Phytoglycogen Improves the Water Solubility and Caco-2 Monolayer Permeation of Quercetin

(Running title: PG Improves QC Solubility)

Authors:

Hua Chen¹, Yuan Yao^{1*}

Affiliations:

¹ Department of Food Science, Purdue University, 745 Agriculture Mall Drive, West Lafayette,
IN 47907

*** Corresponding Author**

Address: 745 Agriculture Mall Drive, West Lafayette, IN 47907

Phone: (765) 494-6317

Fax: (765)-494-7953

Email: yao1@purdue.edu

Abstract

The study examined the capability of phytoglycogen (PG) to improve the water solubility of quercetin (QC). PG-QC formulations were prepared by mixing a QC ethanol solution with a PG aqueous solution followed with vacuum drying of the supernatant. PG-QC formulations with various PG to QC ratios were prepared; the solubility of QC reached 241.76 $\mu\text{g/mL}$ at PG/QC ratio of 30/1 compared with approximately 4.32 $\mu\text{g/mL}$ of QC alone. The X-ray powder diffraction and FTIR analyses showed a significant reduction of QC crystallinity upon formulating with PG that was associated with the intermolecular hydrogen bonding between the hydroxyl groups of QC and PG. The Caco-2 cell monolayer permeation tests showed that PG-QC formulations resulted in substantially enhanced cellular uptake and transepithelial permeation of QC, which was related to the much-enhanced QC solubility. This study showed the potential of using PG to formulate poorly water-soluble ingredients such as QC.

Keywords: Quercetin; phytoglycogen; water solubility; Caco-2 permeation

1. Introduction

Quercetin (QC), or 3, 3', 4', 5, 7-pentahydroxyflavone, is one of the most common plant-derived flavonoids widely distributed in normal human diet, especially in lettuce, onions, apples, wine, berries, and tea (Gormaz, Quintremil, & Rodrigo, 2015). The estimated average dietary intake of QC is 6~18 mg/day in the United States and China (Sampson, Rimm, Hollman, de VRIES, & Katan, 2002; Guo & Bruno, 2015; Kim, Vance, & Chun, 2015). QC aglycone and its more abundant glycoside forms have gained enormous attention due to the emerging evidence for their biological activities. Epidemiological studies have associated QC with antioxidant, anticarcinogenic, anti-inflammatory, cardioprotective, antitumor, and antiviral activities. Recently, emerging interest has been attracted to its chemoprotective effects. However, despite its wide spectrum of health benefits, application of QC has been largely impeded by: 1) poor aqueous solubility (0.165 ~ 10.28 µg/mL in water); 2) chemical instability to heat and light, and in physiological media (Althans, Schrader, & Enders, 2014); 3) poor intestinal absorption when administered orally (Scalia & Mezzena, 2009; Blasina, Vaamonde, Silvera, Tedesco, & Dajas, 2015); and 4) extensive xenobiotic metabolism (i.e., glucuronidation or sulfation) before reaching the systemic circulation due to its polyphenolic nature (Khaled, El-Sayed, & Al-Hadiya, 2003; Ratnam, Ankola, Bhardwaj, Sahana, & Kumar, 2006). These attributes collectively contribute to the poor and highly variable bioavailability of QC. Therefore, there exists a critical need to design formulations for optimal QC bioavailability and total absorption, thus to better realize its diverse health benefits.

Substantial efforts have been taken to prepare QC delivery systems for enhanced oral bioavailability through improving QC solubility and dissolution rate. Self-emulsifying drug delivery system (Tran, Guo, Song, Bruno, & Lu, 2014), liposomes (Rezaei-Sadabady, Eidi,

Zarghami, & Barzegar, 2014), nanoemulsions (Dora et al., 2012; Guo, Tran, Lu, & Bruno, 2014), solid lipid nanoparticles (Aditya et al., 2014), and nanostructured lipid carriers (Sun, Wang, Nie, & Zhang, 2014) have met with varying degrees of success. Polymer-based QC-loaded hydrogels (Althans, Schrader, & Enders, 2014), micellar systems (Araujo & Araujo, 2014), complexation with β -maltodextrins, and solid dispersions (Kakran, Sahoo, & Li, 2011; Li et al., 2013) are also studied for their potential to improve QC bioavailability. However, there is a lack of approach of using naturally occurring, food-based carriers to improve QC solubility and bioavailability in a relatively simple way.

Phytoglycogen (PG) is a highly branched water-soluble α -D-glucan (Huang & Yao, 2011). The largest source of PG is the kernel of the maize mutant sugary-1 (*su1*), a primary genotype of commercial sweet corn. The *su1* mutation leads to a deficiency in SU1, an isoamylase-type starch debranching enzyme (DBE) and resulted in the generation of PG particles instead of starch granules. In PG, there is no long chain that connects individual clusters as in the case of amylopectin, implying a fundamental structural variation between PG and amylopectin (Huang & Yao, 2011). The particle size of PG ranges from 30 to 100 nm, with a density increment toward the outer surface of the particulate (Huang & Yao, 2011). The particulate characteristics of PG allowed us to hypothesize that the spacing between the branches of PG particulate is capable of harboring and dispersing insoluble bioactive, small molecule component such as QC. In addition, PG nanoparticles are digestible, making it ideal for designing delivery systems in food systems. The aim of the present study was to investigate the feasibility of using native PG to enhance QC solubility and to understand if the enhanced QC solubility may increase its permeation through the epithelial membrane with Caco-2 monolayer as the model.

