



**EFFECT OF GELLED INNER AQUEOUS PHASE RHEOLOGY ON THE COLOUR DEGRADATION OF MUILTE AQUEOUS EXTRACTS INCORPORATED INTO WATER-IN-OIL-IN-WATER DOUBLE EMULSIONS**

**EFFECTO DE LA REOLOGÍA DE LA FASE ACUOSA INTERNA GELADA EN LA DEGRADACIÓN DE COLOR DE EXTRACTOS DE MUILTE INCORPORADOS EN EMULSIONES AGUA-EN-ACEITE-EN-AGUA**

L.M.A. Pavón-García<sup>1</sup>, C. Pérez-Alonso<sup>1</sup>, M.E. Rodríguez-Huezo<sup>2</sup>,  
R. Jiménez-Alvarado<sup>3</sup>, L. Alamilla-Beltrán<sup>4</sup>, A. Román-Guerrero<sup>1,5\*</sup>

<sup>1</sup>Facultad de Química, Depto. Ingeniería Química, UAEM, Estado de México, 50120, México.

<sup>2</sup>Departamento de Ingeniería Química y Bioquímica, Tecnológico de Estudios Superiores de Ecatepec, México.

<sup>3</sup>Universidad de la Cañada, Instituto de Farmacobiología, Teotitlán de Flores Magón, Oaxaca, México.

<sup>4</sup>Departamento de Graduados e Investigación en Alimentos, ENCB, IPN, México.

<sup>5</sup>Departamento de Biotecnología, UAM-Iztapalapa, México

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**Abstract**

The aim of this work was to study different  $W_1/O/W_2$  double emulsions in preserving color muitle aqueous extract (MAE), for which the work was divided into two fold, formulation and analysis of primary emulsion  $W_1/O$  and  $W_1/O/W_2$  double emulsions. Sodium alginate (SA), xanthan gum, guar gum, locust bean gum were used as gelling agents of MAE, and it was found that SA produced inner aqueous phase with enhanced viscoelastic properties, resulting in a  $W_1/O$  primary emulsion with more uniform mean droplet size and distribution than when using the other gelling agents or ungelled MAE. Subsequently  $W_1/O/W_2$  double emulsions were produced containing MAE gelled with SA or ungelled in the inner aqueous phase stabilized using pure gum Arabic (GA) or a blend of GA-mesquite gum (MG) in a 70:30 ratio in the outer aqueous phase. The double emulsion formulated with gelled MAE and 70:30 GA-MG blend exhibited more uniform mean inner water and outer oil droplet sizes, and protected best the anthocyanins contained in MAE to preserve its color when exposed to 8 h sunlight, providing a half-time life ( $t_{1/2}$ ) of 55.23 h. The double emulsion formulated with ungelled MAE and stabilized with pure GA displayed a  $t_{1/2}$  of 7.40 h.

**Keywords:** double emulsions, muitle aqueous extract, gelling agents, viscoelastic properties, droplet size, half-time life.

**Resumen**

El objetivo de este trabajo fue estudiar diferentes emulsiones dobles  $W_1/O/W_2$  en la preservación de color de extracto acuoso de muitle (MAE), para ello el trabajo se dividió en dos apartados, formulación y análisis de emulsiones primarias  $W_1/O$  y, emulsiones dobles  $W_1/O/W_2$ . Se empleó alginato de sodio (SA), goma xantana, goma guar, goma de algarrobo como agentes gelantes de MAE, y se encontró que el SA produjo fases acuosas internas con mejores propiedades viscoelásticas, dando como resultado emulsiones  $W_1/O$  con tamaños de gota y distribuciones más uniformes que cuando se usan otros agentes gelantes o el MAE sin gelar. Posteriormente se formularon emulsiones dobles  $W_1/O/W_2$  que contenían MAE gelado con SA o sin gelar en la fase acuosa interna estabilizadas con goma Arábiga (GA) o con una mezcla GA-goma de mezquite (MG) en una relación 70:30 en la fase acuosa externa. La emulsión doble formulada con MAE gelado y con la mezcla 70:30 GA-MG mostraron tamaños de gota promedio internas y externas más uniformes, y brindaron mejor protección a las antocianinas contenidas en MAE para preservar su color cuando fueron expuestas a 8 h de luz solar directa, proporcionando una vida-media ( $t_{1/2}$ ) de 55.23 h comparado con la emulsión doble formulada con MAE no gelado y estabilizada con GA que mostró una  $t_{1/2}$  de 7.40 h.

**Palabras clave:** emulsiones dobles, agentes gelantes, extracto acuoso de muitle, propiedades viscoelásticas, tamaño de gota, vida-media.

\*Corresponding author. E-mail: aroque@xanum.uam.mx  
Phone: +52 55 5804 2847; fax: +52 5804 4712

## 1 Introduction

Mutile (*Justicia spicigera*) is a native plant of southern Mexico, which grows in tropical regions. Aqueous extracts of Mutile produce a deep purple-reddish hue because of its anthocyanins contents (Pavón-García *et al.*, 2011) with high potential for being used as natural colourant in foods. However, anthocyanins as many other phenolic compounds can be quite chemically unstable during processing and storage (Fennema, 2008), their stability depends on the flavonoids 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-Casals and Cisneros-Zevallos, 2004; Vatai *et al.*, 2009; Tonon *et al.*, 2010; García-Márquez *et al.*, 2012). On the other hand, anthocyanins are also considered as good natural antioxidants which may provide an array of health promoting benefits (Tsuda *et al.*, 2000). Almajano *et al.* (2008) reported that W/O emulsions containing tea extracts showed 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 present a number of potential benefits over the conventional oil-in-water emulsions for certain applications such as reducing fat content (Lobato-Calleros *et al.*, 2008) or encapsulation of the functional food components (Benichou *et al.*, 2004) and active molecules (Laugel *et al.* 1996; Kanouni *et al.*, 2002) in the inner aqueous phase. Recently, these systems have raised interest for the micro carrying of hydrophilic or lipophilic ingredients such as food fortificants (Bonnet *et al.*, 2009; Choi *et al.*, 2009; Jiménez-Alvarado *et al.*, 2009), hydrophilic vitamins (Carrillo-Navas *et al.*, 2012), probiotics (Pimentel-González *et al.*, 2009) and natural colorants (Rodríguez-Huezo *et al.*, 2004), which are entrapped in their internal droplets allowing to mask odour or taste and protect them against oxidation by light or enzymatic degradation, prolonging the shelf-life of food products, and performing controlled release of the active ingredients (Pays *et al.*, 2001; Kanouni *et al.*, 2002; Muschiolik, 2007; Bonnet *et al.*, 2009; Carrillo-Navas *et al.*, 2012). Thus, multiple emulsions are widely recognized as a suitable way for protecting bioactive compounds in foods.

