Journal of Food Engineering 103 (2011) 188–196

Contents lists available at ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng

Flavonoid microparticles by spray-drying: Influence of enhancers of the dissolution rate on properties and stability

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ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form 18 October 2010
Accepted 20 October 2010
Available online 25 October 2010

Keywords:
Quercetin
Naringenin
Spray-dried gastroresistant microparticles
Enhancers of the dissolution rate
Storage stability
Antioxidant activity

ABSTRACT

Naringenin (Nn) and Quercetin (Q) have numerous health benefits particularly due to their antioxidant properties. However, their low solubility, bioavailability and stability limit their use as components for functional foods, nutraceuticals and pharmaceutical agents. In this research, Nn- and Q-microparticles were produced by a spray-drying process using a combination of cellulose acetate phthalate (CAP) as coating gastroresistant polymer and swelling or surfactant agents as enhancers of dissolution rate. Raw materials and microparticles produced were all characterized by particle size analysis, differential scanning calorimetry, X-ray diffraction, and imaged by electron and fluorescence microscopy. During 12 months, storage stability was evaluated by analyzing drug content, HPLC and DSC profiles, as well as antioxidant activity (DPPH test). *In vitro* dissolution tests, using a pH-change method, were carried out to investigate the influence of formulative parameters on flavonoid release from the microparticles. Presence of a combination of CAP and surfactants or swelling agents in the formulations produced microparticles with good resistance at low pH of the gastric fluid and complete flavonoid release in the intestinal environment. The spray-drying technique and the process conditions selected have given satisfying encapsulation efficiency and product yield. The microencapsulation have improved the technological characteristics of the powders such as morphology and size, have given long-lasting storage stability and have preserved the antioxidant properties.

1. Introduction

Flavonoids are benzo-γ-pyrone derivatives belonging to the polyphenolic class widespread in vegetables. Food and medicinal plants contain amount of flavonoids ranging from traces to several grams per kilogram of fresh weight. Due to their biological activity (Peterson and Dwyer, 1998), flavonoids find use in herbal medicinal preparations and food supplements marketed in Europe (Monthey and Guthrie, 2002; Boots et al., 2008). Naringenin (Nn) is the predominant flavanone in grapefruit whereas Quercetin (Q) is the major dietary flavonol. Both can be largely found in onions, broccoli, apples, grapes, beans, orange, black and green teas. Epidemiological studies suggest that a diet rich in these micronutrients may be associated with decreased cardiovascular damages and cancer risks (Somerset and Johannot, 2008; Benavente-Garcia and Castillo, 2008). The beneficial effects on human health are particularly due to their antioxidant activity and ability to chelate metals, to scavenge oxygen free-radical, to inhibit enzymes and to prevent oxidation of low density lipoproteins (LDL) (Madsen et al., 2000; Yu et al., 2005). As a drawback, Q and Nn, as much as other polyphenols, are easy degradable because of their sensibility to environmental factors such as light, heat and oxygen. Moreover, the health benefit of Nn and Q are unfortunately limited after their oral administration. In fact, they have a very slightly water solubility (few mg/L), and can undergo to degradation in the drastic acidic stomach environment (Chebil et al., 2007; Srinvas et al., 2010). These properties cause low flavonoids dissolution rate from oral solid dosage forms such as capsules and tablets or from meal as well as the partial degradation in the harsh pH conditions of gastric environment, resulting in a low absorption and bioavailability (Hu, 2007; Erlund, 2004). Nevertheless, relatively high levels of Q and Nn can be adsorbed through the small intestine (Spencer et al., 1999; Hsiu et al., 2002).

Thus, to encapsulate flavonoids in gastroresistant polymers, carrying them directly to the intestine, may improve their bioavailability after oral administration. In addition, the microencapsulation, depending on the polymers and technology used, may stabilize these labile compounds and extend their shelf-life. From an industrial perspective, microencapsulated powders would be easy to handle and to use in food and pharmaceutical processing keeping their initial flavonoid content, bioactivity, and safety in case of prolonged storage. In microsystems, stabilization occurs because the wall/coating material acts as a physical and permeable

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0260-8774/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jfoodeng.2010.10.015

barrier to oxygen and small molecules, for this reason the shelf-life of the encapsulated drugs is consequently prolonged. Moreover, polymers may modulate the release rate and solubility in intestinal fluid (Manach et al., 2005). Among the preparation methods of microparticles, spray-drying is widely used in pharmaceutical and biochemical fields and in food industry due to large availability of equipments and easiness of industrialization. It is also a mild "one-step" processing operation to move from a liquid feed into a powder product (Cal and Sollohub, 2010; Sollohub and Cal, 2010). Since the fast solvent evaporation keeps droplets temperature far below the drying air temperature, spray-drying is strongly recommended for heat sensitive materials such as flavonoids (Ersus and Yurdagel, 2007). In our previous works, the aglycones Nn and Q, and their corresponding glycosides, Naringin and Rutin, having different structures and physicochemical characteristics, were microencapsulated by spray-drying using cellulose-derived polymers (Lauro et al., 2005, 2007). Protective power of cellulose derivatives is based either on the ability to form amorphous matrices loading drugs during the spray-drying process and on their pH-dependent solubility. Cellulose acetate phthalate (CAP) is insoluble and stable in acidic gastric fluid as in its non-ionized form. It becomes soluble, swellable and disintegrable as the phthalic acid groups ionize above pH 6.0 in intestinal fluid (Béchard et al., 1995). However, results showed that satisfying gastroresistant microsystems were produced only in case of more soluble glycosides such as Rutin and Naringin (Lauro et al., 2005, 2007). An incomplete flavonoid release, in the simulated intestinal fluid, was observed in the case of very slightly water soluble aglycones such as Q and Nn (Lauro et al., 2005, 2007).

Thus, the aim of the present work was to produce Nn and Q gastroresistant microparticles by spray-drying studying the influence of various enhancers of dissolution rate on their properties. Since the design and development of gastroresistant microparticles containing low-solubility ingredients requires a compromise between the enhancement of dissolution rate and protection in the gastric environment, we have investigated the effects of the combined use of CAP as coating polymer and three different materials able to enhance the dissolution rate. Two surfactants, Sodium dodecylbenzensulfonate (SDBS) and Tween 85 (Tween) or a swelling agent, Sodium carboxymethylcellulose crosslinked (CMC), were tested as enhancers. The influence of formulative parameters on yield of the process and microparticle properties such as morphology, size, thermal behaviour was investigated. Particular stand out has been made on the dissolution/release rate as well as on the storage stability. Last one was evaluated either in term of flavonoid content and bioactivity.