2. Materials and Methods

2.1 Chemicals

Quercetin hydrate ($C_{15}H_{10}O_7 \cdot XH_2O$) was purchased from TCI America (Portland, OR, USA). HPLC analysis showed that the purity of quercetin was 93.0%, leading to a calculated X value of 1.264. All other chemicals used for the study were of analytical grade unless specified. Double-distilled water was freshly prepared whenever required.

2.2 Methods

2.2.1 *Extraction of phytoglycogen*

To extract phytoglycogen (PG), 320 g of dry kernels were ground and soaked in ten times their weight of deionized water. The suspension was homogenized using a high-speed blender (VARING Laboratory). The homogenates were collected and passed through a 270-mesh sieve, and the solids were further extracted twice using additional deionized water. All of the extracts were combined, the pH was adjusted to 4.8 ~ 4.9 using acetic acid and stored at 4°C for 2 h to induce protein precipitation. The extracts were centrifuged for 20 min at 4°C and $10,000 \times g$. The supernatants were re-centrifuged until no precipitate was present. The supernatants were combined, and the pH was adjusted to 7.0, followed by autoclaving at 121°C for 50 min. The denatured, insoluble proteinaceous material was removed by centrifugation. To precipitate polysaccharides, three volumes of ethanol were added to the supernatant, followed by centrifugation. The precipitate was re-suspended in ethanol and the supernatant was removed after centrifugation; the suspension-centrifugation operation was repeated for three times. The bulk ethanol was removed afterwards by vacuum filtration, and the solid pellets were placed in the hood to remove residual ethanol and used as PG.

2.2.2 Preparation of PG-QC formulations

Solid formulations that contain both PG and QC were prepared as shown in **Figure 1**. A 20 wt% dispersion of native PG was prepared with sodium acetate buffer (0.05 M, pH 6.8) by incubating at 70°C for 5 min with magnetic stirring. Accurately weighed amounts of quercetin (QC) hydrate were suspended in ethanol at concentrations of 20, 26.67, 40, 80, 160, and 400 mg/mL, respectively. The QC hydrate suspensions were incubated at 70°C for 5 min under constant stirring and sonicated 3 times for 30 seconds at power output 8 using an ultrasonic probe (Sonifier 150, Branson Ultrasonics Corp., USA). Subsequently, aliquots (1 mL) of the QC dispersion were dropwise added into 4 mL of the PG aqueous dispersion respectively in the presence of sonication (six 30-second bursts at setting 80) followed by vigorous vortexing for 1 min. The mixed solvent comprised of 20vol% ethanol (1 mL) and 80 vol% aqueous buffer (4 mL) was chosen based on its relative high solubilizing capacity for QC without causing PG precipitation. The resulting dispersions had different weight ratios between PG and QC hydrate (2:1, 5:1, 10:1, 20:1, 30:1 and 40:1; w/w) and were subjected to solvent removal at 40°C using a vacuum concentrator (SpeedVac, Thermo Scientific, USA), yielding solids of PG-QC formulations (termed as PG-QC-#1S). PG-QC-#1N was prepared according to the above-mentioned procedure without using sonication at any stage.

The soluble portion of PG-QC-#1S was isolated using the following procedure and termed as PG-QC-#2. 20 mg/mL PG-QC-#1S was equilibrated in ddH₂O under constant stirring (4 h, at room temperature). Subsequently, the soluble supernatant was separated from the rest of PG-QC-#1S by centrifugation at 5,000 × g for 5 min. The resultant supernatant was transferred to a glass vial and dried under vacuum (SpeedVac) to collect PG-QC-#2 powder.

Physical mixtures of PG and QC were prepared through grinding mixtures of various ratios between PG and QC hydrate (2/1, 5/1, 10/1, 20/1, 30/1 and 40/1; w/w) using a ceramic mortar and pestle. All samples were stored in capped glass vials at room temperature.

2.2.3 QC Quantification

A QC standard curve using ethanol-water mixed solvent (80:20, v/v) was generated for the measurement of QC concentration by the UV-Vis spectrophotometer (DU 730, Beckman Coulter, USA). A standard stock solution of QC hydrate (1.0 mg/mL) was prepared by dissolving accurately weighed QC hydrate in 80vol% ethanol under intermittent vortexing until a clear solution was obtained; working standard solutions were prepared by serial dilution of the stock solution with the 80vol% ethanol. The UV-Vis absorption of the diluted clear solutions was measured at 374 nm. Absorbance at 374 nm was used for determination of QC in the diluted clear solutions. The assay was linear ($r^2 = 0.999$) over the QC concentration range of 1~ 12 $\mu\text{g/mL}$. The QC amount thus determined was the equivalent amount of QC hydrate.

2.2.4 Solubility Studies

20 mg PG-QC formulations with different weight ratios of PG/QC were dispersed into 1 mL ddH₂O in a 2 mL microcentrifuge tube, respectively. The tube was vortexed for 3 min and then equilibrated at room temperature for 24 h on a bi-directional rotator (Lab-line 4631, Maxi Rotator, USA). To avoid photo-degradation, the tubes were wrapped securely with aluminum foil. The dispersions were subsequently centrifuged at $5,000 \times g$ for 5 min to remove any insolubles. After that, 0.2 mL of the supernatant (containing soluble QC) was carefully withdrawn and added into 0.8 mL absolute ethanol. The mixture was then vortexed for 1 min and centrifuged at $10,000 \times g$ for 5 min to separate PG precipitate. The amount of QC remaining in the supernatant represented the soluble QC in the sample and was determined using the standard curve. Proper

dilution was performed using 80vol% ethanol if needed. The QC amount thus determined was the equivalent amount of QC hydrate.