Despite the advantages of multiple emulsions as bioactive carriers and structure agents in food products, they are hindered by their structure

instabilities (Pawlik *et al.* 2010). Multiple emulsions not only display the well-known instability mechanisms of simple emulsions, they also exhibit diffusion of water and/or water-soluble materials through the oil phase because of unbalanced osmotic pressures between the internal and external aqueous phase where oil layer acts as a membrane separating both aqueous phases (ElShafei *et al.*, 2010). The migration of water and/or water-soluble materials through the oil film are dominated by two major release mechanisms: (1) the “swelling-breakdown” mechanism due to the coalescence of the thin liquid film separating the internal droplets and the globule surfaces, and (2) diffusion/permeation through the oil membrane, where molecules either pass through thin lamellae of surfactant which partially form in the oil layer due to fluctuation of its thickness, or diffuse across the oil layer via incorporation in “reverse micelles” (Pays *et al.*, 2002; Cheng *et al.*, 2007; Pawlik *et al.*, 2010).

To overcome these drawbacks, some alternatives have been applied such as balancing the osmotic pressure with glucose (Pawlik *et al.*, 2010), adding a thickening agent in the outer aqueous phase (ElShafeia *et al.*, 2010), using emulsifiers or combinations of emulsifiers with HLB values close to the required HLB of the oil phase (Schmidts *et al.*, 2009), adding sweet whey in the inner aqueous phase (Pimentel-González *et al.*, 2009), using biopolymer blends or protein-polysaccharide complexes as stabilizing agents in the outer aqueous phase (Carrillo-Navas *et al.*, 2012; Jiménez-Alvarado *et al.*, 2009), and gelling the inner aqueous droplets (Surh *et al.*, 2007). The latter alternative combines the properties of a gel-like structure and emulsion properties, and they are commonly referred as “emulgels”. Emulgels allow the obtainment of stable emulsions by decreasing surface and interfacial tension, controlling the osmotic pressure gradient between both aqueous phases, and at the same time increasing the viscosity of the inner aqueous phase making slower the rate of diffusion of the inner to the outer aqueous phase (Khullar *et al.*, 2012; Ajazuddin *et al.*, 2013).

Thus the objectives of this work were to: a) produce water-in-oil-in-water ( $W_1/O/W_2$ ) multiple emulsions using gum Arabic (GA) alone or blended with mesquite gum (MG) as stabilizing agents in the outer aqueous phase; b) produce primary emulsions ( $W_1/O$ ) with gelled aqueous phase incorporating Mutile aqueous extracts (MAE) and ascorbic acid (AA) as bioactives; c) evaluate the effect of the dynamic rheology of  $W_1$  on primary emulsion droplet

size change and stability; d) evaluate the inner and multiple droplet size variation in  $W_1/O/W_2$  emulsion made with the most stable  $W_1/O$ .

## 2 Materials and methods

### 2.1 Materials

Ascorbic acid (AA) was acquired from Quimica Integral de Mexico S.A. de C.V. (Mexico, D.F., Mexico). Sodium alginate (SA; Grindsted alginate BC 113B), the water-soluble surfactant Panodan SDK (PAN; esters of monoglycerides and diglycerides of diacetyl tartaric acid) and the oil-soluble surfactant Grindsted PGPR 90 (GRIN; esters of polyglycerol and polyricinoleate fatty acids) were purchased from Danisco Mexicana S.A. de C.V. (Mexico, D.F., Mexico). Canola oil (Unilever, Mexico) was used as the oil phase of the emulsions. Mesquite gum (MG) was hand collected in the form of tear drops from *Prosopis laevigata* trees in the Mexican State of San Luis Potosi and purified as the procedure described by Vernon-Carter *et al.* (1996). Gum Arabic (GA; *Acacia senegal*), xanthan gum (XG), guar gum NF (GG) and locust bean gum (LG) were purchased from Industria Ragar, S.A. de C.V. (Mexico City, Mexico). Muitle (*Justicia spicigera*) leaves were purchased from a greenhouse (Toluca, State of Mexico, Mexico). All the water used in the experiments was bidistilled.

### 2.2 Muitle aqueous extract (MAE) preparation

Muitle leaves were sun-dried at room temperature (~ 20 °C) for a week. The dried leaves were ground with a mortar and pestle and the pulverized material was passed through a 0.25 mm porous sieve. Twenty grams of dried material was soaked in 300 mL of bidistilled water at 40°C during 1 h in order to obtain the muitle aqueous extract (MAE). The resulting MAE was vacuum-filtered using Whatman No. 1 filter paper and stored in amber jars at 4 °C until used (Sepúlveda-Jiménez *et al.*, 2009).

### 2.3 Preparation of MAE gels

Aqueous dispersions of GG (2% w/w), XG (2% w/w), LG (3% w/w) and SA (3% w/w) were prepared by adding the respective powder into 1% wt solution of AA in MAE at 45 °C. The gum powders were dispersed with a Silverson L4R homogenizer at 5700

rpm until a homogeneous dispersion was achieved. The concentrations used in making the dispersions were those allowing complete hydration of the gums after 12 h. The gelled dispersions were used as the aqueous dispersed phase of the primary emulsions ( $W_1/O$ ).

