211 explain this. Di Mattia *et al.* 2010) have also shown that the flavonoids catechin and
212 quercetin are capable of decreasing the interfacial tension at the oil-water interface, although
213 the rutinose sugar moiety of rutin will tend to make it more water-soluble, i.e., less surface
214 active.

215 Figure 4 shows d_{32} and d_{43} of the rutin-encapsulated multiple emulsions as a function of
216 storage time at room temperature. A very slight increase in the initial average droplet size
217 was observed over the storage period. Increases in droplet size may be due to the osmotic
218 gradient that causes water to flow from the outer aqueous phase to the inner aqueous phase,

219 swelling the oil globules until they reach a critical size (Geiger, Tokgoz, Fructus, Jager-
220 Lezer, Seiller, Lacomme & Grossiord, 1998). Di Mattia et al. (2010) also observed similar
221 effects; the droplet size of emulsions with phenolic antioxidants (catechin, gallic acid and
222 quercetin) also showed an increase in the droplet size with time. Overall, however, the
223 multiple emulsions produced via the SDR are far more stable than those produced elsewhere
224 via other techniques.

225 The droplet size distributions of the freshly made Rosella encapsulated multiple are
226 shown in Figure 5. The average droplet size (d_{43}) of the emulsions as function of time is
227 shown in Figure 6. It is seen that as the concentration of lipophilic emulsifier was
228 increased from 1.6 to 4.5 % the initial particle size distribution of the W/O/W droplets
229 shifted from approximately 21 – 26 μm to 11 – 13 μm . Presumably this is because
230 smaller W/O droplets can be accommodated more easily with smaller W/O/W droplets.
231 Rowe (2006) and Kanafusa et al., (2007) reported similar effects. The small error bars (\leq
232 ± 0.1) on Figure 6 should also be noted, indicating that the droplet sizes were quite
233 reproducible. There was a significant increase in d_{43} for the emulsions stabilized by 1.6 or
234 3.0 wt% primary emulsifier (Cithrol), whereas there was very little change for the system
235 with 4.5 wt.% primary emulsifier. Emulsion stabilised with 4.5 wt% was relatively stable
236 over the storage period 15 days.

237 *3.2. Visual assessment of emulsion stability*

238 The creaming profiles of rosella-encapsulated multiple emulsions with varying concentration
239 of PG3PR lipophilic emulsifier are presented in Figure 7. In terms of creaming stability under
240 gravity, there was very extensive serum separation in the emulsion made with 1.6 wt%
241 PG3PR, whereas the emulsion sample stabilized by 4.5 wt% emulsifier exhibited relatively
242 modest serum separation over the same period of 30 days. The stability of multiple emulsions

243 can be affected by the percentage of lipophilic emulsifier used in primary W/O emulsion.
244 Creaming volume is an indicator for the stability of the internal aqueous droplets which are
245 trapped in the multiple droplets (Jiao & Burgess, 2003).

246 As explained earlier, there is diffusion of water through the oil phase and this could lead to
247 changes in the volume fraction of the primary emulsion in the multiple emulsion system. This
248 change in the volume fraction of the primary emulsion alters the rheological properties of
249 multiple emulsions (Jiao & Burgess, 2003).

250 *3.3 Confocal laser scanning microscopy*

251 A typical micrograph of a sample of the multiple emulsions containing Rosella extract is
252 shown in Figure 8. The concentration of lipophilic surfactant was 1.6 wt%. Oil regions
253 appear bright and aqueous regions dark. The image clearly shows a fine dispersion of
254 internal aqueous phase droplets inside large oil droplets, which in turn are dispersed in the
255 outer aqueous phase, confirming the formation of multiple emulsions. The oil droplets are in
256 the size range 8 to 16 μm diameter, which agrees fairly well with the Mastersizer results (see
257 Figure 6).

258

259 *3.4 Encapsulation efficiency*

260 Absorbance measurements on the inner aqueous phase of the multiple emulsions after 10
261 days, separated by centrifugation, showed that the concentrations of rutin and Rosella extracts
262 were 80 ± 2 and $72 \pm 4\%$ of their original values, respectively. The loss of some flavonoid
263 from the aqueous phase of the primary emulsion may occur during the second emulsification
264 step to produce the W/O/W multiple emulsion. However these losses using the SDR are
265 relatively small compared to other homogenization methods and therefore it appeared also
266 that there were little losses due to other chemical or physical degradation mechanisms.