2. Materials and methods

2.1. Chemicals

Naringenin (Nn, CAS No. 67604-48-2) and Quercetin hydrate (Q, CAS No. 849061-97-8) were supplied by Sigma Aldrich (Milan, Italy); Cellulose acetate phthalate (CAP) from Eastman® Kodak (Kingsport, Tennessee, United States); Vivasol® Croscaramellose sodium (carboxymethylcellulose crosslinked, CMC) from J. Rettenmaier & Söhne (Rosenberg, Germany); Sodium dodecylbenzensulfonate – (SDBS) from Sigma Aldrich (Milan, Italy) and Polyoxyethylene Sorbitan Trioleate (Tween 85) from A.C.E.F. s.p.a. (Piacenza, Italy). All other chemicals used were of reagent grade.

2.2. Flavonoids solubility

Solubility of Nn and Q was evaluated according to USP 31 (USP 31, 2008) by UV spectrometry and HPLC at room temperature (25 °C) in distilled water and in simulated biological fluids without enzymes, (Gastric Fluid, GF, pH 1.2, and Intestinal Fluid, IF, pH 7.5). Each analysis was made in triplicate and results expressed as average values in terms of mg/L. An excess amount of each flavonoid was introduced into glass vials containing 50 mL of solvents; samples were shaken and then stored at room temperature. After 3 days, liquid phases were centrifuged for 15 min at 3000 rpm, and supernatants filtered with 0.45 μm filters and analyzed by UV and HPLC.

2.2.1. UV method

Flavonoid concentration in the supernatant was evaluated by measuring absorbance at λ 310 nm for Nn and at 366 nm for Q in 1 cm cell (UV/Vis spectrometer Lambda 25, Perkin–Elmer Instruments, MA, USA). Calibration curves were previously worked out using distilled water and biological fluids without enzymes. Proportionality between absorbance and concentration was verified in the range 5–50 mg/L for both Nn and Q ($R^2 > 0.999$ for both drugs).

2.2.2. HPLC method

Flavonoid concentration was also evaluated by an HPLC apparatus (Agilent 1100 series system) equipped with a Model G-pump, a DAD G-1315 A detector set at λ 310 nm for Nn and 366 nm for Q, loop 20 μL, and a 150 × 3.9 mm i.d. C-18 μ-Bondapack column. The eluent was MeOH/H_2O (50:50 v/v for Nn and 55:45 for Q), flow rate of 1.0 mL min^{-1}. *Linearity*. Reference standard solutions were prepared each in triplicate at three concentration levels (0.05–1.0 mg/mL) and were injected (20 μL) three times each. The standard curve was analyzed using the linear least-squares regression equation derived from the peak areas (Nn regression equation $y = 33483x - 387.99$, $R^2 = 0.999$; Q regression equation $y = 3849.358x - 5349.742$, $R^2 = 0.999$ where y is the peak area and x the concentration used). *Specificity*. Peaks associated with Nn or Q were identified by retention times and confirmed by co-injections, as well as by UV (Perkin–Elmer Lambda 25 spectrometer) and MS spectra compared with standard. The MS instrument used was a Q-TOF Premier Waters triple-quadrupole orthogonal time-of-flight (TOF) having an electrospray ionization source.

2.3. Preparation of feed mixtures

CAP (2 g) was dissolved in 100 mL aqueous buffer feed solution (pH 7.5). CMC was mixed with Q or Nn in 1.5:1 weight ratio using a Galena Top (Ataena, Tecno-Pro srl, Italy) mixer in presence of micronizing spheres until uniformity. The resultant dry mixtures (CMC/flavonoid 1.67 g) were suspended into the CAP liquid feed reaching the 3.67% w/v final concentration (batches #1a and #2a). Q or Nn were previously dampened with SDBS (#1b and #2b) or with Tween 85 (#1c and #2c) in flavonoid/surfactant weight ratio 1:0.75, and then the soaked mixtures (surfactant/flavonoid 1.17 g) were suspended into the CAP liquid feed reaching the final 3.17% w/v content. The obtained feeds were spray-dried.

As a reference, enhancers-free microparticles (#1d and #2d), CAP-free microparticles (#3a–c and #4a–c) and drug-free microparticles (#A, #B and #C) were prepared by spray-drying under the same experimental conditions. The solvent (aqueous buffer solution), CAP concentration (2% w/v), and the polymer/drug weight ratio (3:1) were kept constant for each formulation. For processing 200 mL feeds were prepared under continuous magnetic stirring and sonicated for 10 min. Compositions of the different spray-dried microparticles are reported in Table 1.

Table 1
Composition and characterization of all the microsystems prepared.

Batches #	Aqueous feed composition	Flavonoid concentration (% w/v)	Enhancer concentration (% w/v)	CAP concentration (% w/v)	Yield %	TDC %	ADC %	EE %	d_{50} μm (span)
#1a	CAP/Nn/CMC	0.67	1.0	2.0	76.0	18.0	12.0	67.0	44.20 (3.99)
#1b	CAP/Nn/SDBS	0.67	0.50	2.0	89.0	21.0	13.0	62.0	15.99 (1.96)
#1c	CAP/Nn/Tween	0.67	0.50	2.0	65.0	21.0	17.0	81.0	18.13 (1.82)
#1d	CAP/Nn	0.67	–	2.0	75.0	25.0	17.5	70.0	4.08 (1.04)
#2a	CAP/Q/CMC	0.67	1.0	2.0	82.4	18.0	17.2	94.0	31.45 (5.29)
#2b	CAP/Q/SDBS	0.67	0.50	2.0	88.9	21.0	18.1	86.0	4.54 (1.22)
#2c	CAP/Q/Tween	0.67	0.50	2.0	69.2	21.0	18.9	90.0	8.65 (1.11)
#2d	CAP/Q	0.67	–	2.0	70.0	25.0	22.0	88.0	5.01 (1.01)
#3a	Nn/CMC	0.67	1.0	–	88.6	28.6	28.4	99.3	74.60 (1.74)
#3b	Nn/SDBS	0.67	0.50	–	94.0	57.1	10.0	17.5	20.81 (1.82)
#3c	Nn/Tween	0.67	0.50	–	64.0	57.1	11.0	19.3	17.02 (2.85)
#4a	Q/CMC	0.67	1.0	–	83.4	28.6	26.7	93.3	65.36 (3.40)
#4b	Q/SDBS	0.67	0.50	–	89.1	57.1	23.0	40.3	3.79 (4.04)
#4c	Q/Tween	0.67	0.50	–	50.5	57.1	25.0	43.8	8.14 (3.34)
#A	CAP blank	–	–	2.0	99.8	–	–	–	3.90 (1.39)
#B	CAP/CMC	–	1.0	2.0	90.0	–	–	–	35.86 (1.96)
#C	CAP/SDBS	–	0.50	2.0	99.1	–	–	–	10.12 (1.97)
#D	CAP/Tween	–	0.50	2.0	94.1	–	–	–	6.42 (1.38)