### 2.4 Rheological characterization of MAE gels

Dynamic oscillatory measurements were performed with a MCR 300 rheometer (Physica Meßtechnik GmbH, Stuttgart, Germany) with a cone-plate geometry of 50 mm of diameter, and cone angle of 2°. The MAE gels were carefully loaded into the measuring system, left to rest 5 min for structure recovery and temperature equilibration. Measurements were performed at 25 °C. Amplitude sweeps (0.01-100% strain, 1 Hz) were carried out on the MAE gels after 24 h of preparation. The storage modulus ( $G'$ ) and the loss modulus ( $G''$ ) were obtained from the equipment software in all cases and plots of  $G'$ -strain % and  $G''$ -strain % were obtained from the experimental data with an Origin Scientific Graphing and Analysis Software version 7 (OriginLab Corporation, Northampton, MA), and analyzed to determine the linear viscoelastic region of the curves, where the gels were not strain nor stress dependent (Dzul-Cauich *et al.*, 2013).

### 2.5 Preparation of the primary emulsions ( $W_1/O$ )

MAE gel-in-oil primary emulsions were prepared with 8% w/w total surfactant concentration (4:1 GRIN to PAN ratios) (Jiménez-Alvarado *et al.*, 2009; Carrillo-Navas *et al.*, 2012). The primary emulsions were coded as  $(W_1/O)_x$ , where the subscript x denotes the gum used for gelling the inner aqueous phase (GG, XG, LG, or SA). A control emulsion (NGE) was made with non-gelled aqueous solution. In all the cases, the requisite amount of  $W_1$  was added dropwise into the oil phase (O) at 45 °C, with constant mixing of 7600 rpm for 10 min with the help of an Ultra-Turrax T50 Basic homogenizer (IKA® WERKE Works Inc., Wilmington, NC, USA) in order to obtain a 0.5 dispersed phase mass fraction ( $\phi_{W_1/O}$ ). The primary emulsions were left to cool down to room temperature.

## 2.6 Multiple emulsion formation

The requisite amount of (W<sub>1</sub>/O) emulsion exhibiting the highest stability was re-emulsified into an aqueous solution (20 % w/w) of either pure GA or a blend of GA-MG in a 70:30 ratio, at 6400 rpm for 6 min with a Ultra-Turrax T50 Basic homogenizer (IKA® WERKE Works Inc., Wilmington, NC, USA) at 25 °C. Two multiple emulsions made with NGE were prepared by re-emulsifying into the same biopolymer solutions mentioned above. All the resulting multiple emulsions had a 0.3 dispersed mass fraction ( $\phi_{W_1/O/W_2}$ ) and were coded as GE<sub>GA</sub>, GE<sub>GA-MG</sub>, NGE<sub>GA</sub>, and NGE<sub>GA-MG</sub>, for gelled (GE) and non-gelled inner aqueous phase W<sub>1</sub> stabilized with pure GA or GA-MG blend. Both biopolymer materials were chosen based on preliminary tests where there was not observed phase separation or no interference with MAE color determination.

## 2.7 Emulsions droplet size

Droplet sizes in all the (W<sub>1</sub>/O) emulsions were determined from images of the emulsions obtained with a phase contrast microscope (Olympus model BX45, Olympus Optical Co. Ltd., Tokyo, Japan) and recorded with a digital camera Moticam 2300 (Motic Group Co. Ltd., Xiamen, China). Image processing for droplet size analysis was carried out using image analysis software (Image Pro-Plus version 4.5 software, Media Cybernetics, Inc., L.P., Silver Spring, USA). Briefly, the data processing of the digitalized images consisted of different steps, including contrast enhancement, different filter operations followed by an automatic segmentation. This procedure allowed considerable reduction of the data analysis time without losing accuracy. A substantial number of droplets (N = 1000) were counted to obtain statistical estimates of the droplet size distribution in each sample. Number frequency distributions of droplets were generated by grouping the bubbles into classes belonging to a common interval. The number frequency of any class interval was computed using Origin Scientific Graphing and Analysis Software version 7 (OriginLab Corporation, Northampton, MA) as the number of droplets in that class (class frequency) divided by the total number of droplets and expressed as a percentage. The number volume mean diameter (D<sub>3,0</sub>) was calculated using Eq. 1.

$$[D_{3,0}] = \left[ \frac{\sum d_i^3}{n_i} \right] \quad (1)$$

where  $d_i$  is the  $i$ th droplet diameter size and  $n_i$  is the number of  $i$ th droplet with diameter  $d_i$  ( $\mu\text{m}$ ). D<sub>3,0</sub> of the (W<sub>1</sub>/O) emulsions was determined over 30 days of storage. The rate of coalescence ( $k_c$ ) of the (W<sub>1</sub>/O)<sub>x</sub> emulsions was determined as reported by Ruíz-Ramos *et al.* (2006).

Droplet sizes in GE and NGE multiple emulsions (D<sub>3,0</sub>) was also determined using laser diffraction with a Malvern particle size analyzer, using water as dispersant (RI 1.333), over 50 days of storage (Jiménez-Alvarado *et al.*, 2009).

## 2.8 Multiple emulsions stability

Differential scanning calorimetry (DSC) technique was applied with a DSC Q1000 (TA Instruments, New Castle, DE, USA) in order to characterize the stability of the (W<sub>1</sub>/O)<sub>x</sub>/W<sub>2y</sub> emulsions. Approximately 10 mg of each (W<sub>1</sub>/O)<sub>x</sub>/W<sub>2y</sub> emulsion was put by separate in aluminium pans, hermetically sealed before being placed in the calorimeter measuring chamber. The samples were submitted to four consecutive regular cooling-heating cycles from 20°C to -60°C and then heated back to 20°C using a heating rate of 5°C/min with 10 min isotherms at the endpoints temperatures. The displacement of the freezing-melting signals of the dispersed phase of emulsions is considered as an indicative of instability in the system (Fouconnier *et al.*, 2012).

## 2.9 Stability of MAE

The stability of MAE alone and gelled with SA, when incorporated into (W<sub>1</sub>/O)<sub>x</sub>, and (W<sub>1</sub>/O)<sub>x</sub>/W<sub>2y</sub> was determined by measuring the decay in colour parameter (**a**) when 5 g samples of each were diluted in 100 ml of water or canola oil and subjected to direct sunlight exposure for 8 h. All the measurements were carried out by using a Hunter Lab (MS-4,500 L. Format 2. 1/4 P65/10, Virginia, USA) colorimeter, and measurements were taken at 1 h intervals. In the Hunter L, **a**, **b** colour space, the **a** scale provides a measurement of hue variation (red-green), where positive values are red and negative values are green (HunterLab, 2012).