267

268 4. Conclusions

269 The SDR technology is capable of producing moderately mono-disperse and stable multiple
270 emulsions as a result of the relatively gentle continuous emulsification processing that can be
271 applied. Using this technology, it has been shown that rutin and Rosella extract flavonoids
272 can be successfully encapsulated within multiple emulsions with a high degree of retention
273 and protection. Thus, using these methods, other flavonoids or nutrients could be
274 encapsulated in order to enhance their bioavailability.

275

276 References

- 277 Akhtar, M., Blakemore, I., Clayton, G., & Knapper, S. (2009). The use of spinning disc
278 reactor for processing ice cream base – effect of ageing in making model ice cream.
279 *International Journal of Food Science and Technology*, 44, 1139-1145.
- 280 Akhtar, M., & Dickinson, E. (2000). Water-in-oil-in-water multiple emulsions stabilized by
281 polymeric and natural emulsifiers. In E. Dickinson & R. Miller, *Food Colloids Fundamentals
282 of Formulation*. Royal Society of Chemistry, Cambridge, UK, pp 133-143.
- 283 Almajano, M.P., Carbo, R., Jimenez, J.A., & Gordon, M.H. (2008). Antioxidant and
284 antimicrobial activities of tea infusions. *Food Chemistry* **108**, 55–63.
- 285 Benichou, A., Aserin, A., & Garti, N. (2004). Double emulsions stabilized with hybrids of
286 natural polymers for entrapment and slow release of active matters. *Advances in Colloids and
287 Interfaces Science*, **108**, 29-41.

288 Bonnet, M., Cansell, M., Berkaoui, A., Ropers, M.H., Anton, M., & Leal-Calderon, F.
289 (2009). Release rate profiles of magnesium from multiple W/O/W emulsions. *Food*
290 *Hydrocolloids*, **23**, 92-101.

291 Burgaud, I., Dickinson, E., & Nelson, P.V. (1990). An improved high pressure homogenizer
292 for making fine emulsions on a small scale. *International Journal of Food Science and*
293 *Technology*, **25**, 39-46.

294 Cevallos, C., Bolyvar, A., & Cisneros-Zevallos. (2004). Stability of anthocyanin-based
295 aqueous extracts of Andean purple corn and red-fleshed sweet potato compared to synthetic
296 and natural colorants. *Food Chemistry*, **86**, 69-67.

297 Di Mattia, C.D., Sacchetti, G., Mastrocola, D., Sarker, D.K., & Pittia, P. (2010). Surface
298 properties of phenolic compounds and their influence on the dispersion degree and oxidative
299 stability of olive oil O/W emulsions. *Food Hydrocolloids*, **24**, 652-658.

300 Fennema, R.O. (2008). *Food Chemistry (3rd edition)*. Boca Raton, London.681

301 Gao, Z., Xu, H., & Chen, H. (2003). Antioxidant status and mineral contents in tissues of
302 rutin and baicalin fed rats. *Life Science*, **73**, 1599-1607.

303 Gaonkar, A.G. (1994). Stable multiple emulsions comprising interfacial gelatinous layer,
304 flavour- encapsulating multiple emulsions and low-no fat products comprising the same. *USA*
305 *Patent number 5332595*.

306 Geiger, S., Tokgoz, S., Fructus, A., Jager-Lezer, N., Seiller, M., Lacombe, C., & Grossiord,
307 J.L. (1998). Kinetics of swelling-breakdown of a W/O/W multiple emulsion: possible
308 mechanisms for the lipophilic surfactant effect. *Journal of Controlled Release*, **52**, 99-107.

309 Guardia, T., Rotelli, A.E., Juarez, A.O., & Pelzer, L.E. (2001). Anti-inflammatory properties
310 of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat.
311 *Farmaco*, **56**, 683-687.

312 Haji F.M., & Haji, T.A.S. (1999). The effect of sour tea (*Hibiscus sabdariffa*) on essential
313 hypertension. *Journal of Ethnopharmacology* **65**, 231–236.

314 Havsteen, B. (1983). Flavonoids, a class of natural products of high pharmacological
315 potency. *Biochem Pharmacol*, **32**, 1141-1148.

316 Janbaz, K.H., Saeed, S.A., & Gilani, A.H. (2002). Protective effect of rutin on paracetamol-
317 and CCl₄-induced hepatotoxicity in rodents. *Fitoterapia*, **73**, 557-563.