CAP: cellulose acetate phthalate. CMC: carboxymethylcellulose crosslinked. SDBS: Sodium dodecylbenzensulfonate. Tween: Polyoxyethylene Sorbitan Trioleate (Tween 85). Q: Quercetin. Nn: Naringenin, TDC%: theoretical drug content, ADC%: actual drug content, EE%: encapsulation efficiency, SPAN: polydispersity index.

2.4. Spray-drying

A series of pilot experiments led to the selection of apparatus and operating conditions for microparticles production basing on an Analytic Hierarchy Process (AHP) method for optimizing the choice among the different suitable alternatives (Saaty, 2003, 2008). The feeds were spray-dried in a Büchi B-191 Mini Spray Dryer (Büchi Laboratoriums-Tecnik, Flawil, Switzerland), using a total amount of 6–8 g of raw materials. The drier conditions were: inlet temperature 125 °C; outlet temperature 78–80 °C; spray flow feed rate 5 mL/min; nozzle diameter 0.5 mm; drying air flow 500 L/h, air pressure 6 bar, aspirator 100%. Each preparation was carried out in triplicate. All the spray-dried microparticles were collected and stored under vacuum for 48 h at room temperature.

Production yields were expressed as the weight percentage of the final product compared to the total amount of the materials sprayed (Table 1).

2.5. Microparticles characterization

2.5.1. Particle size analyses

Particle size analyses of either the commercially available raw materials (Nn and Q) and the microparticles prepared via spray-drying were carried out with a Laser Light Scattering granulometer (Beckman Counter LLS 230, Particle Volume Module Plus, U.K.). The samples (drug or microparticles) were suspended in acid water (pH 3.0); about 50 μL of each sample were poured into the small volume cell to obtain an obscuration between 8% and 12%. Particle size distributions were calculated using the Fraunhofer model. The analyses were made in triplicate. Results were expressed as d_{50} indicating the volume diameter at the 50th percentile of the particle size distribution and span was calculated as $[d(90) - d(10)]/d(50)$.

2.5.2. Morphology

Morphology of the microsystems was examined by scanning electron microscope (SEM, LEO 429 Electron Microscopy Ltd., UK). Microparticles were coated with Au/Pd and eventually observed at different extensions. The fluorescent microscopy assays (FM) were performed observing the samples with a Zeiss Axiophot fluorescence microscope, with 40, 63 and 100 × 1.4 NA plan Apochromat oil immersion objectives (Carl Zeiss Vision, München-Hallbergmoos, Germany) using standard DAPI (4′,6-diamidino-2-phenylindole) optics that adsorb violet radiation (max 372 nm) and emit a blue fluorescence (max 456 nm).

2.5.3. Drug content and encapsulation efficiency evaluation

The flavonoid content in each formulation was assessed by both UV and HPLC. *UV method*. Samples (40 mg) of each batch of microparticles were dissolved in aqueous buffer solution at pH 7.5. The flavonoid content was then determined spectrophotometrically as previously described in Section 2.2.1. *HPLC method*. Samples (15 mg) of each batch of microparticles were dissolved in 15 mL MeOH, sonicated for 5 min, centrifuged for 10 min at 3000 rpm. Nn and Q concentrations were determined in the supernatant solutions using the same chromatographic conditions described (Section 2.2.1). Results were expressed in terms of average values.

The encapsulation efficiency (EE%), was calculated as the ratio of actual (ADC) to theoretical drug content (TDC) in dry microspheres (Table 1).

2.5.4. Differential scanning calorimetry (DSC)

Raw materials, drug-free-, CAP-free- and drug-loaded-microparticles were analyzed by differential scanning calorimetry on an indium calibrated Mettler Toledo DSC 822e (Mettler Toledo, OH, USA). Thermograms were recorded by placing accurately given quantities (8–10 mg weighed with a microbalance MTS Mettler Toledo, OH, USA) of each sample in a 40 μL aluminium pan which was sealed and pierced. The samples underwent two thermal cycles. In the dehydration cycle, the samples were heated from 25 °C to 130 °C at a heating rate of 20 °C/min. Temperature was kept at 130 °C for 15 min to remove the residual solvent. Afterwards, the samples were cooled down to 25 °C and eventually heated up to 350 °C at a heating rate of 10°/min. From this second thermal cycle, melting temperature (T_m), and heats of fusion (ΔH_m) were measured.

2.5.5. Fourier transform Infrared (FTIR) spectroscopy

Fourier transform infrared spectra were obtained using a Jasco FT-300, (Tokio, Japan) Fourier transform IR (FTIR) spectrometer. Samples of each batch of microparticles and of raw materials were analyzed as KBr discs in the spectral region 650–4000 cm^{-1}.

2.5.6. X-ray diffraction on powder (PXRD)

X-ray diffraction on powder spectra were recorded with a Rigaku RINT RAPID microdiffractometer having an imaging plate as detector, using a Ni-filtered Cu Kα radiation (40 kV, 20 mA). A Rigaku imaging plate, mod. R-AXIS DSBC, was used for digitizing the diffraction patterns.