## 2.10 Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA) and a Tukey test for a statistical significance  $p < 0.05$ , using the software SPSS Statistics 17.0 (IBM



Corporation, NY, USA). All experiments were done by duplicate.

### 3 Results and discussion

#### 3.1 MAE gels characterization

Purple-redish colored extracts were obtained from the maceration of vegetal material in water. At the conditions mentioned above, the concentration of total phenolic compounds was determined by the Folin-Ciocalteu's method described by García-Márquez *et al.* (2012) and Cardenas-Sandoval *et al.* (2012) (data not shown), accounting for 58.34 mg of gallic acid equivalents per 100 mL of fresh MAE. The strain sweeps showed differences between the MAE gels, although all of them displayed similar profiles characterized by a linear region exhibiting a constant value of  $G'$  at low strain %, followed by a non-linear region characterized by a strain-thinning behaviour of storage modulus ( $G'$ ) at a large strain % (Fig. 1). At relatively low strain %, the entanglement density of the biopolymers did not change because a balanced state was maintained between the rates of structural breakdown and rebuilding. However, as the strain % was increased, the rate of structural destruction was gradually increased and the structural reformation decreased, with a reduction in the entanglement density (Song *et al.*, 2006). The SA gel had the greatest  $G'$  (2000 Pa) magnitude in the linear viscoelastic region, with the remaining gels showing significant decreasing  $G'$  magnitudes as follows: GG (~ 650 Pa) > XG (~ 490 Pa) > LG (~ 230 Pa). The strain % at which the non-linear viscoelastic behaviour of the gels commenced did not depend on the value of  $G'$  in the linear viscoelastic region.

The  $G''$ -strain % curves of the gels is also shown in Fig. 1. At a given strain % in the linear viscoelastic region the value of  $G'$  was significantly greater than  $G''$ , indicating a dominant elastic character of gels.  $G''$ , as in the case of  $G'$ , showed downward inflections at strains above the linear viscoelastic region. The loss modulus values in the linear viscoelastic region in descending order were: SA (~ 610 Pa) > GG (~ 290 Pa) > LG (~ 180 Pa) > XG (~ 68 Pa). At relatively high strain %, in the non-linear viscoelastic region, all the gels showed a crossover between  $G'$  and  $G''$ . The crossover point indicates that both  $G'$  and  $G''$  have the same value. It is interesting to notice that the SA, GG and XG gels exhibited a  $G''$  overshoot before reaching the crossover point.

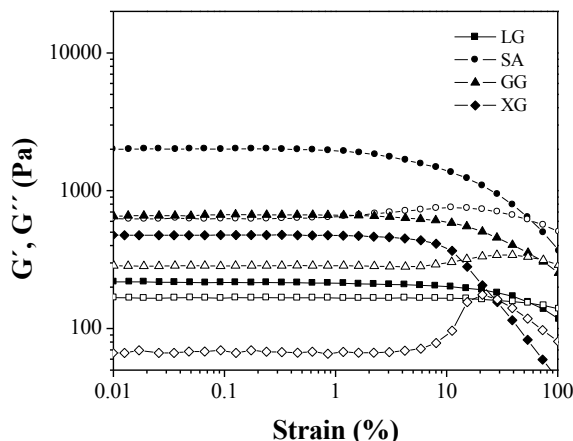


Fig. 1. Dynamic storage modulus ( $G'$ , filled symbols) and dynamic loss modulus ( $G''$ , empty symbols) of MAE gels using different gums.

Gels showing this type of behaviour are classified as type III, weak strain overshoot ( $G'$  decreasing,  $G''$  increasing followed by decreasing). On the other hand, LG gel showed type I behaviour, strain thinning behaviour ( $G'$ ,  $G''$  decreasing). These different types of behaviours arise from complex creation and loss rates of network junctions (Sim *et al.*, 2003) that are beyond the scope of this study.

#### 3.2 Characterization of primary emulsion

Observation of representative micrographs of the fresh  $(W_1/O)_x$  emulsions indicated that the nature of the inner aqueous phase affected the initial droplet size and distribution (Fig. 2). The mean droplet size and span (width) of the droplet distribution went hand in hand, *i.e.*, initial smaller sized globules exhibited narrower droplet distribution and vice versa. The smallest initial  $D_{3,0}$  and size distribution span was exhibited by  $(W_1/O)_{MAE}$ , followed in ascending order by  $(W_1/O)_{SA}$ ,  $(W_1/O)_{LG}$ ,  $(W_1/O)_{XG}$  and  $(W_1/O)_{GG}$  (Table 2). It seems logical that if all the emulsions were made following the same manufacturing procedure, the inner non-gelled aqueous phase (MAE) presented the least resistance to shearing forces applied during homogenization resulting in an emulsion with smaller and more uniformly distributed droplet sizes. The disruption and intermingling of the gelled aqueous phases in the continuous oil phase seem to depend not only on their viscoelastic properties, but on the balance between interfacial forces that tend to hold the droplets together and disruptive forces generated during homogenization that tend to pull them apart (McClements, 2005).