318 Jiao, J., & Burgess, D.J. (2003). Rheology and stability of water-in-oil-in-water multiple
319 emulsions containing Span 83 and Tween 80. *AAPS Pharm Sci*, **5(1)**, Article 7.

320 Kanafusa, S., Boon-Seang, & Naajim, A.K. (2007). Factors affecting droplet size of sodium
321 caseinate-stabilized O/W emulsions containing β -carotene. *European Journal of Lipid*
322 *Science and Technology*, 1038-1041.

323 Kanouni, M., Rosano, H.L., & Naouli, N. (2002). Preparation of a stable double emulsion
324 (W₁/O/W₂): role of the interfacial films on the stability of the system. *Advances in Colloid*
325 *and Interface Science*, **99**, 229-254.

326 Kim, K.H., Lee, K.W., Kim, D.Y., Park, H.H., Kwon, I.B., & Lee, H.J. (2005). Optimal
327 recovery of high-purity rutin crystals from the whole plant of *Fagopyrum esculentum*
328 Moench (buckwheat) by extraction, fractionation, and recrystallization. *Bioresour*
329 *Technology*, **96**, 1709-1712.

330 Kozlov, A.B., Ostrachovitch, E.A., & Afanasev, I.B. (1994). Mechanism of inhibitory effects
331 of chelating drugs on lipid peroxidation in rat brain homogenates. *Biochem Pharmacol*, **47**,
332 795-799.

333 Laugel, C., Chaminade, P., Baillet, A., Seiller, M., & Ferrier, D. (1996). Moisturizing
334 substances entrapped in W/O/W emulsions: analytical methodology for formulation, stability
335 and release studies. *Journal of Controlled Release*, **38**, 59-67.

336 Liu, R., Ma, G., Meng, F. & Su, Z. (2005). Preparation of uniform-sized PLA microcapsules
337 by combining Shirasu Porous Glass membrane emulsification technique and multiple
338 emulsion-solvent evaporation method. *Journal of Controlled Release*, **103**, 31-43.

339 Lobato-Calleros, C., Rodriguez, E., Sandoval-Castilla, O., Vernon-Carter, E.J., & Alvarez-
340 Ramirez, J. (2006). Reduced-fat white fresh cheese-like products obtained from W₁/O/W₂
341 multiple emulsions: Viscoelastic and high-resolution image analyses. *Food Research*
342 *International*, **39**, 678-685.

343 Muller, M.H., & Franz, G. (1992) Chemical structure and biological activity of
344 polysaccharides from Hibiscus sabdariffa, *Planta Medical*. **58**, 60-67.

345 Munin, A., & Edwards-Lévy, F. (2011). Encapsulation of natural polyphenolic compounds; a
346 review. *Pharmaceutics* **3**, 793-829.

347 Muschiolik, G. (2007). Multiple emulsions for food use. *Current Opinion in Colloid and*
348 *Interface Science*, **12**, 213-220.

349 Onyenkwe, P.C. Ajani, E.O., Ameli, D.A., & Garnamel, K.S. (1999). Antihypertensive effect
350 of Roselle (Hibiscus sabdariffa) calyx infusion in spontaneously hypertensive rats and a
351 comparison of its toxicity with that in Wister rats. *Cell Biochemistry and Function*, **17**, 199-
352 205.

353 Pal, R. (2008). Viscosity models for multiple emulsions. *Food Hydrocolloids*, **22**, 428-438.

354 Pierpoint, W.S. (1986). Flavonoids in the human diet. *Progress in Clinical and Biological*
355 *Research*, **213**, 125-140.

356 Reynolds, J.E.F. (1996) *Martindale, The Extra Pharmacopoeia*, 31st ed., London, The Royal
357 Pharmaceutical Society, Council of the Royal Pharmaceutical Society of Great Britain,
358 pp1679-1680.

359 Roger, C.R. (1988). The nutritional incidence of flavonoids: Some physiological and
360 metabolic considerations. *Experientia*, **44**, 725-733.

361 Rowe, E.L. (2006). Effect of emulsifier concentration and type on the particle size
362 distribution of emulsions. *Journal of Pharmaceutical Sciences*, **54**, 260-264.