2.6. Stability Studies

2.6.1. Physicochemical stability

Evaluation of the physicochemical stability was performed according to the conventional method (Long Term Studies – 12 months) reported by the guide lines ICH (International Conference on Harmonization, 2003). Glass vials containing 0.5 g of formulations were stored for 12 months at 25 °C ± 2 °C/60%RH ± 5%RH in a climatic chamber (Climatic and Thermostatic Chamber, Mod.CCP37, AMT srl, Milan, Italy). At given times (0, 3, 6 and 12 months), samples of each batch were collected. Humidity was evaluated gravimetrically, flavonoid content by HPLC method, microscopy and DSC analyses. All measurements were performed in triplicate.

2.6.2. Functional stability

The antiradical activity of pure and encapsulated Q was tested using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH test) according to the procedure previously reported (Mencherini et al., 2007). Briefly, Quercetin and sample of each batch submitted to the same storage conditions were dissolved in MeOH and a part (37.5 μL) of this solution, containing different amounts of the microparticles or pure Q, was added to 1.5 mL of daily prepared DPPH solution (0.025 g/L in MeOH); the maximum concentration employed was 200 μg/mL. An equal volume (37.5 μL) of the vehicle alone was added to control tubes. Absorbance at 515 nm was measured 10 min after starting the reaction on a Shimadzu UV-1601 UV–visible spectrophotometer. The DPPH concentration in the reaction medium was calculated from a calibration curve analyzed by linear regression. The percentage of remaining DPPH ($DPPH_{rem}$) was calculated as

$$\%DPPH_{rem} = [DPPH]_t/[DPPH]_0 \times 100 \quad (1)$$

where T is the experimental duration time and 0 the initial time. The $DPPH_{rem}$ was then plotted against the antioxidant concentration to obtain the mean effective scavenging concentrations (EC_{50}), calculated using the Litchfield & Wilcoxon test (Tallarida and Murray, 1984), as the concentration in μg/mL of Q necessary to decrease the initial DPPH concentration by 50%. All experiments were carried out in triplicate. α-Tocopherol, was used as positive control of the assay.

2.7. In vitro dissolution/release tests

In vitro dissolution/release tests of flavonoids from microparticles were carried out in sink conditions on a SOTAX AT 7 Smart Apparatus (Basel, CH) on line with a spectrophotometer (UV/Vis spectrometer Lambda 25, Perkin–Elmer Instruments, MA, USA). The concentrations of flavonoids released were determined at λ 310 nm for Nn and 366 nm for Q, using USP 31 (USP 31, 2008) dissolution test apparatus no. 2: paddle, 100 rpm at 37 °C. The pH-change method (USP 31, 2008) was used: 750 mL of HCl 0.1 N (pH 1.00) from 0 to 2 h, then the addition of 250 mL of 0.2 M tribasic sodium phosphate solution to give a final pH of 6.8 in a total volume of 1000 mL. All the dissolution/release tests were made in triplicate; only the mean values are reported (standard deviations <5%).

3. Results and discussion

Feed solutions made of CAP, Q or Nn and various enhancer (Tween 85, SDBS or CMC) of dissolution rate were spray-dried with the aim to produce gastroresistant microparticles with improved dissolution rate in intestinal fluid and stable in normal storage conditions. CAP was used as an enteric polymer stable at gastric pH, easy swellable and disintegrable in the neutral and alkaline environment of the small intestine. SDBS has been selected as anionic surfactant, in analogy to SLS – Sodium Lauryl Sulfonate – yet used to enhance the dissolution rate of drugs such as propanolol and theophilline (Gaylord and Schor, 1989); Tween 85 (an esterified and polyethoxylated derivative of sorbitan) as a stable and biocompatible non-ionic surfactant showing minimal binding to proteins (Tommasini et al., 2004). CMC as an insoluble, hydrophilic, highly absorbent material has been chosen because its excellent water swelling and drug dissolution promoting due to its fibrous nature (Sangalli et al., 1989).

3.1. Microparticles production

Drug content of the different powders, production yields and encapsulation efficiency (EE%) are reported in Table 1. The actual flavonoid content values determined by both UV and HPLC analyses were perfectly agreed. EE% was calculated as the ratio of actual to theoretical drug content. Results showed that Q and Nn are efficiently encapsulated in the CAP/enhancer-microsystems. EE ranged from 62.0% to 81.0% for Nn-containing microparticles and from 86.0% to 94.0% for Q-microparticles depending on the enhancer used in the formulation. The largest actual flavonoid content and EE were observed for CAP/Q/CMC (17.2% and 94.0%, respectively, #2a), and CAP/Nn/Tween (17.0% and 81.0%, respectively, #1a) microparticles. These EE values were fairly large and comparable to the values previously obtained for enhancer-free microparticles (#1d and 2d) (Lauro et al., 2005, 2007). It has to be noted that particles composed by flavonoid and surfactants (SDBS and Tween) without CAP give very low EE (17.5–43.8 for batches #3b and 3c, #4b and 4c), whereas EE were remarkably better using CMC (#3a and 4a) able to load and adsorb the drug. Production yields were high for all the SDBS-microparticles (about 89%, #1b and 2b); less marked for CMC – (76.0–82.4%, #1a and 2a) or Tween – (65.0–69.2%, #1c and 2c) particles. These results are fairly satisfying considering the low quantity of materials used for preparing the microparticles, having in mind that smallest and lightest particles are lost with the exhaust of the spray dryer.

