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Encapsulation of flavonoid in multiple emulsion using spinning disc reactor technology

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Abstract

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

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

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

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

obtained from Riedel-de Haen, Germany.

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

2.2 Preparation of rosella extract

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

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

2.3 Preparation of primary W/O emulsions

For encapsulating rutin, the aqueous phase was a pH 7 buffer prepared by combining 195 mL of 0.2 M NaH_2PO_4 with 305 mL of 0.2 M Na_2HPO_4. The oil phase was prepared by

147 dissolving 4 wt% Arlacel P135 into sunflower oil with gentle stirring and heating at 50^{o}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,

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

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

where n_i is the number of droplets of diameter d_i.

2.4 Confocal laser scanning microscopy

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

2.5 Visual assessment of emulsion stability

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

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

2.6 Encapsulation efficiency

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

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

3 Results and discussion

3.1 Particle-size distribution of emulsions with and without flavonoids

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

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

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

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

3.2. Visual assessment of emulsion stability

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

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

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

3.3 Confocal laser scanning microscopy

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

3.4 Encapsulation efficiency

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

4. **Conclusions**

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

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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.