QC solubility of all PG-QC formulations was obtained by determining the amount of QC in the supernatant and was expressed as μg of QC within 1 mL of a 20 mg/mL PG-QC aqueous dispersion. The solubility tests were all based on 20 mg/mL aqueous dispersions of PG-QC formulations. Solubility of QC in the physical mixtures of PG and QC hydrate was also determined using the same procedure. All experiments were carried out in triplicate.

2.2.5 Percentage Solubilization of QC and Mass Ratio of PG/QC in PG-QC Formulations

The percentage of QC solubilized and the mass ratio of PG and QC in PG-QC formulations were determined after the reconstitution of PG-QC in water. A 20 mg/mL PG-QC aqueous dispersion was prepared. After centrifugation at $5,000 \times g$ for 5 min, the supernatant (containing soluble QC) was analyzed by UV-spectrophotometer as described above. Meanwhile, a certain volume of the supernatant was accurately taken, dissolved in 4 times the volume of ethanol to precipitate the PG material. After centrifugation at $10,000 \times g$ for 5 min, the PG pellet (W_{PG}) was separated from the supernatant and accurately weighed after drying. On the other hand, to measure of the total amount of QC in PG-QC formulations, 0.2 mL of the abovementioned 20 mg/mL PG-QC aqueous dispersion was added directly (without any centrifugation) into 0.8 mL ethanol. The resultant mixture was vortexed for 3 min and centrifuged at $10,000 \times g$ for 5 min. The QC dissolved in the supernatant ($W_{\text{QC total}}$) was measured by UV-Vis at 374 nm. The percentage solubilization of QC (%QC solubilized, Eq. (1)) and the mass ratio of PG: QC (Eq. (2)) of the soluble portion of PG-QC formulations were calculated as following:

$$\% \text{ QC solubilized} = \frac{W_{\text{QC soluble}}}{W_{\text{QC total}}} \times 100 \quad \text{Eq. (1)}$$

$$\frac{\text{PG}}{\text{QC}} = \frac{W_{\text{PG}}}{W_{\text{QC soluble}}} \quad \text{Eq. (2)}$$

where $W_{\text{QC soluble}}$ is the amount of QC in the supernatant after the centrifugation of the 20 mg/mL PG-QC aqueous dispersion, and $W_{\text{QC total}}$ is the total amount of QC in the PG-QC formulations. W_{PG} is the amount of PG in the supernatant that contained the soluble QC. All experiments were performed in triplicate.

2.2.6 Solid State Characterization of PG-QC Formulations

2.2.6.1. FTIR Analysis

The FTIR spectra of solid samples were obtained on a Thermo-Nicolet Nexus 470 FTIR spectrometer (Nicolet, Thermo, USA) equipped with a Smart OMNI-sampler. The scanning range was $800 \sim 4000 \text{ cm}^{-1}$, using a resolution of 4 cm^{-1} and 40 accumulations. FTIR spectra of raw QC, PG, and physical mixtures of QC and PG, as well as PG-QC formulations were recorded.

2.2.6.2. X-ray Powder Diffraction Analysis

To study the crystalline characteristics, X-ray diffraction analysis was performed on an X-ray powder diffractometer (XRD-6000, Shimadzu, North America) equipped with a Bragg - Brentano optical setup. Nickel filtered Cu $K\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$) was used with a long fine focus X-ray tube. All samples were scanned from $3^\circ \leq 2\theta \leq 35^\circ$, with a scanning speed of $8^\circ(2\theta)/\text{min}$, at 40 kV and 30 mA. The diffraction patterns of raw QC, native PG, physical mixtures of QC and PG, as well as the PG-QC formulations were recorded.

2.2.7 Determination of QC Permeation with Caco-2 Cell Monolayer

2.2.7.1. Preparation of QC Solutions

5, 10, 50, and 93.1 mM stock solutions of QC hydrate were prepared individually with 50% ethanol in HBSS (pH 6.5). Then a dilution series of working concentrations of 50, 100, 500, and 931 μ M were prepared with HBSS (pH 6.5, final concentration of ethanol as 0.5vol%). The resulting final concentration of ethanol, 0.5vol%, did not affect TEER values and cell viability. To ensure the uniformity of the preparations, all QC HBSS solutions were homogenized at 11,600 rpm for 5 min using a high-speed homogenizer (Ultra-Turrax T 25, IKA). It was noted that high QC concentrations of 500 and 931 μ M led to colored precipitate upon equilibration at room temperature, thus were not used in further experiments.

2.2.7.2. Preparation of PG-QC Formulation Solutions and QC Determination

A screening was performed to select the best PG-QC formulation for the Caco-2 permeation experiment. It was found that the QC solubility in PG-QC-#1S (PG/QC ratio of 30/1, w/w) was the highest (241.76 μ g/mL) comparing to formulations with other PG/QC ratios. Thus this formulation was selected and furthered treated to produce PG-QC-#2 (**Fig. 1**). A stock solution of PG-QC-#2 was prepared at 20 mg/mL with HBSS (pH 6.5, 0.5vol% ethanol). The corresponding soluble QC in PG-QC-#2 was determined. Afterwards, the PG-QC-#2 stock solution was diluted with HBSS (pH 6.5, ethanol as 0.5vol%) to achieve a series of working test solutions (equivalent to 50, 100, 500, and 931 μ M QC hydrate). To ensure the uniformity of these preparations, all PG-QC-#2 test solutions were homogenized at 11,600 rpm for 5 min using high-speed homogenizer. All the preparations were prepared fresh before the Caco-2 permeation experiment and maintained at 37°C.