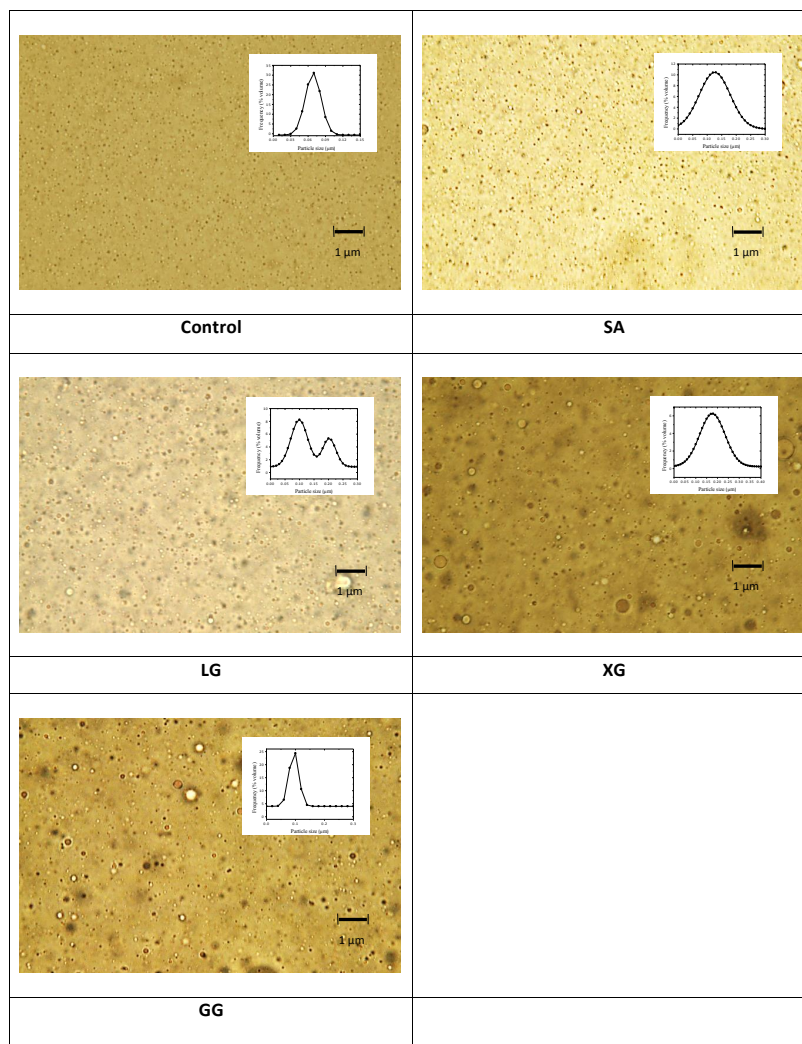


Fig. 2. Representative micrographs and droplet size distribution of fresh  $(W_1/O)_x$  emulsions.

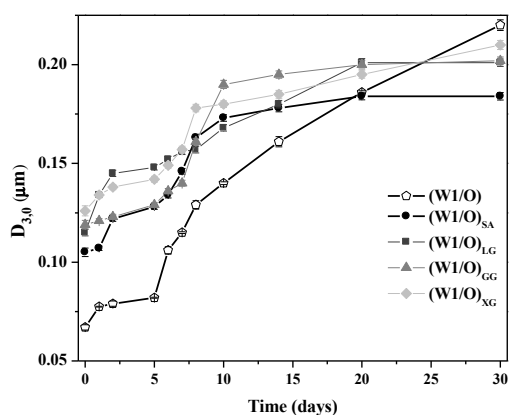


Fig. 3.  $D_{3,0}$  for the  $(W_1/O)_x$  emulsions with aging time of 30 days.

Fig. 3 shows the evolution of  $D_{3,0}$  for the  $(W_1/O)$  emulsions with aging time. The rate of coalescence of the  $(W_1/O)$  droplets largely follow a first-order kinetics (Sherman, 1969; Ruíz-Ramos *et al.*, 2006). Table 1 shows the rate constant ( $k_c$ ) found for the  $(W_1/O)$  experimental data. As can be seen, the  $(W_1/O)_{MAE}$  showed the highest rate of coalescence despite it was also the one exhibiting initial smaller mean droplet size and narrowest droplet span. The lowest  $k_c$  was exhibited by  $(W_1/O)_{SA}$ , followed in ascending order by  $(W_1/O)_{GG} < (W_1/O)_{LG} < (W_1/O)_{XG}$ . Thus, in the case of the  $(W_1/O)$  emulsions it seems that enhanced rheological properties of the inner aqueous phase translate into more stable primary emulsions against droplet coalescence.

Table 1. Changes in droplet size ( $D_{3,0}$ ) and apparent rate of coalescence for the primary emulsions

Primary emulsion	$(D_{3,0})_{t=0}$ ( $\mu\text{m}$ )	$\text{Span}_{t=0}$	$(D_{3,0})_{t=30}$ ( $\mu\text{m}$ )	$\text{Span}_{t=30}$	$k_{C(W_1/O)} \times 10^7$ ( $\text{s}^{-1}$ )
(W <sub>1</sub> /O)	0.067±0.0005 a	1.195±0.081 a	0.223±0.001 a	7.801±0.121 a	14.58
(W <sub>1</sub> /O) <sub>SA</sub>	0.105±0.001 b	1.061±0.064 b	0.174±0.012 a	1.507±0.025 b	5.92
(W <sub>1</sub> /O) <sub>GG</sub>	0.120±0.005	1.227±0.161 c	0.201±0.003 b	2.223±0.154 c	6.20
(W <sub>1</sub> /O) <sub>LG</sub>	0.115±0.003 b,c	1.131±0.094 c	0.202±0.010 c	2.353±0.015 d	7.06
(W <sub>1</sub> /O) <sub>XG</sub>	0.126±0.004 e	1.227±0.001 d	0.210±0.006 c	3.833±0.001 e	7.50

Different letters in the same column means significantly differences ( $p < 0.05$ )

Table 2. Color loss rate of MAE incorporated into emulsions

Sample	$k \times 10^6$ ( $\text{s}^{-1}$ )	$R^2$	$t_{1/2}$ (h)
MAE	26.03	0.944	7.40
SA	20.22	0.931	9.52
(W <sub>1</sub> /O) <sub>SA</sub>	16.85	0.958	11.43
NGE <sub>GA</sub>	12.50	0.940	15.40
GE <sub>GA</sub>	7.89	0.943	24.40
NGE <sub>GA-MG</sub>	9.14	0.941	21.07
GE <sub>GA-MG</sub>	3.49	0.940	55.23

A stable (W<sub>1</sub>/O) is a fundamental and significant step in order to prepare a stable W<sub>1</sub>/O/W<sub>2</sub> multiple emulsion. The inclusion of gelling or thickening agents to the inner aqueous phase of water-in-oil-in-water multiple emulsions enhanced the long-term stability of multiple emulsion since these agents balance Laplace and osmotic pressures among droplets in W<sub>1</sub>. This property could be related to a greater ability to withstand osmotic pressure differences between inner and outer aqueous phases in a multiple emulsion, prolonging its stability. In this case the most appropriate gelling agent was SA, since it displayed de lowest droplet size with higher emulsion stability.

