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Natural dispersion of biflavonoids from *Garcinia madruno* extracts: A green and sustainable processing to improve the solubility and dissolution rate

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ABSTRACT

Garcinia madruno is rich in antioxidant biflavonoids that could be used to treat oxidative-related diseases. Up to now, its commercial application is challenging due to the poor water solubility of biflavonoids. This work aimed to develop a green and sustainable process to improve the solubility and dissolution rate of *G. madruno* biflavonoids by the development of 100% natural nanodispersions using peel extracts of this neotropical fruit. Three hydroalcoholic extracts were elaborated with different concentrations of biflavonoids and epicarp proportions. The biflavonoid natural suspensions were obtained by evaporating the ethanol of the different extracts. As a control, suspensions stabilized with commercial surfactants were compared to each other. Two natural nanosuspensions with different concentrations of biflavonoids and one nanosuspension stabilized with commercial surfactants were obtained. All dispersions had submicrometric particle distribution. M3-S nanosuspension (stabilized by commercial stabilizer) was the smallest dispersion with an average particle size of 256.6 nm, whereas natural suspension (NS-1 and NS 2) had better performance in the short-term stability and improved the solubility and dissolution rate until 400% in comparison of M3-S and isolate morelloflavone. Our results demonstrated that it was possible to formulate a biflavonoid suspension using their own plant compounds as natural stabilizers.

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1. Introduction

Garcinia madruno is an exotic neotropical species with high potential to be used for the development of active ingredients for functional drinks, dietary supplements, and medical foods, among other healthy products, mainly due to their overexpression of biflavonoids (Carrillo-Hormaza et al., 2019). Twenty-one different biflavonoids have been identified in the leaves, stems, and fruits of this species (Carrillo-Hormaza et al., 2016). Specifically, *G. madruno* biflavonoids are highly antioxidant compounds (Carrillo-Hormaza et al., 2016), and recently they have shown promising potential as in vivo atheroprotective and neuroprotective agents (Tabares-Guevara et al., 2017; Sabogal-Guáqueta et al., 2018). The highest content of these compounds is found in the leaves and epicarp. Indeed, the content of biflavonoids is very substantial in both matrices, reaching concentrations above 7% and 20% (dry matter), respectively (Carrillo-Hormaza et al., 2019).

G. madruno biflavonoids are characterized as the covalent union of two monomeric units of flavonoids through C—C bonds between flavanone–flavone (morelloflavone type), flavone–flavone (amentoflavone type) and flavanone–-flavanone (GB 1a type) (Carrillo-Hormaza et al., 2016). Disadvantageously, the monomeric flavonoids, as well as the biflavonoids, are poorly water-soluble, challenging its use as food, dietary supplements or for pharmaceutical purposes. Additionally, the union of two monomeric units of flavonoids found in biflavonoids may promote even more the decrease of solubility, dissolution rate, and bioavailability compared to their monomers and hence the in vivo bioavailability is scarce (Rein et al., 2013). Indeed, their incorporation in beverages and other kinds of aqueous formulations is one of the most critical processes at the moment (Premathilaka et al., 2022).

Multiple strategies are available to improve the incorporation of poorly water-soluble compounds in liquid formulations and enhance their bioavailability. Nowadays, nanotechnology is one of the most employed methods for this purpose. Reducing the particle size causes an increase of the superficial area of particles, improving directly the solubility and the bioavailability (Kesisoglou et al., 2007; Khadka et al., 2014). Especially with flavonoids, different technological approaches and formulations strategies have been applied for nanonisation leading to formulations as nanosuspensions, nanoemulsions, liposomes, solid lipid nanoparticles, among others, (Mishra et al., 2013; Macedo et al., 2014; Mignet et al., 2013; Weiss et al., 2008). Even some of these applications can be found in commercial food products and dietary supplements (Kobierski et al., 2011).

Most applications of the nanosized flavonoids have been developed using pure flavonoids and different commercial stabilizers. The use of pure compounds and the commercial stabilizers permits a better formulation process, improves stability by preventing particle agglomeration, controls the release of the active compound and standardizes the content of the natural actives in the final formulation (Benson et al., 2019). The most common approaches for stabilizing nanoformulations are based on electrostatic or steric mechanisms. Electrostatic stabilization is obtained by adsorbing ionic surfactants onto the particle surface to promote an electrostatic repulsion between the particles (Hong et al., 2014). Meanwhile, steric stabilization is achieved by adsorbing polymers or nonionic surfactants onto the surfaces of particles to form a dynamically rough surface to prevent particle agglomeration (Hong et al., 2014). Thus, the selection of stabilizers is one of the most critical steps for the nanoformulation process. Not only the performance and stability of the final formulation will depend on it, but also the restrictions of its use in the food industry is limited on type and concentration of stabilizers selected (Office of Food Additive Safety, 2018).

In nature, there are multiple sources of potential stabilizers as adsorbing polymers, nonionic type surfactants and ionic-type surfactants, an example of them are gums, saponins, and lecithin, respectively (Sedaghat Doost et al., 2019; Xu et al., 2011; Kontogiorgos, 2019; Taheri and Jafari, 2019). However, there are many stabilizers controlled or prohibited in the food industry (Shah et al., 2017). An attractive alternative is to employ GRAS (Generally Recognized As Safe) natural stabilizers to create nano-formulations (Knoth et al., 2019). However, in most cases, it is challenging to stabilize formulations by using only the available natural or GRAS stabilizers (Karthik et al., 2017). Therefore, reducing the particle size of *G. madruno* biflavonoids, using natural stabilizers seems to be an attractive approach to improve solubility and hence the in vivo performance. However, in the process, green extraction techniques should be also considered, which have gained attention considering that they encourage the decreasing use of volatile organic solvents and the sustainable approach in the use of raw material.

Considering that the main phenolic compounds reported in *Garcinia* genus are water-insoluble compounds with high molecular weight such as biflavonoids, xanthones and polyisoprenylated benzophenones (Carrillo-Hormaza et al., 2016), it is very likely that some *Garcinia* species transport these insoluble compounds through the latex canals. Now, the plant latex is a colloidal system with a particle size distribution even in a nanometric range (Bauer et al., 2014). Additionally, plant latex is a good source of natural stabilizers and biosurfactants such as proteins, gums, saponins, modified lipids, rubber polymers, terpenoids, among others (Agrawal and Konno, 2009). Regarding to *G. madruno,* this species expresses its latex throughout the plant, including the epicarp. Therefore, it is feasible to attribute the self-stabilization process observed in the natural dispersions to the co-extracted natural stabilizer compounds of *G. madruno* latex. This idea is reinforced by previous works where some isolated fractions from *Garcinia* species are rich in cellulose with stabilization properties (Winuprasith and Suphantharika, 2013). To avoid further isolation process of *G. madruno* biflavonoids, the aim of this work was to develop a nanonization process from highly concentrated ethanolic extracts, using the compounds of the same plant as natural stabilizers and promoting the obtention of 100% natural formulations of *G. madruno* biflavonoids.