2.2.7.3. *Cell Culture*

The human intestinal Caco-2 BBE cell line obtained from the American Type Culture Collection (ATCC, Rockville, MD) was cultured with DMEM supplemented with 10% fetal calf serum, 1% sodium pyruvate, and 1% penicillin antibiotic (pH 7.4) in a humidified atmosphere (95% relative humidity) of 5% CO₂ at 37°C, and were sub-cultured at 80% confluency. Caco-2 cells in passages between 57 and 68 were used for all experiments. For the permeability studies, Caco-2 cells were harvested with trypsin-EDTA and seeded onto 12 mm i.d. Transwell inserts (polycarbonate membrane, 0.4 µm pore size, insert membrane growth area: 1.12 cm², Corning Costar Corp. NY, USA) of 24-well plates at a density of 3.5×10^5 /cm² cells/insert. Culture medium (0.5 mL in the insert and 1.5 mL in the well) was replaced every 48 h for the first 6 days and every 24 h thereafter for a total of 22 days. The integrity of the monolayer was checked routinely by measuring the transepithelial electric resistance (TEER) with Millicell-ERS equipment (Millipore, USA). Only monolayers with a TEER of more than 350 Ω cm² were used for the transepithelial permeation experiments. TEER values were measured prior to and during the whole experiment to guarantee the maintenance of the barrier properties.

2.2.7.4. *Cell Viability*

The impact of QC alone and PG-QC formulation test solutions on the viability of Caco-2 cells was evaluated by the colorimetric MTT reduction assay. The amount of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that was oxidized into purple formazan was measured spectrophotometrically. This oxidation takes place only when mitochondrial reductase enzymes are active, and thus conversion is directly related to the number of viable cell counts. For the MTT assay, 3×10^5 cells were seeded in 96-well plate (Corning Costar, USA) in 100 µL of cell culture medium. The cells were then cultured under the same

condition as monolayer preparation for 96 h before use. Immediately prior to each experiment, the medium was removed, and 100 μ L of the QC hydrate solutions (50 and 100 μ M) and PG-QC test solutions (equivalent to 50, 100, 500, and 931 μ M QC hydrate) in HBSS containing 0.5vol% ethanol was added to each well. HBSS was used as a control and monitored in parallel with the QC formulations. After 2 h incubation, medium was removed, and 10 μ L 5 mg/mL MTT solution (in PBS) was added to each well; the cells were incubated for another 2 h (37°C, 5% CO₂) to allow MTT to be metabolized. The medium was then removed and the formazan crystals (product of MTT metabolism) were dissolved in 300 μ L DMSO. Absorbance values were read at 595 nm on a microplate reader (iMark, Bio-Rad Instruments, Inc., USA). The percent cell viability was calculated from the equation below:

$$\text{Cell viability (\%)} = \frac{\text{QC}_{\text{signal-background}}}{\text{HBSS}_{\text{control-background}}} \times 100 \quad \text{Eq. (3)}$$

where QC_{signal-background} was obtained by measuring the absorbance of QC-containing solutions (i.e., QC alone and PG-QC formulation solutions) and subtracting each absorbance by that of DMSO; and HBSS_{control-background} was obtained by subtracting the absorbance from the HBSS control by that of DMSO.

2.2.7.5. *Stability QC in HBSS*

The stability of QC in QC alone and PG-QC formulation test solutions upon the 2 h exposure to HBSS media (pH 6.5 and 7.4) was evaluated. The test was carried out by incubating QC and PG-QC test solutions in HBSS at pH 6.5 and 7.4 respectively in cell-free environment in a shaker bath at 37°C, 60 rpm and thereafter measuring QC concentration after 2 h using a UV-Vis spectrophotometer at 374 nm.

pH of 6.5 on the apical side was chosen since it is close to that of the *in vivo* intestinal lumen, whereas pH 7.4 on the basolateral side corresponds to the blood circulation. Stability of QC in the HBSS media was expressed as the percentage of QC remained soluble after incubation in HBSS (pH 6.5 and 7.4) at 37°C for 2 h.

2.2.7.6. Cellular Uptake and Transepithelial Permeation of QC

To characterize the *in vitro* cellular uptake of QC from QC alone and PG-QC formulation solutions, spent medium was replaced with fresh medium 24 h before the permeation experiments. One hour before experiments, spent media from the apical and basolateral sides were aspirated. The monolayers were quickly rinsed twice with warm PBS (pH 7.4, 37°C) and the monolayers were incubated with HBSS (pH 7.4) twice for 30 min each time in a CO₂ incubator at 37°C. Thereafter, the HBSS buffer was gently removed by aspiration before the test solutions were loaded to the triplicate wells of 24-well Transwell[®] plates. To measure the apical-to-basolateral permeability, 0.5 mL aliquots of individual test solutions (QC alone solutions with 50 and 100 µM QC hydrate and PG-QC solutions with 50, 100, 500, and 931 µM QC hydrate, all in HBSS containing 0.5vol% ethanol, pH 6.5) were loaded to the apical side (donor compartment) of the transwell respectively; the basolateral side (receiving compartment) received 1.5 mL HBSS (0.5vol% ethanol, pH 7.4, 37°C). As a control, the insert was incubated with 0.5 mL prewarmed blank HBSS (0.5 vol% ethanol, pH 6.5, 37°C) alone on the apical side, while the basolateral side received 1.5 mL of prewarmed HBSS (0.5 vol% ethanol, pH 7.4, 37°C). All cultures were incubated in a CO₂ incubator for 2 h.