### 3.3 Characterization of multiple emulsions

All the multiple emulsions formed were type C systems (Fig. 4), i.e. they were made up by spherical oil droplets containing within them a large number of water droplets.  $D_{3,0}$  of inner water droplets and of the oil droplets was monitored during 50 days of aging. In Fig. 5a, the droplet size evolution of (W<sub>1</sub>/O)<sub>MAE</sub> is compared to that of the inner aqueous droplets in NGE<sub>GA100</sub> and NGE<sub>GA-MG</sub>. The relative increase

in droplet size was larger in the multiple emulsions than in the primary emulsion, specifically at relatively short aging times, with NGE<sub>GA100</sub> showing the largest inner droplet size increase. Fig. 5b compares the relative droplet size evolution of (W<sub>1</sub>/O)<sub>SA</sub> with GE<sub>GA100</sub> and GE<sub>GA-MG</sub>. The overall largest droplet size increase with aging time occurred for GE<sub>GA100</sub> followed by (W<sub>1</sub>/O)<sub>SA</sub>, with GE<sub>GA-MG</sub> showing the smallest droplet change. At the end of the 50 days storage time, the inner droplet size of GE<sub>GA-MG</sub> was significantly smaller than for that of NGE<sub>GA70</sub>, despite that the latter multiple emulsion had much more smaller initial inner droplet size (Fig. 5a). These results indicate that gelling of the inner aqueous phase in multiple emulsions enhances the stability of inner droplet size. Fig. 5c shows the multiple emulsions oil droplet size change, whether or not the inner aqueous phase was gelled or not. In all cases the droplet size of multiple emulsions tended to decrease, achieving a minimum after about 27 days of aging, afterwards showing a droplet size increase with further aging time. However, oil droplet size of the multiple emulsions experienced significantly lower fluctuations when inner aqueous phase was gelled than when it was not. The outer biopolymer composition also had an effect on oil droplet size variation. The blend GA-MG in a 70:30 ratio provided better stability than pure GA, indicating that a positive synergistic effect occurred. It is beyond the scope of this manuscript to try to offer a detailed explanation of the mechanisms giving rise to the complex droplets variation with aging time. Suffice that destabilization processes available to multiple emulsions are necessarily much more varied than those for simple emulsions. For example, the outer droplet interface may coalesce with one or more multiple emulsion droplets; the individual internal aqueous droplets can be expelled sequentially from the multiple emulsions droplets; gradual shrinkage of the internal droplets is possible due to osmotic

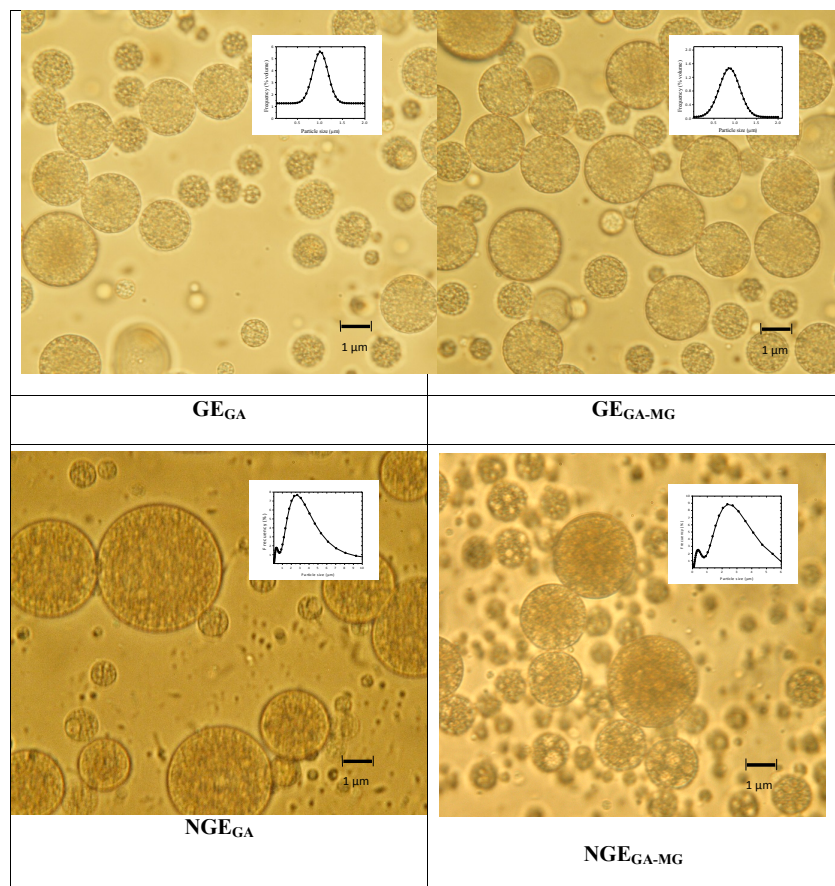


Fig. 4. Micrographs of fresh ( $W_1/O/W_2$ ) multiple emulsions containing gelled (GE) and non-gelled (NGE) MAE, stabilized with gum Arabic (GA) and GA: mesquite gum (MG) (70:30 ratio).