3.2. Microparticles characterization

3.2.1. Particle size analyses and morphology

Fig. 1a and b shows flavonoids commercially available in a crystalline state with a needle shape (d_{50} 40.64 μm and 22.61 μm respectively, by LLS). Micrographs of CAP and CMC as raw materials showed flakes-like (CAP, Fig. 1c) or an irregular and lamellar (CMC, Fig. 1d) structures. LLS analysis indicated micronized SDBS- and Tween-microparticles with very narrow size distribution (Table 1). Mean diameter of 15.99 and 18.13 μm for Nn-microparticles (#1b and #1c, respectively), and 4.54 and 8.65 μm for Q-microparticles (#2b and #2c, respectively) were observed. The extent of this variation depends on the considered flavonoid, the lowest volume median diameter being registered for Q-containing particles. Similar sizes were observed for CAP-free microsystems; mean diameters ranged from 20.81 μm, (#3b) and 17.02 μm (#3c) to 3.79 (#4b) and 8.14 μm (#4c). On the contrary, CMC-microparticles had much larger particle sizes both for CAP-containing, (44.20 μm, #1a, and 31.45 μm, #2a) and for CAP-free microparti-

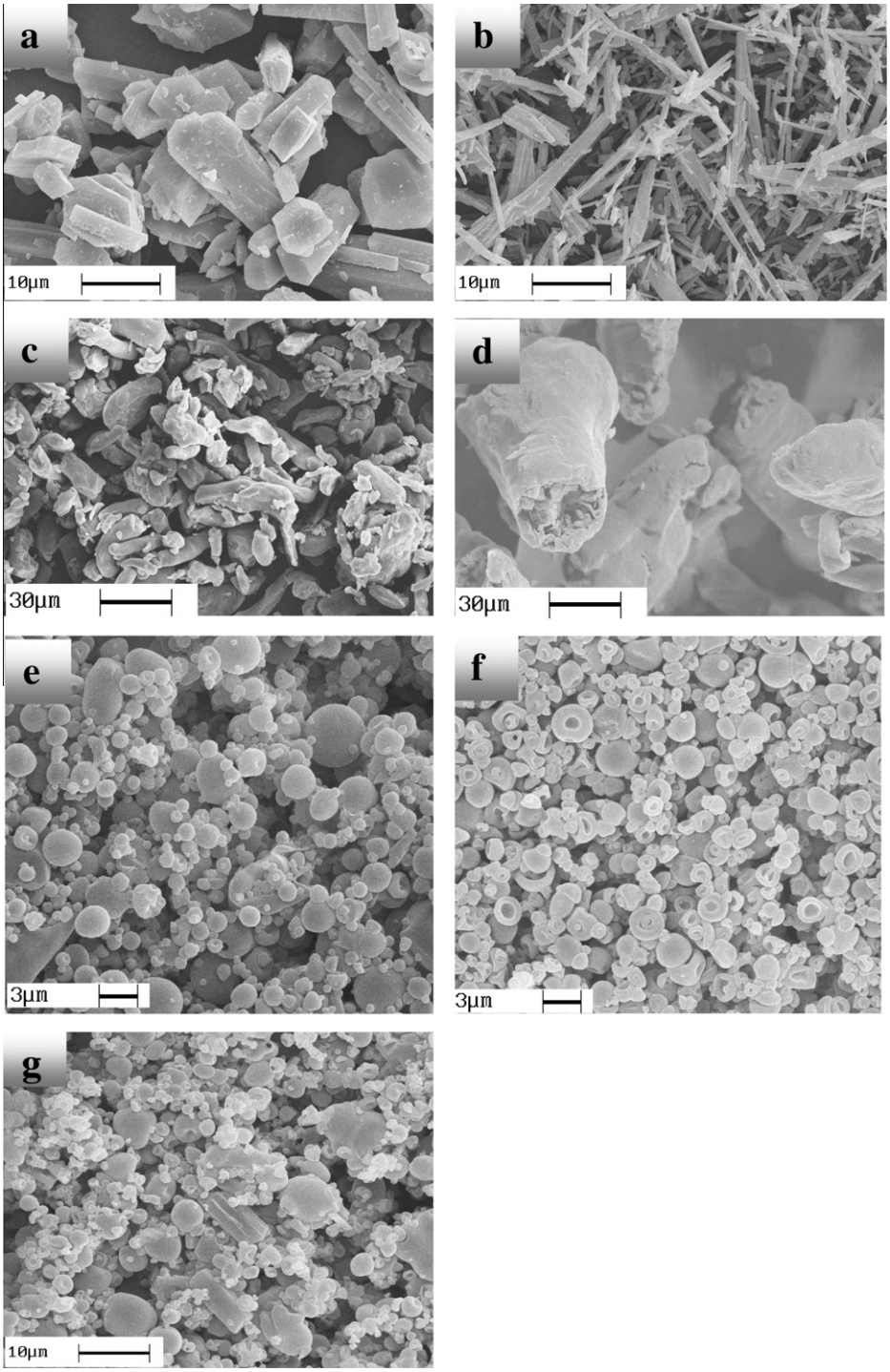

Fig. 1. Scanning electron microscopy images of – Nn (a), Q (b), CAP (c), CMC (d) raw materials, CAP/Nn/SDBS (batch #1b) (e), CAP/Nn/Tween (batch #1c) (f), CAP/Nn/CMC (batch #1a) (g) microparticles.

cles (74.60 μm, #3a, and 65.36 μm, #4a). The small and comparable dimensions exhibited by SDBS- and Tween-microparticles may be due to the arrangement of both surfactants and flavonoids in micelles of colloidal size in which the drug is homogeneously distributed. This effect turned the feed suspension in a solution easier to be sprayed. As previously reported (Naseem et al., 2004; Liu and Rong, 2006a,b), the solubilisation capacity of the surfactant increases in form of micelle and the hydrophobic forces become the main driving force locating flavonoid inside the micelles as shown by FM images (#3c, Fig. 2a).

SEM analysis supported this observation. In fact, micrographs showed small and well formed microparticles, spherical in shape, for both Tween and SDBS-containing microparticles. In addition, the SDBS-microparticles (#1b, Fig. 1e) showed few uncoated flavonoid-crystals embedded on the surface, whereas, images of Tween-microparticles (#1c, Fig. 1f) displayed the complete absence of crystals and aggregates. These results confirmed the major interaction and solubilisation of flavonoids in the non-ionic surfactant (Tween) micelles with respect to the anionic surfactant (SDBS), according to the observations of Liu and Rong (2006a,b) and Ka-

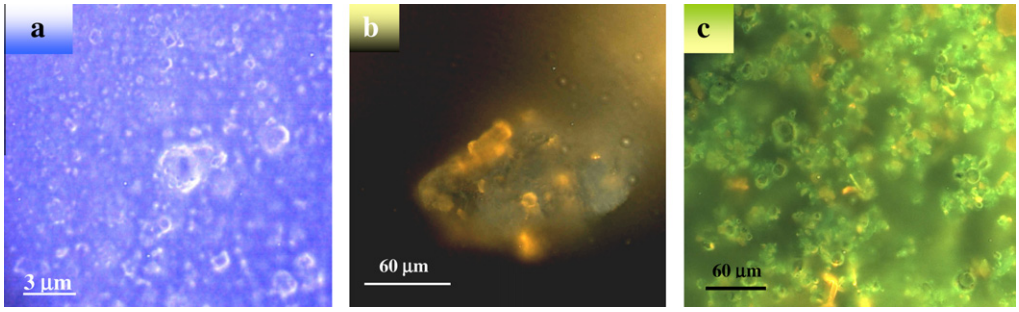

Fig. 2. Fluorescence microscopy image of Nn/Tween-microparticles (batch #3c) (a), Nn/CMC physical mixture (b); CAP/Nn/CMC-microparticles (batch #1a) (c).