After the incubation, the apical and the basolateral solutions were collected respectively. In the meanwhile, cellular extracts were prepared by rinsing the inserts three times with 0.5 mL ice-cold PBS, and incubating with methanol containing 0.5vol% acetic acid for 30min. Then the

cells were scraped off and the resulting mixture was sonicated in a sonication bath for 10 min at room temperature and then centrifuged at $1,600 \times g$ for 5 min. The supernatant was collected; the cells were rinsed two more times with 1 mL methanol, vortexed for 1 min, and centrifuged at $1600 \times g$. All the supernatants were combined. The apical and basolateral solutions (in HBSS) and the cellular extracts were evaporated to dryness under vacuum. The residues were reconstituted in 250 μ L methanol for HPLC analysis. In all cases, TEER values were monitored before and after the experiments. The incubation did not adversely affect TEER values, confirming that the integrity of the cell monolayers was well preserved. All measurements were performed in triplicate.

2.2.7.7. HPLC Analysis of QC

The amount of QC transported (expressed as nmol/well) was determined using reversed-phase HPLC composed of an Ace C8 *column* (4.6×75 mm, i.d., 3 μ m), a G1312A binary pump, a G1329A auto-sampler, and a G1315B diode array (DAD) UV detector set at 370 nm. The mobile phase consisted of 35vol% acetonitrile with 1vol% formic acid and 65vol% water with 1vol% formic acid. Isocratic elution was performed at ambient temperature at a flow rate of 0.8 mL/min, and injection volume was 30 μ L. The total chromatographic run time was 6 min for all analyses. The retention time of QC was 2.6 min. The QC calibration curve prepared in methanol was linear ($r^2 = 0.999$) over the concentration range of about 0.1 ~ 300 μ g/mL. The software used for chromatographic data acquisition and analysis was Agilent ChemStation, version B.01.03.

2.2.8 Statistic analysis

Experiments were performed at least three times. Results were expressed as mean $\pm$ standard deviation. The differences between the mean values were analyzed for significance using one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered as being statistically significant.

3. Results and Discussion

3.1 Effect of Ratio between PG and QC on QC Solubility

PG is a naturally occurring and biodegradable plant-based food material. Compared with the synthesized materials used in the pharmaceutical industry, PG is a much-preferred carrier to improve the solubility of poorly water-soluble compounds for food applications. In our study, to evaluate the effect of the ratio between PG and QC on QC solubility, varying concentrations of QC hydrate were incorporated with PG aqueous dispersion. In addition, the impact of ultrasonication on the fabrication and solubility of PG-QC formulations were also evaluated. In this study, we defined that the QC in the supernatant of QC-containing aqueous dispersion was soluble QC. As shown in **Figure 2**, similar trends were observed for PG-QC-#1S and PG-QC-#1N. An increase in the PG/QC ratio (i.e. the mass ratio between PG and QC hydrate) from 2/1 to 30/1 resulted in a progressive increase in QC solubility, reaching a highest value of 241.76 $\mu\text{g/mL}$ for PG/QC 30/1. However, further enhancement of QC solubility was restricted when the PG/QC ratio increased from 30/1 to 40/1. Nevertheless, in the case of physical mixtures, there was no apparent enhancement of QC solubility at all PG: QC ratios, with QC's highest solubility of 4.32 $\mu\text{g/mL}$ for the physical mixture PG/QC 20/1.

The effect of sonication on QC solubility was shown in **Figure 2**. PG-QC-#1S formulations showed remarkably higher solubility enhancing effect for QC, as opposed to PG-

QC-#1N prepared in the absence of sonication. An explanation of this is that sonication facilitates the interactions between QC and PG by mediating fast local micromixings. Processes like ultrasonication help in rapid mixing the solvent (ethanol phase) and the antisolvent (aqueous phase) due to generation of cavitation bubbles. The acoustic cavitation (the formation, growth, and implosive collapse of bubbles in a liquid) may create extreme conditions inside the collapsing bubble, which induces rapid mixing. Additionally, acoustic cavitation produced by ultrasonic waves can strongly increase the surface area and the mass transfer between the two phases (Freitas, Hielscher, Merkle, & Gander, 2006; Ghows & Entezari, 2011).

Figure 3 explains the mechanism of forming PG-QC complexation. At the initial stage of PG/QC mixing, QC would exist in 3 forms: (1) supersaturated solution of individual molecules, (2) crystallites (nuclei) that contains multiple molecules, and (3) molecules adsorbed with PG particulates individually (or possible in aggregates). Over the mixing and solvent-removal process, the QC molecules in the supersaturated solution would further partition between crystallites and PG particulates. There are likely two factors that allow the PG particles to impede the re-crystallization or crystal growth of dispersed QC molecules: (1) hydrogen-bonding between the glucan chains of PG and OH groups of QC, which enables the anchoring of QC molecules on the surface or within a certain depth of PG particulate; and (2) space-limiting effect due to the physical trapping of QC molecules in a certain depth within the PG particulate, thereby obstructing QC molecules from coming into close proximity. As a result, QC may exist in an amorphous form or with reduced crystallinity in the water-dispersible PG-QC complexes, which may attribute to the improved QC solubility compared with crystallized QC. PG particulate is highly hydrophilic and has sufficient rigidity to maintain its particulate structure (Chen, Narsimhan, & Yao, 2015), which make it advantageous in dispersing QC in an aqueous

phase without deformation or collapse. This is especially important given that PG-QC dispersions would undergo a drying process when the removal of solvent may enhance the crystallization of dispersed QC molecules.