2. Material and methods

2.1. *Chemical and reagents*

HPLC grade acetonitrile and acetic acid were purchased by Merck (Merck Chemicals, Darmstadt, Germany). Morelloflavone and fukugiside were isolated using an High-Performance Thin Layer Chromatography (HPTLC) method previously reported (Carrillo-Hormaza et al., 2016) from a biflavonoid fraction obtained as described previously (Osorio et al., 2013). The chromatographic purity of morelloflavone and fukugiside

was > 97%. Soy lecithin (Lipoid S 75, Lipoid GmbH, Ludwigshafen, Germany), Plantacare 2000 (Alkylpolyglycoside C8/C10, BASF, Ludwigshafen, Germany), Span 80 (Sorbitan monooleate, Croda, Snaith, England), Poloxomer 188 (Kolliphor® P 188, BASF, Ludwigshafen, Germany), Polysorbate 80 (Tween® 80, Croda, Snaith, England), Polysorbate 60 (Kolliphor, BASF, Ludwigshafen, Germany), Polysorbate 20 (Caelo, Heilen, Germany), TPGS (D-α-tocopherol polyethylene glycol 1000 succinate, Gustav Parmentier GmbH, Frankfurt, Germany) and three mixtures of these surfactants were used as stabilizer for the production of the nanosuspensions.

2.2. Plant material

A pool of healthy fruits of G. madruno were collected between September 2016 to September 2017 in farms located in the Andean region of Colombia. The fruits were manually separated to obtain the epicarp, which was homogenized in a blender by adding a 10:1 (w/w) ratio of epicarp and water, respectively. The resulting epicarp paste was freeze-dried (Eyela Freeze Dryer FDU-1200) and the lyophilizate was ground in a hammer mill (IKA A11 Basic) and sieved through a mesh 30 and 80. Afterwards, the epicarp powder fractions obtained between both sieves were collected, stored for short periods of time at room temperature, and protected from light and moisture until the extraction processes.

2.3. Extraction treatments

For all extractions performed, an LSP-500 ultrasonic liquid processor (Sonomechanics, New York, USA) equipped with an ultrasonic generator of 500 W, an air-cooled piezoelectric transducer (ACT-500), a full-wave Barbell Horn™ (FBH, 21 mm tip diameter) and a reactor chamber (304 stainless steel) were used. For the continuous flow mode, a Masterflex L/S digital peristaltic pump system with Easy-Load® II pump head, a glass jacketed beaker of 1 L and a Polystat Cooling Circulating Bath were coupled to the ultrasonic system. All extraction processes were carried out by using previously established conditions (Carrillo-Hormaza et al., 2019, 2016): constant frequency of 20 kHz (± 0.5) with a batch size of 500 mL of a mixture of ethanol/water 50:50 (V/V) as extraction solvent, a pump flow of 400 µL/min, an amplitude of 50% and an extraction time of 10 min. All the extracts obtained were filtered under vacuum using a paper filter (10 µ) and Büchner funnel.

A total of four different liquid extracts were obtained applying the general extraction conditions described above with the specific conditions shown in Table 1: Extract 1 (E1) was the filtered liquid obtained after one extraction process using epicarp powder as plant material; Extract 2 (E2) was the filtered liquid obtained after two successive extraction processes using the same extraction liquid with two parts of epicarp powder as plant material; Extract 3 (E3) was obtained in the same way of E2, but changing the starting plant material of the second extraction step, where a previously washed epicarp powder was used instead; and Extract 4 (E4) was obtained in the same way of E1, but using washed epicarp. The powder epicarp was subjected to a clean-up process to eliminate the most polar compounds (washed epicarp). For this step, a controlled sonication bath (Elma P60H, Singer, Germany) was used with a fixed power of 760 W, a frequency of 37 kHz, and using water as a solvent with a solid to liquid ratio of 1:10 (w/w). After filtering, the liquid was discarded, and the powder obtained was dried at 40 °C for three days in a drying oven. The resulting epicarp was used in the corresponding extraction procedures as “washed epicarp” in E3 and E4 (Table 1). Each extraction treatment specified in Table 1 was carried out at least in triplicate, and the liquid extracts obtained were stored in the dark at 4 °C until the evaporation step.

2.4. Preparation of suspensions

Two different procedures were applied to prepare G. *madruno* biflavonoid suspensions:

2.4.1. Natural suspensions of biflavonoids from liquid extracts

The four liquid extracts were pressure-controlled evaporated using a rota-evaporator (Hei-VAP Core, Heidolph, Germany). The evaporation process was carried out until reaching 50% of the initial volume of each extract applying the following conditions: bath temperature of 40 °C, 150 rpm of rotation speed and a vacuum ramp from 120 to 65 mbars in 20 min. Each evaporation process was performed at least in triplicate, and the suspensions obtained were stored in the dark at 4 °C until further analysis.

2.4.2. Biflavonoid suspensions formulated with commercial stabilizers

NS-3 was freeze-dried (Eyela Freeze Dryer FDU-1200) and the powder obtained, freeze-extract (F-E) was ground in a hammer mill brand Actum (Medellin, Colombia). Different aqueous suspensions with 20 mL of total volume were prepared using 5% (w/v) of F-E and 1% (w/v) of different stabilizers. Under continuous stirring at 700 rpm, the amount of required stabilizer was added to the total volume of water. F-E was added slowly after the complete incorporation of stabilizer and the suspension obtained was stirred for 30 min. A

Table 1 – Specific experimental conditions of the different extracts performed.

Experimental conditions		Extract 1 (E1)	Extract2 (E2)	Extract 3 (E3)	Extract 4 (E4)
First extraction	**Solvent**	Ethanol/water 50:50			
	Plant material	Epicarp powder	Epicarp powder	Epicarp powder	Washed epicarp
	Plant material (%)	7%	7%	7%	7%
Second extraction	**Solvent**	Not performed	First extract	First extract	Not performed
	Plant material		Powder epicarp	Washed epicarp	
	Plant material (%)		7%	7%	
After evaporation step (Code)		Natural suspension 1 (NS-1)	Natural suspension 2 (NS-2)	Natural suspension 3 (NS-3)	Natural suspension 4 (NS-4)

total of 11 suspensions were prepared using the following stabilizers and mixtures: soy lecithin (SL), Plantacare 2000, Span 80, Poloxomer 188, Polysorbate 80 (Ps 80), Polysorbate 60 (Ps 60), Polysorbate 20 (Ps 20), TPGS, Mix 1 composed of Ps 20 and Ps 60 (70:30), Mix 2 (Ps 20 and Ps 60 55:45) and Mix 3 (Ps 20, Ps 60 and TPGS 49:21:30).