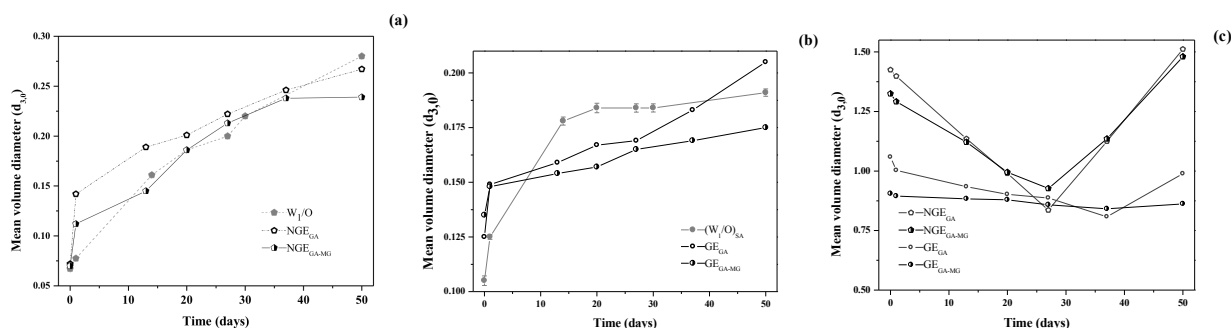


Fig. 5. Droplet size evolution of: a)  $(W_1/O)_{MAE}$  and inner aqueous droplets of  $NGE_{GA}$  and  $NGE_{GA-MG}$ ; b)  $(W_1/O)_{SA}$  and inner aqueous droplets of  $GE_{GA}$  and  $GE_{GA-MG}$  and c) outer droplet size of  $W_1/O/W_2$  emulsions.

gradient between the inner and continuous aqueous phases, when net mass transport of water occurs from the inner phase to the outer continuous phase through the oil film acting as a “semi-permeable membrane”; and conversely, when the osmotic gradient acts in the opposite direction, water diffusion through the oil

film from the continuous phase into the encapsulated aqueous droplets will produce swelling of these inner droplets (Hernández-Marín *et al.*, 2013). Thus, the strategy to follow for achieving this aim is to balance the osmotic gradients between the inner and outer aqueous phases and to structure the interfacial



membranes in order to minimize the diffusion effects through them.

The thermograms showing the thermal events occurring during the cooling and heating of  $W_1/O$  and  $W_1/O/W_2$  emulsions are shown in Fig. 6. On first cooling of  $W_1/O$  emulsion, there is a crystallization peak due to oil at  $-32^\circ\text{C}$  (confirmed by cooling free oil, data not shown) and the aqueous phase around  $-46^\circ\text{C}$  (Fig. 6a), in agreement with those reported by Clause (1998). When this emulsion was submitted to cooling-heating cycles, there was noticed the presence of three different phases: (I) crystallization peak of water in the disperse phase  $W_1$ , (II) crystallization peak of the oil phase, and (III) crystallization peak of water at the bulk, the latter observed from the third cooling cycle at around  $-20^\circ\text{C}$ , it could be related with the expulsion of drops of water to the oil continuous phase and the emulsion breaking (Clause *et al.*, 2005).

Fig. 6b-d, shows the thermograms obtained for the  $W_1/O/W_2$ , it is observed the presence of exothermic peaks at  $-43^\circ\text{C}$ ,  $-32^\circ\text{C}$ , and  $-15^\circ\text{C}$ , which correspond to freezing of  $W_1$ , O, and  $W_2$

phases respectively, these temperatures agree with those peaks found in Fig. 6a. In all the multiple emulsions, during cooling cycles the intensity of the peak at  $-43^\circ\text{C}$  seems to increase as more cooling cycles are applied, this could be related to high inner aqueous phase viscosity due to the presence of SA, which acts as a barrier against energy transfer delaying the complete  $W_1$  icing and therefore making this peak almost indiscernible. Nonetheless, as more cooling cycles are applied this signal is likely to increase not only on the heat flow intensity but also on the onset temperature range; this phenomenon could be related to the mechanism described by Cramp *et al.* (2004) and Bibette (1992), where, if the inner aqueous phase and oil phase are both solid, the surfactants that are anchored at the interface seems to be squeezed out of the gap separating two droplets, due to solid droplets are probably not able to deform somewhat to accommodate the pressure applied by the expansion of water and oil phases on freezing, as liquid droplet would do, creating water-to-water contact that would lead to partial coalescence of inner aqueous droplets.

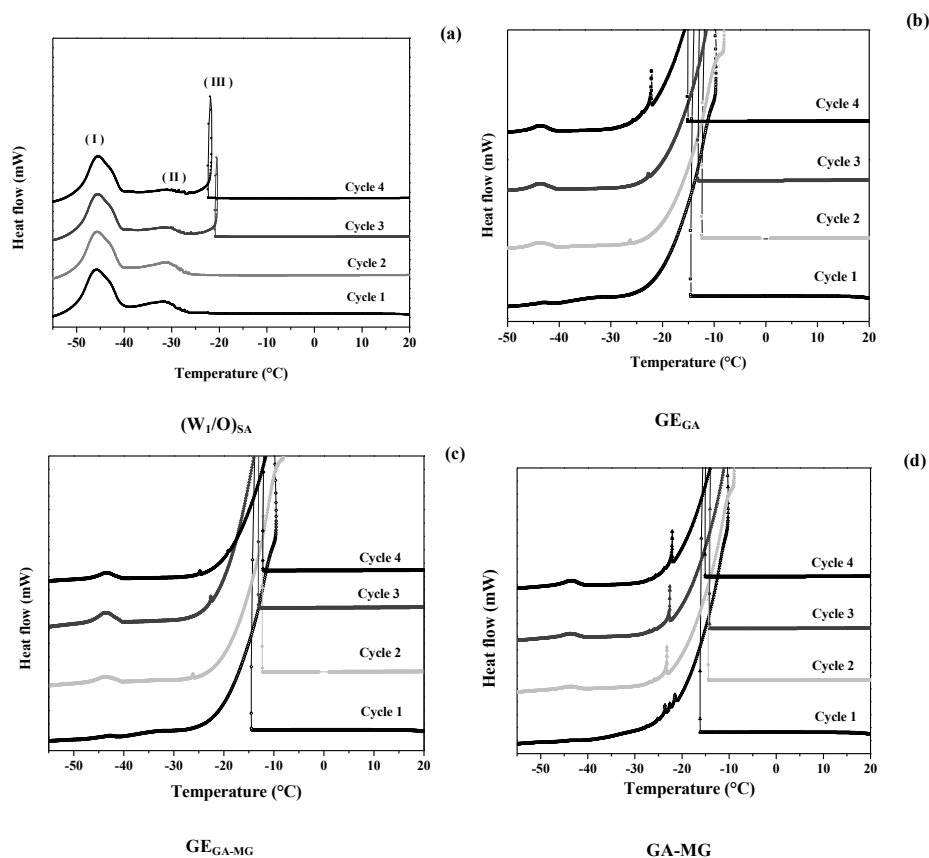


Fig. 6. DSC profiles of the simple and multiple emulsions subjected to four cooling cycles.