As shown in **Figure 2**, the highest QC solubility occurs with the PG/QC ratio of 30/1 for both the sonicated and non-sonicated group (241.76 and 112.41 $\mu\text{g/mL}$, respectively). In contrast, the QC solubility of PG/QC ratio of 2/1 was only 40.48 $\mu\text{g/mL}$. This result was counterintuitive but can be explained with **Figure 3**. In this study, the amount of PG in the mixture was constant (0.8 g in a solvent formed by 1 mL ethanol and 4 mL aqueous buffer); therefore a low PG/QC ratio corresponds to high dose of QC. At the initial stage of mixing, the number of nuclei (for QC crystallization) was much greater for the low PG/QC ratio formulation than the high PG/QC ratio formulation (**Fig. 3**). When the rate of QC crystallization during the mixing, stabilization, and solvent removal is greater than QC adsorption to PG, the partitioning favors the crystallization. This may result in low QC adsorption and consequently low solubility at low PG/QC ratio. With a further increase of PG/QC ratio from 30/1 to 40/1, however, the solubility of QC decreased from 241.76 to 132.01 $\mu\text{g/mL}$, possibly due to a reduced total QC available for PG adsorption. Apparently, an optimal QC feeding amount and sufficient PG concentration are both important for achieving a high QC solubility.

3.2 Solid State Characterization

3.3.1 XRD Analysis

The water solubility of QC is in general adversely affected by the crystallinity of QC, because QC molecules must overcome the crystal lattice energy to become dissolved. Therefore, X-ray diffraction analysis was performed to evaluate the crystalline state of QC in PG-QC

formulations. As seen in **Figure 4**, between 5-35° of 2θ, QC hydrate exhibited peaks at 2θ of 10.76°, 12.48°, 15.88°, 24.48°, and 27.48°, indicative of its highly crystalline nature. In contrast, PG is amorphous with no prominent peaks appeared in its diffraction spectrum. For processed QC without PG, the recrystallized QC displayed the same spectrum profile as that of QC hydrate with a slightly higher crystallinity.

For the physical mixture: PG/QC 2/1, the crystalline structure of QC persisted as evidenced by the presence of peaks at 2θ of 10.76°, 12.48°, 24.48°, and 27.48°. In contrast, these peaks were negligible with PG-QC-#1S: PG/QC 2/1, suggesting that the crystallinity of QC was nearly absent due to the complexation with PG. A similar comparison can be found between physical mixture: PG/QC 5/1 and PG-QC-#1S: PG/QC 5/1 (**Fig. 4**). The presence of a polymeric matrix has been shown to inhibit the crystallization of poorly water-soluble drugs when they are introduced from organic solution to an aqueous solution (Hecq, Deleers, Fanara, Vranckx, & Amighi, 2005; Dalvi & Dave, 2009; Kumari, Yadav, Pakade, Singh, & Yadav, 2010). Polymers can interact and associate with drugs through hydrogen bond formation, thus disrupting the original crystalline structure of drugs and allowing conversion of the drugs to a more amorphous state (Scarfato et al., 2008; Wu et al., 2008; Zhang, Yang, Tang, Hu, & Zou, 2008). In addition, polymers may assume the role of mechanical barriers against crystallization, hindering the incorporation of drug molecules into crystal lattice and preventing crystal growth and agglomeration.

3.3.2 FTIR Analysis

FTIR has been successfully used to characterize intermolecular interaction in various QC delivery systems, such as QC-loaded chitosan nanoparticles, starch-QC solid complex, and solid cyclodextrin inclusion complex of QC (Zhang, Yang, Tang, Hu, & Zou, 2008; Koontz, Marcy,

O'Keefe, & Duncan, 2009; Zhang, Yang, Li, & Gao, 2011). The FTIR spectra of PG, QC hydrate, physical mixtures of PG and QC hydrate, and two PG-QC-#1S solids are shown in **Figure 5**. The major characteristic absorption bands corresponding to –OH phenolic stretching (around 3400 and 3210 cm^{-1}), aromatic C=O stretching (1660 and 1603 cm^{-1}) that appeared as a very strong doublet, benzene ring bond stretching (1560 and 1520 cm^{-1}), =C–O–C antisymmetry deformation vibration (1313 and 1167 cm^{-1}), aromatic bending (around 1100 cm^{-1}), –OH phenolic bending (around 1200 and 1400 cm^{-1}), and aromatic C–H bending (997 cm^{-1}) were present in the spectrum of QC hydrate. These distinctive absorption bands of QC hydrate are in the spectral region where PG absorption is limited, thus allowing identification of possible interactions between PG and QC. The spectrum of PG featured a series of overlapping bands located at 1180 ~ 900 cm^{-1} , which were due to vibration modes such as stretching of C–C and C–O and bending of C–H, and were often referred to as the “saccharide bands”. The broad band at 3280 cm^{-1} was attributed to the O–H stretching of intermolecular and intramolecular associations, and the small band at 3000 ~ 2800 cm^{-1} was assigned to C–H asymmetric stretching of C6 in glucose. The band at 1633 cm^{-1} was due to C=O stretching of C1. The peaks stretching over 1178 ~ 1450 cm^{-1} was due to C–H angle vibration.