2.5. *Characterization of suspensions*

2.5.1. *Particle size, zeta potential, and microscopic characterization*

Nanosuspensions were characterized regarding size by using a combination of laser diffraction, light microscopy, and Dynamic Light Scattering (DLS). Laser diffraction (LD) was performed for all samples during the development process and the short-term stability study by using Mastersizer 3000 (Malvern Instruments, Germany). LD data are given as volume-based d(v)0.1, d(v)0.5 and d(v)0.9 values, i.e., the 10%, 50%, and 90% of the volume of the particles in the corresponding fraction have this size or less. Based on the pure morelloflavone as reference, LD data were analyzed using the Mie theory with real and imaginary refractive indices of 1.79 and 0.01, respectively. Light microscopy was used to further prove the data obtained by LD and to determine the shape of the particles throughout the study. A test with Sudan III red dye was performed as well to evaluate the presence of lipids in samples. Light microscopy was performed with an Olympus BX53 light microscope (Olympus Cooperation, Tokyo, Japan), which was equipped with an Olympus SC50 CMOS color camera (Olympus soft imaging solutions GmbH, Münster, Germany). DLS was used to assess the mean hydrodynamic particle size and the size distribution (z-average and polydispersity index) of the final selected suspensions. DLS data were analyzed by using the general-purpose mode of the Zetasizer Nano ZS (Malvern Instruments, Germany).

2.5.2. *Zeta potential*

The zeta potential (ZP) of the selected dispersions were determined (Zetasizer® Nano ZS, Malvern Panalytical GmbH, Germany) in two different dilutions medium, i) in water adjusted to a conductivity of 50 µS/cm with sodium chloride, and ii) in the original dispersion medium (supernatant of natural dispersions). Both samples of each dispersion were evaluated at the same dilution.

2.5.3. *Particles isolation*

For a comprehensive HPLC characterization, the particles of the different suspensions were isolated by centrifugation at 20 °C. An aliquot of 1 mL of each suspension was centrifuged three times at 14.600 rpm for 15 min with a rest period of 5 min among each cycle. The supernatant was collected for further HPLC-DAD analysis, and the precipitate was washed with 1 mL of pure water and centrifuged again under the same conditions. The final precipitates (isolate particles) were dried at 40 °C for three days in a drying oven, weighted and stored at 4 °C. The precipitates were dissolved in 2.0 mL of an ethanol/water 70:30 (v/v) mixture before the HPLC-DAD analysis.

2.6. *Saturation solubility determination*

An equivalent of 30 mg of total biflavonoids of each sample (pure morelloflavone, pure fukugiside, natural suspension 1, natural suspension 2, Mix 3 suspension, and freeze-dried powder extract) was added to 10 mL of deionized water (n = 3). The tubes were vortexed for 1 min and then equilibrated at 37 ± 0.5 °C for 48 h in a temperature-controlled shaking water bath. The same procedure was applied at 20 °C. Afterwards, the samples of both temperatures were centrifuged at 14,600 rpm for 15 min three times, and the concentrations of total biflavonoids, aglycones, and glycosides in the supernatant were determined by HPLC-DAD. Solubility augmentation was determined by the next equation:

$$\%K_{sp} = \frac{K_{sp}\,[BF]\ Nanosuspensions}{K_{sp}\,[BF]\ Isolated} \times 100\%$$

Where K_{sp} is saturation solubility and [BF] is the concentration of biflavonoids for the different nanosuspensions compared to isolated actives.

2.7. *Dissolution profile*

The dissolution profile of pure fukugiside and morelloflavone powders, the suspensions and the freeze-dried powder extract were performed on a Pharma Test D-63512 (Hainburg, Germany) using a USP Apparatus 2 (paddle method) in triplicate. A paddle rotation speed of 100 rpm and a temperature of 37 ± 0.5 °C were used in each study. An aliquot of the suspensions or powder (equivalent to 12 mg of total biflavonoids) were dispersed into the dissolution vessels containing 500 mL of dissolution medium (Buffer phosphate pH 6.8). Then, 2 mL of the solution were withdrawn after 5, 10, 20, 40, 60, 120, and 240 min, and replaced with an equal volume of fresh medium to maintain a constant total volume. The samples were centrifuged at 14,600 rpm for 15 min three times, and the concentrations of total biflavonoids, aglycones, and glycosides in the supernatant were determined by HPLC-DAD.

2.8. *HPLC-DAD determination*

The HPLC-DAD analysis of the liquid extracts, suspensions, supernatants and dissolved isolated particles were carried out using an Agilent 1260 Series LC system, equipped with a vacuum degasser, an autosampler, a quaternary pump and a Diode Array Detector (DAD). Separation of the compounds was performed using an Agilent Zorbax SB Rapid Resolution High Throughput® (RRHT) C18 (50 mm × 4.6 mm, with a 1.8-µm particle size) column, with a flow rate of 1.2 mL/min at 30 °C and isocratic mode composed by 0.5:39.5:60 of acetic acid, water and acetonitrile, respectively. The run time was 1 min, and the injection volume was 3 µL. The peaks were monitored using the DAD set at 290 nm, and spectra were recorded between 200 and 400 nm.

An external standard method was used for the relative quantification of the previously identified *G. madruno* metabolites (Carrillo-Hormaza et al., 2016). Two groups of compounds were included in the study: total glycosides were quantified in terms of fukugiside and total aglycones were quantified in terms of morelloflavone. From the stock solutions (2 mg/mL) of each standard, calibration curves were established with six levels in the ranges between 0.5 and 100 µg/mL for fukugiside and morelloflavone. The content of total aglycones and total glycosides were expressed as milligrams equivalent of morelloflavone or

fukugiside, respectively, by a gram of dried sample (mg/g sample) or 100 mL (mg/100 mL). The total content of biflavonoids were expressed as the sum between total aglycones and total glycosides.

2.9. Short term stability study

A short-term stability test was performed to evaluate the effects of natural and commercial stabilizers on the physical stability of the nanosuspensions stored at 4 °C. Small aliquots of suspensions were periodically withdrawn to characterize the particle size and shape up to 4 weeks of storage, i.e., at the day of production (d0), after 7, 14, 21 and 28 days of storage.

2.10. Statistical and data analysis

All the experiments were carried out at least in triplicate, and the results are expressed as the mean ± standard deviations. The dissolution profile plots and the Analysis of variance (ANOVA) were performed by using the Graph Pad Prism® version 5.00 for Windows (Graph Pad Software, Inc.- San Diego CA 2007) software. Analysis of variance (ANOVA) was carried out for each variable to test the statistical significance using a P-value at 5% level.