363 Selim, K.A., Khalil, K.E., Abdel-Bary, M.S., & Azeim, A. (2004). Extraction, Encapsulation
364 and Utilization of Red Pigments from Roselle (*Hibiscus sabdariffa* L.) as Natural Food
365 Colourants. *Food Science and Technology. Faculty of Agriculture, Fayoum University.,*
366 *Fayoum, Egypt.*

367 Tokimitsu, I., Kobayashi, K., Uzu, A., & Arisawa, M. (1990). Cosmetic composition of
368 double emulsion type. *European Patent* EP0391124.

369 Tseng, T.H., Kao, T.W., Chu, C.Y., Chou, F.P., Lin, W.L., & Wang, C.J. (2000). Induction of
370 apoptosis hibiscus protocatechuic acid in human leukaemia cells via reduction of
371 retinoblastoma (RB) phosphorylation and Bcl-2 expression. *Biochemical Pharmacology*. **60**,
372 307–315.

373 Tsuda, T., Kato, Y., & Osawa, T. (2000). Mechanism for the peroxynitrite scavenging
374 activity by anthocyanins. *FEBS letters*, **484**, 207-210.

- 375 Van der Graaf, S., Schroen, C.G.P.H., & Boom, R.M. (2005). Preparation of double
376 emulsions by membrane emulsification-a review. *Journal of Membrane Science*, **251**, 7-15.
- 377 Wang, C.J., Wang, J.M., Lin, W.L., Chu, C.Y., Chou, F.P., & Tseng, T.H. (2000). Protective
378 effect of Hibiscus anthocyanins against tertbutyl hydroperoxide induced hepatic toxicity in
379 rats. *Food and Chemical Toxicology*, **38**, 5, 411 – 416.
- 380 Luo, Z., Murray, B.S., Ross, A.L., Yusoff, A., Morgan, M.R.A., Povey, M.J.W. & Day, A.J.
381 (2012). Effects of pH on the ability of flavonoids to act as Pickering emulsion stabilizers.
382 *Colloids Surfaces B*. **92**, 84-90.
- 383 Luo, Z., Murray, B.S., Yusoff, A., Morgan, M.R.A., Povey, M.J.W. & Day, A.J. (2011).
384 Particle-stabilizing effects of flavonoids at the oil water interface. *Journal Agriculture Food*
385 *Chemistry.*, **59**, 2636-2645.

386 **Figure Legends**

387 **Figure 1.** (a) The UV-visible spectrum of Rosella extract in buffer pH 2 and scanned at speed
388 of 40 nm/min; (b) a standard calibration curve of the Rosella extract, absorbance measured at
389 wavelength of 519 nm.

390 **Figure 2.** The droplet-size distributions of freshly made 20 vol% primary W/O emulsions
391 stabilized by 1.6 wt% polymeric emulsifier (arlacel) with and without rutin encapsulated at
392 room temperature.

393 **Figure 3.** The droplet-size distributions of W/O/W emulsions stabilized by 1 wt% Brij 78 (20
394 vol% primary emulsion) with and without rutin encapsulated at room temperature.

395 **Figure 4.** The effect of storage time on the average droplet-size distributions of W/O/W
396 emulsion with rutin encapsulated. (20 vol% primary emulsion (1.6 wt% polymeric
397 emulsifier) dispersed in the secondary aqueous phase (1wt% Brij 78). The error bars are
398 based on standard deviations for sets of at least three measurements.

399 **Figure 5.** The droplet-size distribution of W/O/W emulsion, 20 vol% primary emulsions
400 with varying concentration of Cithrol dispersed in the outer aqueous buffer containing 1wt%
401 Synperonic with different wt. % of lipophilic surfactant used in primary W/O emulsion.

402 **Figure 6.** The effect of storage time on the average droplet-size distributions of W/O/W
403 emulsion with rutin encapsulated. (20 vol% primary emulsion (1.6 wt% polymeric
404 emulsifier) dispersed in the secondary aqueous phase (1wt% Brij 78). The error bars are
405 based on standard deviations for sets of at least three measurements.

406

407 **Figure 7.** The creaming profile for W/O/W emulsions (20 vol% Cithrol-stabilised primary
408 emulsion; 1 wt% Synperonic) with primary emulsions stabilized by different wt% of

409 lipophilic emulsifier. The values of % creaming used are based on the average of sets of three
410 measurements. The error bars show standard deviation of 3 sets of measurement.

411 **Figure 8.** The confocal laser scanning image of W/O/W emulsion (20 vol% primary
412 emulsion with 0.1% Rosella extract stabilized by 1.6 wt% Cithrol) stabilized by 1wt%
413 Synperonic at pH 2.