In the case of physical mixtures, superimposition of respective absorption bands associated with PG and QC hydrate was evident with the peak intensity of individual QC peaks remained almost unchanged at PG/QC of 2/1. This finding was indicative of the absence of intermolecular interaction between PG and QC molecules. In contrast, the spectra of the corresponding PG-QC-#1S samples showed a significant suppression of the absorption bands of QC within 1700 ~ 1200 cm^{-1} . Noticeably, while the C=O stretching bands of QC hydrate at 1660 and 1603 cm^{-1} remained in its physical mixtures with PG; both bands decreased with PG-QC-

#1S: PG/QC 2/1 and disappeared with PG-QC-#1S: PG/QC 20/1. The absence of the bands at 1660 and 1603 cm^{-1} combined with the disappearance of 1633 cm^{-1} of PG indicated that the conversion of QC to an amorphous state correlated with the H-bonding formation between QC and PG in PG-QC-#1S: PG/QC 20/1. Comparing to native PG, the weakening of the band at 3000 ~ 2800 cm^{-1} in the PG-QC-#1S also suggested that the stretching vibration of $-\text{CH}_2-$ of C6 in the glucosyl units was suppressed, indicating that the $-\text{OH}$ associated formed H-bonding with other $-\text{OH}$ groups of PG or QC in the formulation.

Furthermore, the characteristic $-\text{OH}$ phenolic stretching (around 3400 cm^{-1}) that appeared as a small shoulder in the spectrum of QC emerged as a single broad peak centered around 3300 cm^{-1} in the PG-QC formulations, similar to the spectrum of pure PG. This distinct peak broadening may result from possible intermolecular H-bonding between PG and QC, which could contribute to the disruption of the crystalline structure of QC and allow its entrapment in and/or outside of PG matrix.

3.3 QC Cellular Uptake and Transepithelial Permeation

3.4.1 Cell Viability affected by QC

The cytotoxicity of QC alone and PG-QC solutions on Caco-2 cells was examined by direct measurement of cell viability using MTT assay (**Supplementary data Fig. 1**). Cultures were incubated with varying concentrations of QC hydrate (50 and 100 μM) and PG-QC-#2 (50, 100, 500 and 931 μM of QC hydrate equivalence) test solutions for 2 h. Results for the MTT assay were expressed as a percentage of control cells. Cell viability of > 80% was considered as non-toxic (Tian, Yang, Yang, & Wang, 2009). A high cell viability of > 94% was observed in all cases, indicating that the working test solutions of QC and PG-QC were non-toxic to the cells over the concentration ranges used in permeability study. Previous reports also indicated that 90%

of the cells were viable up to 100 μM of QC, suggesting that no significant cytotoxicity occurred (Chabane, Ahmad, Peluso, Muller, & Ubeaud-Séquier, 2009).

3.4.2 Evaluation of Caco-2 Cell Monolayer Integrity

The TEER measurements were employed as an indicator of the integrity of the Caco-2 cell monolayers. Caco-2 monolayer integrity was controlled by ensuring a TEER value of $> 350 \Omega \text{ cm}^2$. This assured that the Caco-2 cells had formed a proper monolayer with efficient tight junctions. The TEER values measured prior to and after the permeability test were all above $350 \Omega \text{ cm}^2$, indicating that the integrity of the monolayers was well maintained during the 2 h *in vitro* permeability test.

3.4.3 Stability of QC in HBSS at Different pH values

The stability of QC in cell-free HBSS media adjusted at pH 6.5 and 7.4 was evaluated and expressed as the percentage of QC retained after incubation at 37°C for 2 h, the same condition as used for the permeability experiment. The results indicated that the stability of QC in the PG-QC solutions was slightly higher in pH 6.5 than pH 7.4, but the difference was not significant (**Supplementary data Fig. 2**). Both QC and PG-QC formulations exhibited high stability in HBSS. It is known that QC has two pKa values, namely 7.3 and 8.4 (Date et al., 2011); it also contains phenolic groups capable of becoming ionized ($\text{pKa} \approx 6.5$) at higher pH values (Pool, Mendoza, Xiao, & McClements, 2013). According to Chabane et al. (2009), the quantities of non-ionized QC are 98.26% and 87.61% at pH 6.5 and 7.4, respectively. Thus, the slightly greater stability of QC in pH 6.5 HBSS was likely due to the deionization of QC at lower pH values, which makes it less susceptible to hydrolysis. In general, our results were in accordance with reports supporting that QC was stable in HBSS at pH 6.5 and 7.4 (Chabane, Ahmad, Peluso, Muller, & Ubeaud-Séquier, 2009; Tian, Yang, Yang, & Wang, 2009).