3. Results and discussions

3.1. Development of natural suspensions of G. madruno biflavonoids

The first step to obtaining natural suspensions of *G. madruno* biflavonoids was to characterize and define the starting liquid extract. Four extraction treatments were performed applying the conditions described in Table 1. According to the results (Fig. 1A), E3 was the extract with the highest content of total biflavonoid, followed by E2, E4 and E1. The total biflavonoid content obtained for each one of the treatments applied was 1600, 1310, 878 and 720 mg/100 mL, respectively, using for the double extractions (E2 and E3) a total of 70 g of plant material and 35 g for the single extraction treatments (E1 and E4) maintaining the same epicarp/solvent ratio for each process. Applying double extraction processes (E2 and E3) increased almost proportionally the content of biflavonoids in comparison with the single extractions. Considering that it was not possible to increase the plant material proportion during the first extraction step without compromising the fluidization of the whole system, the double extractions turned into a feasible technological approach to concentrate the content of biflavonoids in the liquid extracts. Likewise, the use of washed epicarp in E3 and E4 increased significantly the biflavonoid content in the extracts because some water-soluble hydrophilic

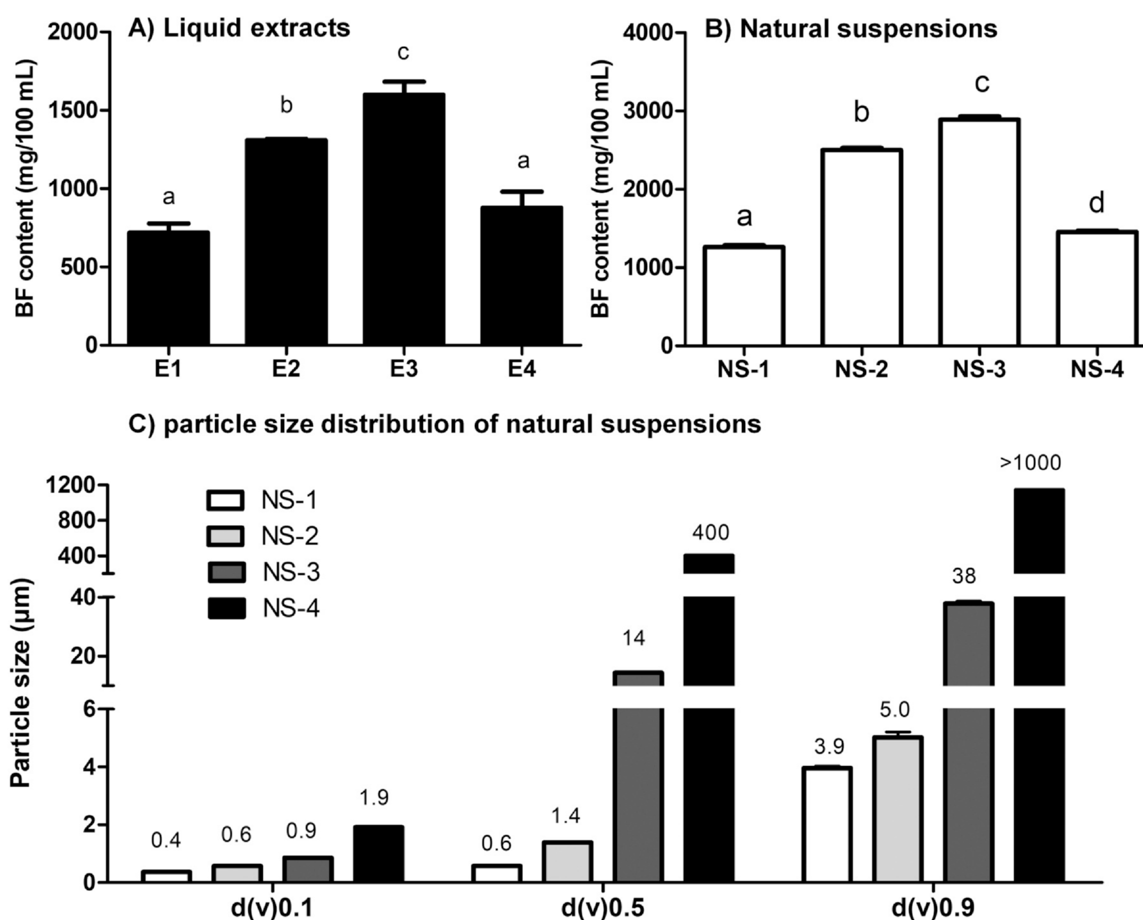

Fig. 1 – Biflavonoid content and particle size distribution of extracts and natural suspensions. A) Total biflavonoid content in the extracts (BF, biflavonoids). B) Total biflavonoid content in the natural suspensions obtained after evaporation of extracts. C) The particle size distribution of natural suspensions. Data are presented as mean ± SD (n = 5). Values within each plot, followed by the same superscript letters, are not significantly different at the 0.05 level, according to ANOVA (P > 0.05).

compounds (interferences) like sugars, organics acids, amino acids among others were removed while bioflavonoids were retained.

The extracts obtained were subjected to an evaporation process of ethanol. As result, aqueous suspensions with the half volume of the extracts, i.e. almost the double content of biflavonoids were obtained (Fig. 1B). Fig. 1C shows the particle size distribution obtained for each suspension. In general, the suspensions obtained from extracts produced with washed epicarp (NS-4 and NS-3) presented the largest particles. Considering that no stabilizers were added and no fragmentation techniques were applied, the particle sizes obtained for NS-1 and NS-2 were small. Indeed, NS-1 presented a d(v)0.5 in the nanometric range (580 nm), and the 90% of the volume of the total particles of this suspension possessed a size of 3.95 µm and below. On the other hand, significant differences were found between NS-1 and NS-4. More aggregates were observed, and a d(v)0.5 near to 800-fold higher was obtained for NS-4 when water-soluble compounds were removed from the plant material. This suggests the presence of natural compounds in *G. madruno* epicarp stabilizing the biflavonoid suspensions (NS-1 and NS-2). Thus, among the natural suspensions obtained, NS-1 and NS-2 were selected to continue the characterization process and the stability and solubility studies.