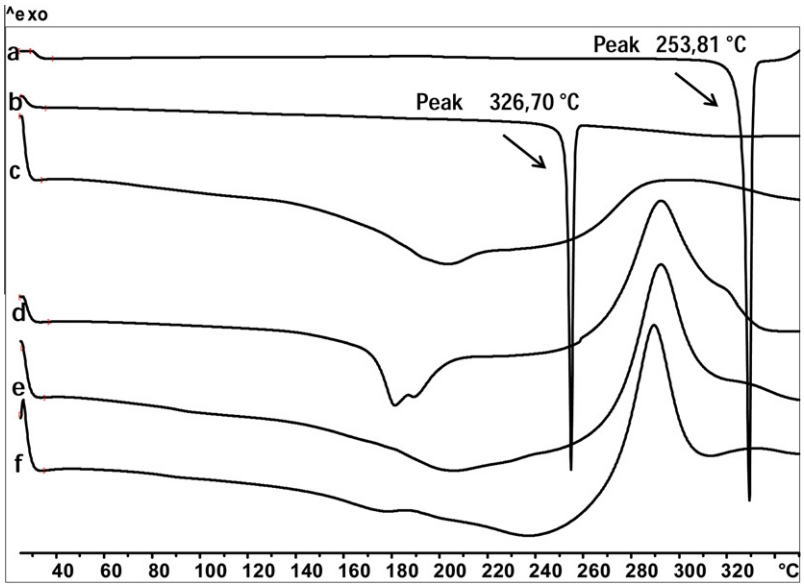

Fig. 3. Differential scanning calorimetry thermograms of Q (a), Nn (b), CAP (c), CMC (d) as raw materials, and CAP/Nn/CMC (batch #1a) (e), CAP/Q/CMC (batch #2a) (f) microparticles.

naze et al. (2006). Different results were obtained for CMC-microparticles. In fact, inspection of their micrographs, revealed an evident size increase (as also indicated by LLS), a more irregular shape and the presence of partially collapsed particles pierced by few uncoated flavonoid crystals (SEM Fig. 1g). FM micrographs confirmed that flavonoids produced a solid physical mixture with CMC keeping their crystalline state (Fig. 2b). The presence of crystals and aggregates explains the larger sizes obtained by Coulter Counter analysis for CMC-microparticles (44.20 μm for Nn-particles #1a, and 31.45 μm for Q-particles #2a). As a matter of fact, the laser diffraction technique is based on the assumption of spherical particles, leading to an heterogeneous particle distribution for batches containing aggregates and crystals (Fig. 2c).

3.2.2. Differential scanning calorimetry (DSC)

Thermal profiles of the neat flavonoids showed their melting point, respectively at about 253.81 °C (Nn) and 326.70 °C (Q) (Fig. 3). The absence of these melting peaks in the thermal profiles (Fig. 3) of all microparticles confirmed that both flavonoids were well encapsulated/embedded in the polymeric matrices, as previously shown by SEM and FM analyses. Moreover, absence of new peaks suggested that no chemical interactions occurred between coating/loading materials and the flavonoids. Only in Tween-microparticles thermal profiles (#1c and #2c), a shift to higher temperature (from 199.8 °C, raw material, to 228.3 °C) of the CAP melting point was observed (Fig. 4). This thermal behaviour is referable to a physical interaction between the particle components (Liu and Rong, 2006a,b).

These results do not exclude the presence of encapsulated flavonoid in residual crystalline state. The DSC technique could not detect any residual crystalline structure in the presence of low-melting-point polymers acting as solvents for the encapsulated or embedded material, as also previously reported elsewhere (Kanaze et al., 2006). In fact, CAP which melts at lower temperature than flavonoids, by increasing the temperature during the DSC experiment, starts to melt at first, and may dissolve residual crystals present in the microsystems. For this reason, a PXRD analysis was necessary for further investigation on crystalline state.

3.2.3. X-ray diffraction on powder

All PXRD profiles of CAP/flavonoids/enhancer-microparticles (Fig. 5) showed a strong reduction in crystallinity state of flavonoids. In particular, Tween-containing powders resulted completely amorphous (#1c and #2c) confirming the full flavonoid encapsulation, according to SEM and DSC results.

3.2.4. Fourier transform Infrared (FTIR) spectroscopy

FTIR spectra of all microparticles did not show any differences in the peak patterns with respect to raw materials. Only IR spectra of Tween-microparticles (#1c and #2c) displayed an amplification

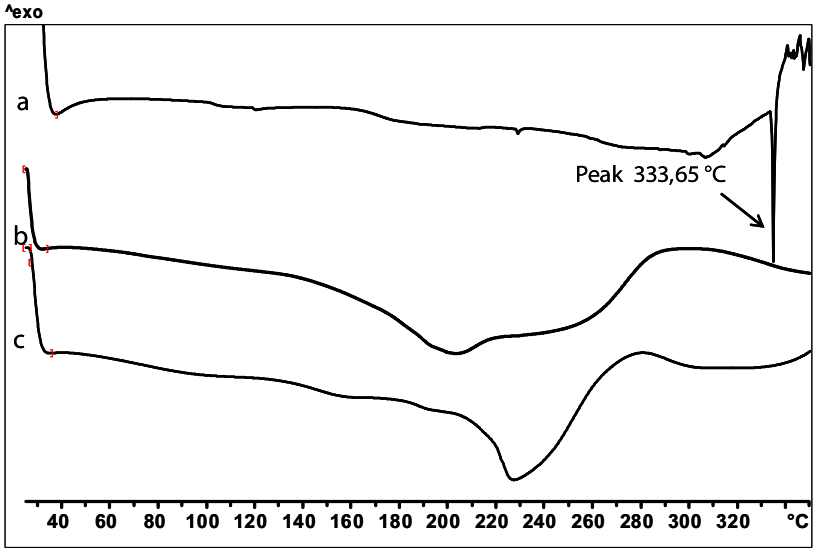

Fig. 4. Differential scanning calorimetry thermograms of Tween 85 (a), CAP (b) as raw materials, and CAP/Nn/Tween (Batch #1c) (c) microparticles.