On the other hand, during cooling cycles the transfer of water from the internal to the external aqueous phase of multiple emulsions was observed, causing the appearance and size increase of one or more peaks in the region of  $-15\text{ }^{\circ}\text{C}$  as the number of cooling cycles increased (Clausse, 1998). This transfer could be attributed to the difference of vapour pressures between the ice and undercooled water at the same temperature (Clausse *et al.*, 1999). GEGA (Fig. 6b) and  $\text{NGE}_{\text{GA-MG}}$  (Fig. 6d) exhibited new and increasingly larger sized peaks as each cooling cycle proceeded, while  $\text{GE}_{\text{GA-MG}}$  (Fig. 6c) withstood the four cooling cycles without exhibiting new peaks, confirming that this system was more stable than the rest. This stability could be due to the higher the molecular weight of the biopolymer blend used as stabilizer, the stronger the steric effect provided that makes the biopolymer slower to diffuse from the interface and reduce the instability of the system (Palanuwech *et al.*, 2003).  $\text{NGE}_{\text{GA}}$  did not resist the four cooling cycles and the DSC profile is not shown. Consequently, multiple emulsions stabilized with GA-MG displayed better properties than those with GA. Therefore, it can be inferred that the increase in the solid concentration of the inner  $W_1$  aqueous phase, and the upswing in the viscosity of this phase as well as the combination in the use of biopolymer blends in the external aqueous phase as stabilizing agents provide better structuring and stabilizing properties.

### 3.4 Stability of MAE in emulsions

The stability of ungelled and gelled MAE entrapped in the inner aqueous phase of the multiple emulsions was monitored and compared to that of their free counterparts, when exposed to 8 h of direct sunlight (Fig. 7). The decay in  $a/a_0$ , where  $a_0$  is the initial reading obtained on the  $a$  scale at time = 0, and  $a$  is the reading obtained on the  $a$  scale at time =  $t$ , was used as an indicative of the pigment stability. Parameters  $b$  and  $L$  showed negligible changes in time so their data are not reported. The ungelled and gelled MAE in SA showed a significantly greater decay in  $a/a_0$  than when incorporated into the multiple emulsions. The multiple emulsions made with gelled inner aqueous phase showed significantly lower decay than those made with ungelled MAE. Furthermore, the  $\text{GE}_{\text{GA-MG}}$  showed a significantly lower drop in  $a/a_0$  with aging time than  $\text{GE}_{\text{GA}}$ . These results confirm that both the gelling of the inner aqueous phase and the use of a biopolymer blend in the outer aqueous phase had a positive impact on pigment degradation. The impact

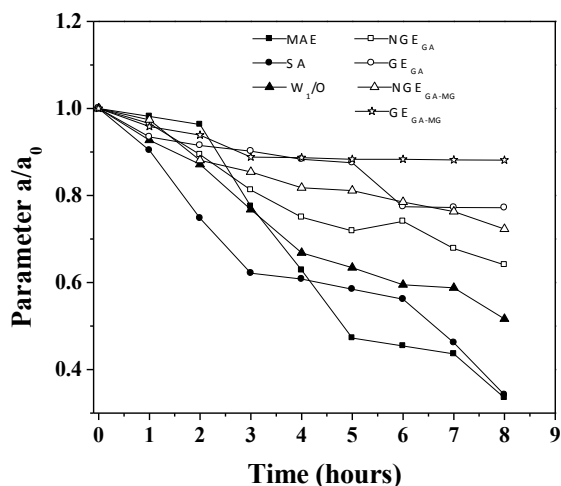


Fig. 7. Decay in  $a/a_0$  of ungelled (MAE) and gelled MAE (SA) entrapped in single emulsion  $(W_1/O)_{\text{MAE}}$  and in the inner aqueous phase of the multiple emulsions  $(W_1/O/W_2)$  exposed to 8 h of direct sunlight.

of both these factors can be best appreciated in Table 2. The data of Fig. 7 were adjusted to a first order kinetic model (all  $R^2$  were higher than 0.931), in order to obtain the rate loss constant ( $k$ ) and the mean half-life time ( $t_{1/2}$ ) of the samples. The comparison in  $t_{1/2}$  between  $\text{GE}_{\text{GA-MG}}$  and free MAE is dramatic, being of 55.23 h for the former and of only 7.40 h for the latter. The  $t_{1/2}$  for  $\text{GE}_{\text{GA}}$  was less than half (24.40 h) of that for  $\text{GE}_{\text{GA-MG}}$ .

## Conclusions

The presence of gelling agents in the inner aqueous phase of water-in-oil-in-water multiple emulsions helps to stabilize the primary water-in-oil emulsion, condition that is fundamental in order to prepare stable water-in-oil-in-water multiple emulsions. The proper selection of biopolymers in the outer aqueous phase also contributed to increase the stability of the multiple emulsions. Both factors have bearing on the stability of bioactives contained in the inner aqueous phase, providing them with longer half-time life, and on providing multiple emulsions with a greater ability to withstand destabilizing factors, contributing to achieve longer-term stability. In agreement with data, the use of sodium alginate as gelling agent promotes the stability of inner  $W_1/O$  emulsion, mainly due to its better viscoelastic properties that reduces the inter droplet interactions leading to a decrease in coalescence phenomena. On the other hand, the use of

biopolymer blends like gum Arabic and mesquite gum in a 70:30 ratio, provided multiple emulsions more stable than those stabilized with pure biopolymers, showing a synergistic effect. The high molecular weight of the biopolymer blends and their stronger steric effect, in combination with a gelled emulsion are capable for slowing the loss of color of the bioactive compound entrapped in the inner phase, prolonging the shelf life of anthocyanins in muiltle extracts.

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