3.2. Development of *G. madruno* biflavonoids suspensions with commercial stabilizers

A reference suspension was developed from the F-E powder using different commercial dispersion stabilizers to be compared against the performance of NS-1 and NS-2. A pool of NS-3 was selected to obtain F-E due to its high amount of biflavonoids (Fig. 1A) and because only with this suspension, a stable and fine powder after freeze-drying was obtained. With F-E, eleven different suspensions were formulated. According to the results (Fig. 2), only the suspensions prepared with stabilizers that contain PEG in their chemical structures (Poloxamer 188, Ps 80, Ps 60, Ps 20 and TPGS) reduced the particle size distribution compared with the re-suspended F-E in water. In the case of the dispersions obtained with surfactants without PEG (soy lecithin, Plantacare 2000 and Span 80), the particle size obtained was larger in comparison with the re-suspended F-E, especially for soy lecithin. Among PEG surfactants, the median particle size (d(v)0.5) obtained for the suspensions decreased in the following order: Poloxamer 188 > TPGS > Ps 60 > Ps 80 and Ps 20 in a range from 4.1 to 0.85 µm. However, for the smallest particles (d(v)0.1), the suspension stabilized with TPGS had the lowest particle size (70 nm). Overall, polysorbates showed the best performance. Suspensions stabilized with Ps 20 presented the smallest median particle size and suspensions with Ps 60 the narrowest particle distribution. Among polysorbates, the effectivity as stabilizer was related to the chain length of fatty acid: the smaller the chain, the smaller the particle size distribution of the natural suspensions as well. This might be correlated with the intermediate lipophilicity of biflavonoids and the possibility to interact in a better way with medium-chain fatty acids, like the one found in Ps 20.

The use of surfactant mixtures has been widely used in the nano-dispersions formulation. When mixtures are used, it is possible to improve the physical stability (Cerdeira et al., 2013), the particle size distribution (Niwa et al., 2011) and even the dissolution profile (Hong et al., 2014) of nano-formulations. Therefore, three mixtures were prepared using the stabilizers that provided the narrowest and smallest particle size distribution (Ps 20, Ps 60, and TPGS- Fig. 2). Mix-1 and Mix-2 were Ps 20 and Ps 60 mixtures at 70:30 and 55:45 proportions, respectively. No differences were found between these two mixtures. However, both mixtures reduced the particle size slightly in comparison with the suspensions stabilized by only one surfactant, guaranteeing to be in a submicrometric range (Fig. 2). On the other hand, the suspension formulated with the combination of the three surfactants (M3-S) reached the smallest d(v)0.5 particle size (122 nm). The addition in small proportions of TPGS to the Ps 20 and Ps 60 mixture could reduce significantly the particle size of the biflavonoid particles validating the results obtained in the experiments with only one surfactant. Thus, Ps 20 and TPGS promote a particle size reduction in M3-S whereas Ps 60 maintain the particle size distribution.

3.3. Characterization of suspensions

As shown in Table 2, the mean particle sizes of NS-1, NS-2 and M3-S were all in the submicrometric scale (< 1000 nm). The smallest particle size was obtained in M3-S, following by NS-1 and NS-2. The particle size was directly correlated with the total content of biflavonoids. The most concentrated suspension (NS-2) had the largest median particle size and polydispersity index (PdI). Synergies between natural

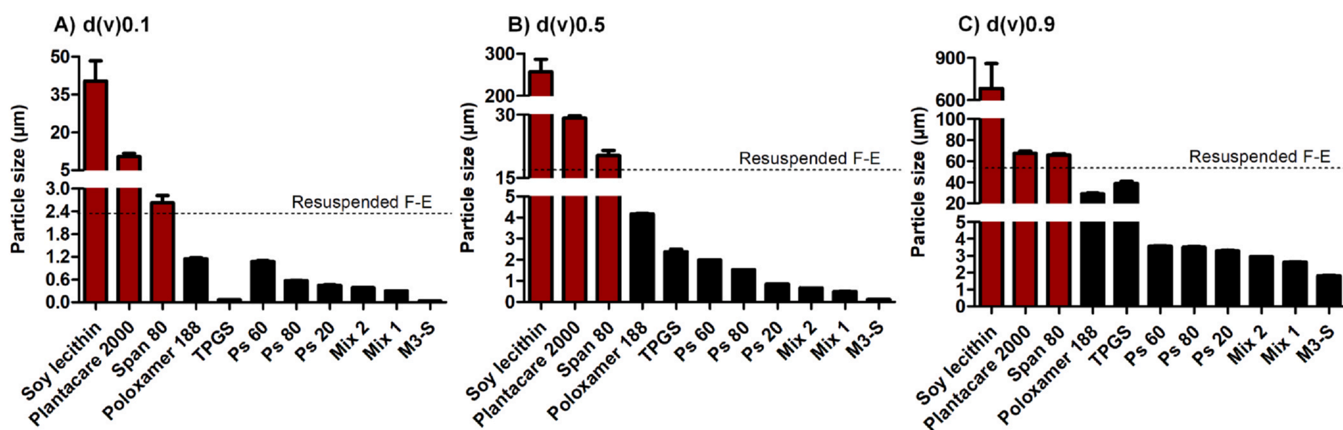

Fig. 2 – Effect of different stabilizers on the particle size of suspensions obtained from freeze-dried powder extract (F-E). All treatments were made with 5% of F-E and 1% of stabilizer.

Table 2 – Zeta potential, particle size and biflavonoid content of suspensions.

Suspensions	Zeta potential[a] (mV)	pH[a]	Zeta potential[b] (mV)	pH[b]	Particle size (z-average nm)	PdI	Biflavonoid content (mg/100 mL)
NS-1	-19.9 ± 1.3	4.16 ± (0.045)	-1.8 ± 0.2	2.86 ± (0.026)	608.5 ± 20.1	0.141 ± 0.100	1303.0 ± 56.4
NS- 2	-18.4 ± 1.1	4.05 ± (0.022)	-1.6 ± 0.2	2.74 ± (0.038)	880.0 ± 50.8	0.414 ± 0.083	2430.1 ± 106.3
M3-S	-23.5 ± 1.2	4.03 ± (0.037)	-4.6 ± 0.7	2.71 ± (0.021)	256.6 ± 4.6	0.159 ± 0.044	1237.6 ± 35.7

[a] Measured in water.
[b] Measured in the own supernatant.

stabilizers and the surfactants used in M3-S reduced the particle size compared with natural suspensions. Nevertheless, the particle size achieved for NS-1 and NS-2 was appropriate as well, considering that, no fragmentation procedure was applied and no commercial stabilizers were added.

Wet millings, high-pressure homogenization (HPH), and ultrasounds are some of the available techniques more used for the reduction of particle size at a lab, pilot, and industrial scale. For instance, a nanosuspension of quercetin formulated with 2% of Ps 80 and obtained after 30 cycles of HPH reached a particle size of 412 nm (Karadag et al., 2014). A similar result was obtained for a myricetin nanosuspension formulated with a mixture of cyclodextrin / TPGS (2%) and 20 HPH cycles to obtain a final particle size of 316 nm (Hong et al., 2014). However, all these fragmentation processes consume a lot of time and energy to achieve the rupture of the particles by the shearing and collision forces generated by the equipment. Thus, when these techniques are used the manufacturing cost of products increase significantly and also, they require meaningful investments and high maintenance costs for their operation (Cheow et al., 2013; Fontana et al., 2018). Therefore, with the natural suspensions, the fragmentation step and the use of commercial stabilizers are avoided. This contributes to the sustainability of the process by saving energy and time, which reduces the manufacturing cost and making an eventual scaling process more feasible. Additionally, green and 100% natural formulations are obtained.