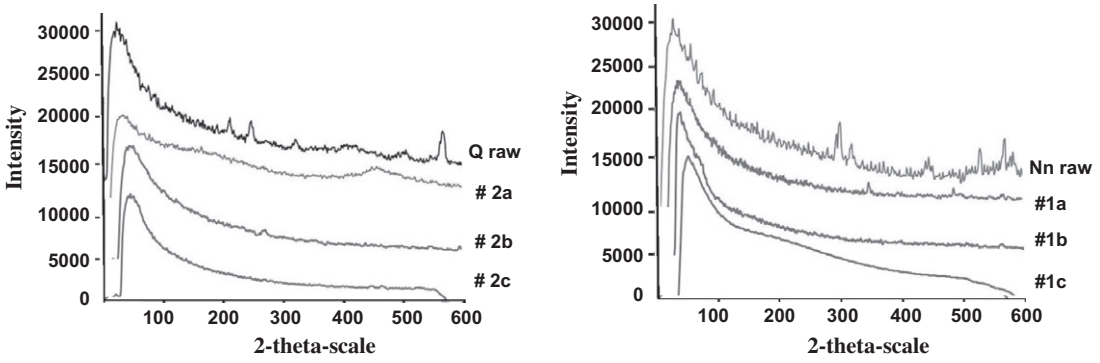

Fig. 5. X-ray diffractogram of produced microparticles (batches #1a–c and 2a–c) and, Q and Nn as raw materials.

of OH band (3500 cm^{-1}) (data not shown), confirming that some physical interactions occur between flavonoids and the non-ionic surfactant. According to literature (Naseem et al., 2004; Liu and Rong, 2006a,b), in some conditions hydrophobic phenyl aglycones can just bind with non-ionic surfactant molecules through intermolecular H-bond with their hydroxyl-groups.

3.3. Dissolution/release studies

At first, to evaluate the dissolution/release profile of flavonoids from the microparticles, their solubility in each dissolution medium has been detected as described in Section 2. The solubility values of Nn and Q (mg/L) resulted 35.0 and 7.0 mg/L in water; 15.0 and 5.4 mg/L in simulated gastric fluid; 41.0 and 29.0 mg/L in simulated intestinal fluid, respectively. Then, the sink conditions, which describe a dissolution system sufficiently dilute so that the dissolution process is not impeded by saturation of the solution were evaluated resulting about 15 mg/L of Naringenin and 5 mg/L of Quercetin. The dissolution/release profiles of Nn and Q from the microparticles are reported in Figs. 6 and 7 in comparison with the neat flavonoids and gastroresistant microparticles prepared without the enhancers.

After 2 h a little quantity of flavonoids (about 15.4–21.8% of Nn, Fig. 6, #1a, #1b, #1c; and 6.2–14.8% of Q, Fig. 7, #2a, #2b and #2c) were released/dissolved in simulated gastric fluid (GF, pH 1.0) confirming the gastro-resistance of the CAP/flavonoids/enhancer-microparticles. At the same time, about 12.3% and 7.5% of Nn and Q, respectively, were released/dissolved from CAP/Nn (Fig. 6, #1d) and CAP/Q (Fig. 7, #2d) microparticles formulated without the enhancers. These results showed that Nn and Q were less released at pH 1.0 by CAP-enhancer-free microparticles than from the CAP-enhancer-containing microparticles. Nevertheless, after pH change (pH 6.8), flavonoid release from the enhancers-free formulations was incomplete (54.1% Nn and 50.9% Q) and similar to those of neat Nn (46.3%) and Q (43.1%) (Figs. 6 and 7). Interestingly, a large and considerable improvement of the drug dissolution rate was obtained from all enhancer-containing microparticles. About 100% of Nn and Q dissolved/released from CMC – (#1a, 99.7% and #2a, 99.1%) and Tween – (#1c 99.8% and #2c 99.5%) microparticles and about 80% from SDBS-microparticles (#1b, 86.5% and #2b, 86.3%) (Figs. 6 and 7) in 45 min after pH change in intestinal environment. This excellent behaviour may be explained by an increase of the flavonoid–water interaction due to the enhancers able to improve flavonoid wettability and solubility in the simulated intestinal fluid, with slight differences due to structure and properties of flavonoids and the enhancers (Liu and Rong, 2006a,b).

3.4. Stability studies

3.4.1. Physicochemical stability

Long-term stability studies (International Conference on Harmonization, 2003) were performed on all microsystems to examine

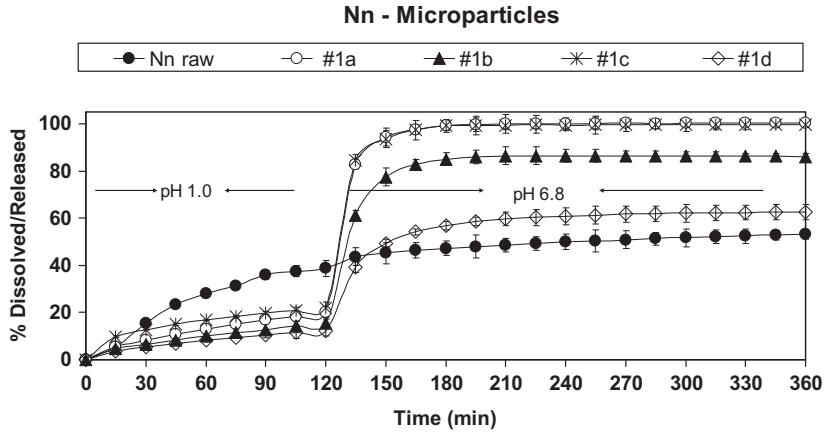

Fig. 6. Release profile of CAP/Nn/enhancers microparticles (CAP/Nn/CMC #1a, CAP/Nn/SDBS #1b, CAP/Nn/Tween #1c) compared to Nn as raw material, and CAP/Nn (batch #1d) microparticles.

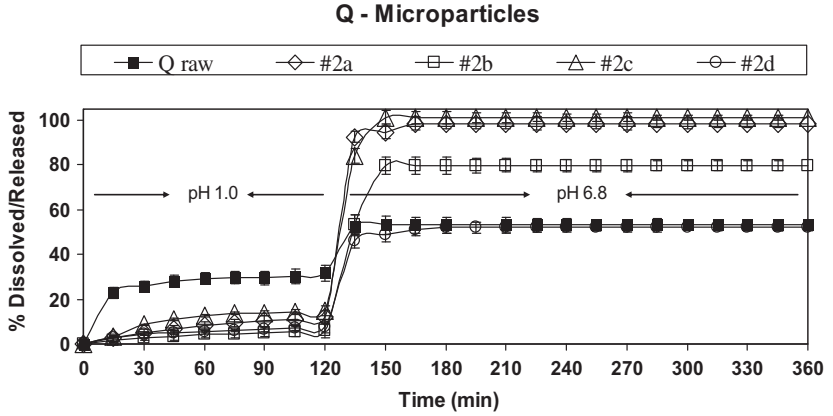

Fig. 7. Release profile of CAP/Q/enhancers microparticles (CAP/Q/CMC #2a, CAP/Q/SDBS #2b, CAP/Q/Tween #1c) compared to Q as raw material, and CAP/Q (batch #2d) microparticles.

the effect of the spray-drying process, surfactants and swelling materials on stability and quality of the produced microparticles. The physicochemical and functional stability evaluation in the normal storage conditions provides information concerning shelf-life and efficacy of a product with a specific function. Nn and Q content of the encapsulated powders were analyzed over 12 months, including four time points (0, 3, 6 and 12 months) by microscopy, DSC and HPLC. At the end of 12 months storage period at 25°, the material was stable; no significant variation in the weight was found indicating that microparticles are not hygroscopic. Regarding the flavonoids, no degradation products or decrease of concentration which can be considered significant in term of stability (<1%) were recorded by HPLC (International Conference on Harmonization, 2003).

3.4.2. Functional stability

Functional stability was evaluated for Q-microsystems as free-radical scavenging activity using the DPPH test and the strong antioxidant, α-tocopherol, as positive control of the assay (Mencherini et al., 2007). The antioxidant activity of flavonoids is related to different factors as the number and nature of the substituents (mainly –OH) on the backbone of the molecule, as well as its backbone structural characteristics such as its ability to assume a planar conformation (Hotta et al., 2002). Due to flavon-3-ol planar structure supporting a series of phenolic –OH (di-*ortho*-OH on ring B, two *meta*–OH on ring A, one –OH at C-3 position, the 2,3-double bond in conjugation with 4-oxo-function of the benzo-γ-pyrone skeleton), Q exhibits the highest antiradical activity compared to other flavonoids (Hotta et al., 2002; Tabart et al., 2009) in *in vitro* tests. Results indicated that there was no detectable loss of activity during 12 months for all the prepared Q-microsystems at the test conditions. The strong antioxidant activity of encapsulated Q, expressed as EC_{50}, was at about the same level at 3 and 6 months and remained quite unaltered during 12 months of storage (Table 2). During the same time, the value of inhibition of Q unencapsulated varied from EC_{50} 2.3 (0 month) to 5.1 (12 months). Our results are in agreement to those reported by Vicentini et al. (2008) about the study of stability on lamellar liquid crystalline formulation containing Quercetin.

Table 2
Free-radical scavenging activity of encapsulated and unencapsulated Quercetin during 12 months of storage.

Materials	Months			
	0	3	6	12
DPPH test EC_{50} μg/mL[a]				
Q	2.3 ± 0.4[a]	2.5 ± 0.2	4.5 ± 0.5	5.1 ± 0.4
CAP/Q/CMC	2.8 ± 0.3	3.0 ± 0.3	3.0 ± 0.4	3.2 ± 0.3
CAP/Q/SDBS	2.7 ± 0.2	2.8 ± 0.3	2.8 ± 0.3	3.1 ± 0.2
CAP/Q/Tween	2.5 ± 0.3	2.6 ± 0.3	2.6 ± 0.2	2.8 ± 0.3
α-Tocopherol[b]	10.1 ± 1.3	10.2 ± 1.3	10.1 ± 1.6	10.3 ± 1.3

[a] Mean ± standard deviation.
[b] Positive control of the assay.

4. Conclusion

Slightly water-soluble flavonoids Nn or Q were microencapsulated by spray-drying using a combination of gastroresistant polymer CAP and a series of enhancers of the dissolution rate. The aim of the study was to produce microparticles with enhanced dissolution rate in intestinal fluid able to improve the absorption and bioavailability of flavonoids after the oral administration. The spray-drying technique and the process conditions selected were effective in microencapsulating and stabilizing the sensitive flavonoids. Satisfactory encapsulation efficiency and product yield were obtained. A good compromise between the improvement of the dissolution rate in intestinal fluid (pH 6.8) and protection in gastric environment (pH 1.0) of both flavonoids was reached. This result was achieved by using either CAP as gastroresistant polymer and a swelling agent such as CMC, or surfactant agents in the formulations. The best results were obtained with Tween-containing microparticles showing high encapsulation efficiency, low and homogeneous dimensional distribution, trendy spherical shape and absence of uncoated flavonoid crystals. All the produced CAP-enhancers microparticles gave high flavonoid release (from 80% to 100%) in simulated intestinal fluid without altering CAP gastro-resistance at pH 1.0. The protective effect resulted to be associated not only with CAP coating properties and its pH-dependent solubility, but also with the physical state of the matrices as demonstrated by solid state study. In addition, it was observed that microencapsulation improved the stability avoiding the loss of the Quercetin strong antioxidant activity during 12 months of storage. This approach could be suitable to obtain stable and handling flavonoid powders with extended shelf-life and improved dissolution/release rate, which could find application in food and pharmaceutical field.

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