Further information about the size, distribution, and shape of the micro and nanodispersions produced by different techniques, were visualized by microscopy. Hence, a kind of spherical particles was observed for all suspensions (Fig. 3A, B and C). The aspect and shape seemed to be more liquid drops or vesicles than solid particles. Biflavonoids are very slightly water and oil-soluble and therefore with the evaporation of ethanol, suspended solid particles of these compounds were expected. Under the extraction procedure applied (50% ethanol), the amount of extracted oil would be too low to create an emulsion, because very non-polar compounds are not extracted under these conditions. Also, the test with Sudan III red reactive was negative for the natural dispersions, indicating the absence of lipids (data not shown). Therefore, a natural vesicular system with the biflavonoids dissolved inside the drops could have been obtained as well.

By applying a high-speed centrifugation process, the dispersions were separated in a precipitate on the bottom and the aqueous supernatant above. The content of total biflavonoids was quantified in both fractions (Fig. 3D and E). The total biflavonoids content in the precipitate of NS-1, NS-2, and M3-S were 20.8%, 33.6%, and 33.2%, respectively. Non-significant differences were found between NS-2 and M3-S, whereas NS-1 presented the lowest amount of biflavonoids in the isolated particles. Around 70% of the isolated particles of NS-2 and M3-S corresponded to unknown compounds bonded to the biflavonoid core, whereas the particles obtained for NS-1 had even a higher amount of these compounds (≈80%). The unknown compounds bonded to the biflavonoids core would be creating the corona layer involved with the stabilization of these dispersions and these are proposed as the natural stabilizers of the formulations. Unlike the particle composition, the content of total biflavonoids in solution (supernatants) showed a remarkable

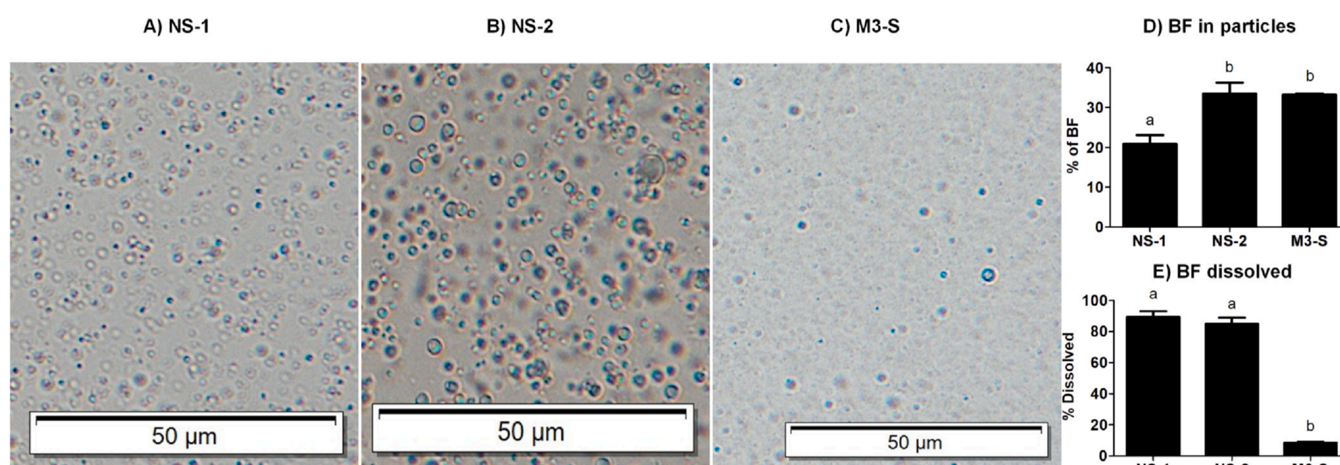

Fig. 3 – Morphological and chemical characterization of particles. Microscopic pictures of NS-1 (A); NS-2 (B); and M3-S (C). Biflavonoid content in isolate particles (D). Total biflavonoids dissolved in the supernatant (E). Data are presented as mean ± SD (n = 6). Values within each plot, followed by the same superscript letters are not significantly different at the 0.05 level, according to ANOVA (P > 0.05).

difference between natural suspensions and M3-S (Fig. 3E). Around 85% of the total biflavonoid content found in NS-2 and NS-1 were dissolved in the supernatant. In contrast with M3-S, where only 8.5% of biflavonoids was solubilized in the aqueous medium. This result indicates that the natural compounds involved in the stabilization process of the natural dispersions not only influenced on the surface of the particles, but they also promote a co-solvent effect, increasing the proportion of compounds in solution in the aqueous medium.

The zeta potential gives a hint of the long-term physical stability of a dispersion (Rachmawati et al., 2013). The zeta potential was measured by diluting the sample in water and the original dispersion medium (Table 2). The results in water showed similar effects for the three formulation, wherein all cases negative values around – 20 mV were obtained, indicating the presence of a negatively charged layer at the particle surface. All formulated dispersions were complex samples with probably more than 20 biflavonoids in the particles and many other unknown compounds loosely bound on their surfaces and dissolved in the supernatant. Therefore, high chemical diversity can create different types of particle-particle and particle-media interactions. Thus, for these types of samples, an alternative to infer the electric phenomena of the original undiluted system, is to measure the zeta potential using the original dispersion medium obtained after a centrifugation process of each dispersion (Cano-Sarmiento et al., 2018). The zeta potential value obtained for each formulation diluted in the corresponding supernatant was drastically reduced, reaching values near to zero (between –1.8 and –4.6 mv, Table 2). Taking into account that all dispersion formulated had a pH of around 2.8 due to the high content of organic acids found in *G. madruno* epicarp (Carrillo-Hormaza et al., 2016), the zeta potential change observed among both medium could be caused by the acidic environment of the natural dispersion medium, where the high content of hydrogen ions (H^+) could be attracted, promoting a neutralization of the charge and a shift of the zeta potential to values near to zero. Similar dependence on pH has been reported in a dispersion of quercetin stabilized by electrostatic repulsion with negative charges (Son et al., 2019). Additionally, this could be a hint of a growing corona layer, i.e. a strong tendency to likely absorb on the crystal surface. Hence, a large layer such as observed for non-ionic polymer surfactants, shifts the plane of shear to larger distance, which in turn led to reduced zeta potential measurement (Kakran et al., 2012).

Although there is no available information about any natural stabilizers or biosurfactant isolated or identified in *G. madruno*, even the information about this genus is very scarce. There are only some reports about saponins from *G. kola* roots, however, without an in-depth chemical study have been published (Elekofehinti, 2015). Several studies correlate the latex of *Garcinia* species with the expression of phenolic compounds (Hemshekhar et al., 2011). For instance, the latex of *G. mangostana* is secreted throughout the plant, including the whole fruit, leaves, stem, and roots (Tjitrosemito and Juliarini, 2008). In some species of the genus, the latex may contain more than 20% of phenolic compounds (Murthy et al., 2017).

3.4. *Short term stability*

The changes in particle size of the three formulations after four weeks of storage at 4 °C are shown in Fig. 4. In general, no significant changes in the particle size were observed for the natural dispersion through all the period evaluated at d (v) 0.1 and 0.5 level. On the other hand, significant changes were observed for M3-S, mainly during the first week. The particle size (d(v) 0.5) of this suspension increased from 122 nm to 398 nm after 7 days of storage. After this time, the particle size continued increasing, but the changes were not significant. The high content of dissolved biflavonoids in NS-2 and NS-1 (Fig. 3E) supposed a potential instability of these formulations by particle growth with the available compounds in solution (Ostwald ripening). This effect could be observed for NS-1 and NS-2 after 4 weeks of storage for the largest particles (d(v) 0.9). The corona layer formed around the particles with the own natural stabilizers helped to prevent the growth and aggregation of particles between the natural dispersions and M3-S. NS-2 had a higher concentration of biflavonoids and less amount of stabilizer compounds bonded to the biflavonoid core compared with NS-1 (Fig. 3). Probably this contributed to increasing the attraction forces

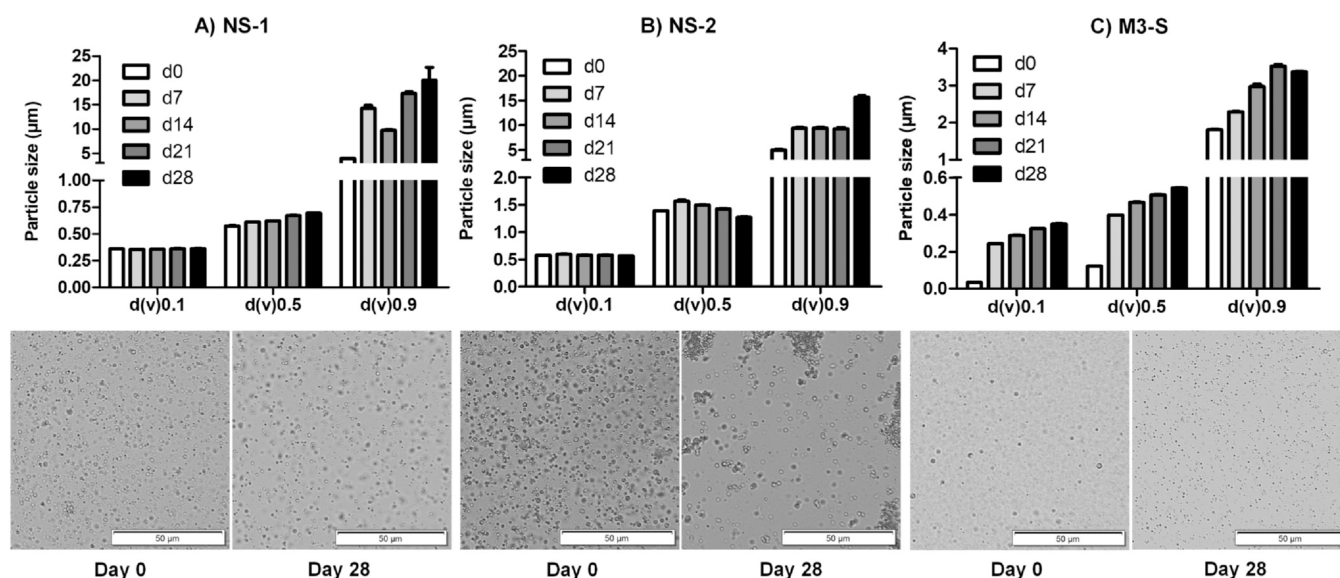

Fig. 4 – Short-term stability at 4 °C of NS-1 (A), NS-2 (B) and M3-S (C).

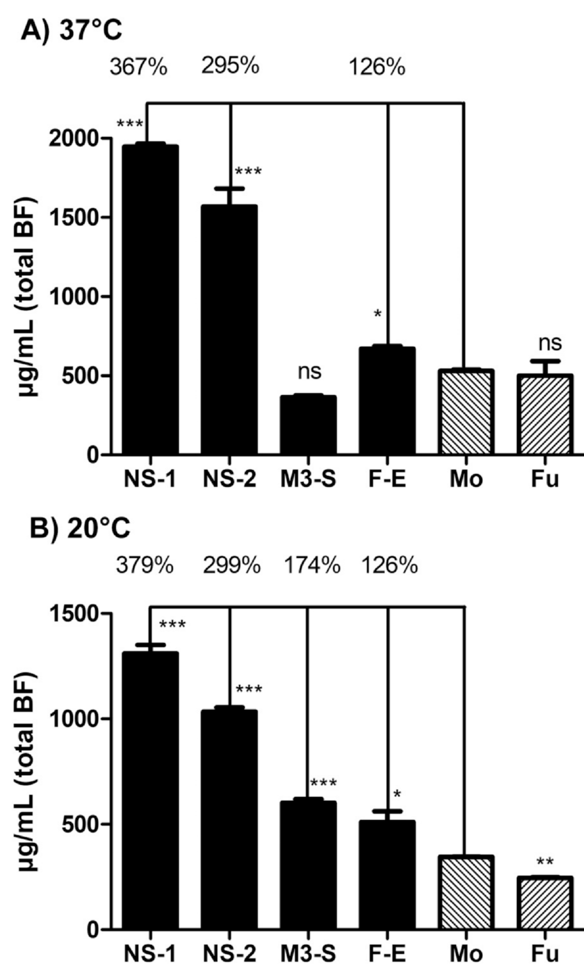

Fig. 5 – Saturation solubility at 37 °C (A) and 20 °C (B) of isolate biflavonoids (morelloflavone-MO, Fukugiside-FU) natural dispersions (NS-1 and NS-2), Mix 3-supension (M3-S) and freeze-dried powder extract (F-E). Data are presented as mean ± SD (n = 3). Analysis was performed using ANOVA and the Bonferroni test. p < 0.05 significant (*), p < 0.01very significant (**), and p < 0.001 extremely significant (***).

among particles in the undiluted system that had a zeta potential near to zero, promoting the formation of the agglomerates observed in the microscope after 4 weeks of storage (Fig. 4B).

Considering that non-ionic surfactants were used in M3-S and the chemical nature of the natural stabilizers is unknown for the natural suspensions, the zeta potential in both mediums and the short-term stability results suggest a mixed mechanism of stabilization involving a steric impediment and moderate electrostatic repulsion for all suspensions formulated. Probably, saponin glycosides and some carbohydrates as cellulose are the natural polar compounds that promoting the stabilization of the dispersions. However, further characterization experiments are required with the particles to identify the compounds involved with the direct stabilization and dissolution of the biflavonoids.

3.5. *Solubility studies*

NS-1, NS-2, M3-S, F-E and the pure compounds morelloflavone and fukugiside, were evaluated in terms of their saturation solubility (Fig. 5) and biflavonoid dissolution profile (Fig. 6). The solubility at 37 °C of pure morelloflavone and fukugiside was 531 and 501 µg/mL, respectively. No significant differences were found among them, and according to the values obtained, both would classify as "very slightly soluble compounds". Regarding the formulations, the solubility at 37 ° of total biflavonoids increased in the following order M3-S < F-E < NS-2 and NS-1. Only M3-S had a lower solubility in comparison with the pure compounds, whereas NS-1, NS-2, and F-E achieved to increase in 367%, 295% and 126% the saturation solubility at 37 °C compared with the value of pure morelloflavone. The behavior obtained at 20 °C was similar to 37 °C. Only M3-S showed a different result. The saturation solubility increased significantly, even the value obtained overcome F-E and pure compounds at 20 °C (Fig. 5B). The low solubility observed for M3-S at 37 °C was mainly due to the instability of this formulation with temperature promoting a high adhesiveness to the glass surfaces. Only under this condition, some sticky aggregates were seen around the flask walls, and some biflavonoids could get trapped there. In general, the M3-S had a low performance in comparison with the natural suspensions (NS-1 and NS-2), considering that M3-S formulation had the smallest particle size. This result seemingly contradicts the Ostwald–Freundlich equation, which describes that the saturation solubility is inversely proportional to the particle size (Arnold, 1995). On the other hand, small particles possess a much higher adhesiveness which led to the observed sticky aggregation. This in turn reduces the total surface area and thus the dissolution rate. However, the high amount of biflavonoids found in solution and the higher content of natural stabilizers and co-solvents, could also explain the better results obtained for natural suspensions.

Unlike the saturation solubility results, the dissolution profile of aglycones and glycosides differed significantly among them. Around 80% of the total fukugiside was dissolved in four hours (Fig. 6B), whereas only 25% of the available morelloflavone was dissolved (Fig. 6C). On the other hand, NS-1 was the only suspension that solubilized all its biflavonoids (Fig. 6A). In general, the main achievement of natural suspensions was to increase the dissolution rate of aglycones. For instance, all the aglycones of NS-1 solubilized before 10 min, whereas only less than 10% of the pure morelloflavone was dissolved in the same period of time (Fig. 6C). This result is of high relevance when it is analyzed the bioavailability of these compounds. In general, as pure compounds, the flavonoid glycosides are more soluble and bioaccessible than aglycones. However, for the glycosides, the body's absorption by simple diffusion in the stomach and intestine is limited (Antunes-Ricardo et al., 2017). The glycosides require an extra step of deglycosylation by the human small intestine and liver beta-glucosidase, decreasing the absorption rate and the bioavailability of the aglycones bonded (Lewandowska et al., 2013). With our natural suspensions, the dissolution rate and solubility of its aglycones improved significantly. Therefore, the bioaccessibility should increase as well, resolving the main problem of bioavailability of these compounds.

The dissolution profiles obtained were highly correlated with the result of saturation solubility. Hence, NS-2 showed slightly lower performance than NS-1, with around 80% of biflavonoid released, followed by F-E (70%), and the lowest value was for M3-S (50%). M3-S presented the same incompatibility with the temperature observed in the saturation solubility test, limiting its potential as a scaling prototype (Fig. 6).

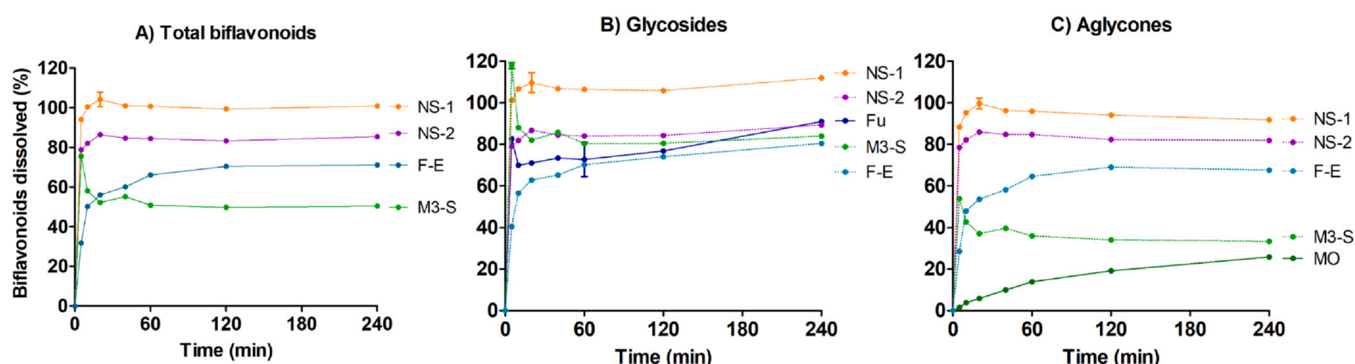

Fig. 6 – Dissolution profile of total biflavonoids (A), total glycosides (B) and total aglycones dissolved (C) for the isolated biflavonoids and formulation elaborated.

Overall, the increment of the solubility and the dissolution rate of biflavonoids formulated by natural suspensions were due to the following factors: i) decreasing the size of biflavonoid particles to the submicrometric scale. Although this was not the main factor because M3-S had the smallest particle size and the worst solubility; ii) a high amount of biflavonoids in solution in the dispersion medium. This was the main factor involved with the high performance of the natural suspensions, already almost 85% of total biflavonoids found in the undiluted suspensions were in solution; and iii) the presence of natural stabilizers, these compounds were directly responsible for reducing the particle size, stabilize the formulation, and improve the solubility of biflavonoids.

4. Conclusions

This study allowed developing for the first time 100% natural nanosuspensions of *G. madruno* biflavonoids without adding any commercial stabilizer and without using high energy consumption fragmentation techniques. The natural dispersions presented a suitable stabilization mediated by the own compounds of *G. madruno* peel. These compounds had a dual effect as natural stabilizers and as natural co-solvents, increasing significantly the amount and dissolution rate of biflavonoids in solution. The natural suspensions were obtained by the co-extraction of biflavonoids with the natural stabilizers present in *G. madruno* peel and their followed reconstitution of particles through the solvent evaporation step. Undoubtedly, our results propose to the natural dispersions of *G. madruno* biflavonoids as a novel and natural active ingredient to incorporate in a wide range of functional products. Likewise, future researches are required to evaluate the chemistry and potential of new *G. madruno* biosurfactants to stabilize other dispersions with different active compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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