3.4.4 Recovery and Permeation of QC

Several important results can be seen from **Figure 6**. First, after 2 h incubation, QC molecules could be recovered from apical compartment, basolateral compartment, and Caco-2 cells. The total recovery was fairly consistent among different types of QC dispersion, ranging from 76.4% (QC 25) to 83.5% (PG-QC 465.5). It appears that a higher initial apical QC loading would lead to higher recovery. The imbalance of QC (i.e. loss from recovery) is indicative of the combined effect of chemical degradation and metabolic conversion of QC that could occur during the transepithelial permeation process. The 2 h incubation in the cell-free HBSS resulted in only minor degradation of QC (<10%) at apical (6.5) and basolateral (7.4) pH values (**Supplementary data Fig. 2**); therefore, the metabolic conversion of QC, while slightly, occurred over the duration of 2 h permeation. QC has been indicated to undergo metabolic conversion when incubated with Caco-2 cells. Murota *et al.* (2000) indicated that substantial accumulation of QC conjugates (glucuronide and/or sulfate) was found in Caco-2 cellular extracts and basolateral solution following incubation with QC. They also detected the appearance of QC conjugates in the apical solution due to transcellular efflux (Murota, Shimizu, Chujo, Moon, & Terao, 2000). Several other studies have also reported the detection of QC metabolites. Walgren *et al.* (1998) reported an average recovery of 67% from the Caco-2 apical and basolateral sides without taking into account the cellular uptake and possible metabolism. However, Boyer *et al.* (2004) failed to detect conjugation, in particular, glucuronidation in the intracellular Caco-2 extracts, which was ascribed to the lack of glucuronidation in their Caco-2 cell lines or the inadequate detection limit for the conjugates. In our study, we speculate that the loss of minor amount of QC was attributed to the metabolic conversion in addition to chemical degradation.

Compared with the similar QC recovery percentages among various samples, a more significant result was the QC distribution largely affected by the samples loaded, in particular between the QC alone and PG-QC dispersions. For example, the QC recovery percentages of the apical, cellular, and basolateral portions were 58.7, 15.6, and 4.6% for sample QC 50 and 38.8, 21.6, and 17.9% for PG-QC 50. We consider that such a difference in QC distributions was caused by the difference of stability of QC dispersions over the 2 h incubation. For QC 50 dispersion, its QC concentration was 100 μ M, corresponding to about 30.2 μ g/mL. Considering the low QC solubility, there was a possibility that such a high QC dose led to the supersaturation over the 2 h incubation that generated crystalline particles not accessible to the trans-membrane permeation. In contrast, the QC molecules associated with PG particulates were stable against recrystallization, able to desorb and release, and therefore accessible to the permeation through Caco-2 cell monolayer.

The association with PG particulates was able to realize much higher QC doses in PG-QC dispersions, which allowed for more effective QC delivery to the trans-membrane permeation. The PG-QC 250 dispersion was able to realize a cellular and basolateral concentration of 58.2 and 49.2 μ g/mL, respectively, with the recovery percentages of the apical, cellular, and basolateral portions of 36.4, 23.3, and 19.7%, respectively (**Fig. 6**). Such a high loading and permeation was not achievable using QC-alone dispersions. For PG-QC 465.5, the basolateral QC concentration after 2 h incubation reached 75 μ g/mL, with the recovery percentages of the apical, cellular, and basolateral portions of 45.2, 22.2, and 16.1%, respectively, with the total recovery rate of 83.5%. It appears that such a high initial QC dose not only enhanced QC permeation but also improved the recovery rate of total QC – possibly due to a relatively low percentage of QC metabolic conversion during the permeation process.

The differences in QC solubility should be a primary factor that governs its permeated amount through the Caco-2 cell monolayers. It has been suggested that the intestinal permeation of QC occurs by two mechanisms: (1) passive diffusion controlled by the affinity of the hydrophobic molecules with the hydrophobic phospholipid bilayer membranes and (2) pH-dependent transport mediated by influx protein carriers belonging to the soluble carrier (SLC) family, in particular the organic anion transporting protein OATP-B. Upon gaining access to the cytosol, QC may undergo metabolism by a variety of enzymes (Murota, Shimizu, Chujo, Moon, & Terao, 2000; Spencer, Kuhnle, Williams, & Rice-Evans, 2003; Mullen, Edwards, & Crozier, 2006; Mukai et al., 2013) or it may cross the basolateral membrane of the enterocytes, resulting in absorption (Walgren, Walle, & Walle, 1998; Walgren, Lin, Kinne, & Walle, 2000). By increasing apical concentration of QC, it is possible to achieve a higher concentration *gradient* for the apical-to-basolateral transport, thus promoting passive diffusion. Additionally, the PG-QC particles have a large surface/volume ratio, which might lead to the adherence of particulates to the membrane surface that improved the accessibility of membrane for QC permeation.

However, once entering the cells, QC molecules can be subsequently effluxed across the apical membrane. It has been revealed that intestinal absorption of QC is superimposed by the efflux from the basolateral to the apical side, as evidenced by the higher permeability in the basolateral-to-apical direction (Walgren, Walle, & Walle, 1998; Chabane, Ahmad, Peluso, Muller, & Ubeaud-Séquier, 2009). The interplay between the uptake and the efflux appears to govern the efficiency of QC transcellular absorption across the apical membrane. MRP2, an apically expressed multidrug resistance-associated protein transporter, has been widely implicated to assume an important role in the transcellular efflux of QC by opposing transcellular uptake and intracellular accumulation (Walgren, Lin, Kinne, & Walle, 2000). However, it was found that QC

was not among the highest affinity substrates for MRP2. Also it was observed that the MRP2-mediated transcellular efflux is a saturable process. These phenomena implied that the increased QC dosage could elevate transcellular absorption to a point where MRP2-mediated efflux is unable to outweigh such an added dosage.

4. Conclusions

In this study, phytoglycogen (PG) was used to improve the solubility and Caco-2 cell monolayer permeation of quercetin (QC). The dendritic structure of PG allowed the adsorption and stabilization of QC molecules with the particulates, thus improving the dispersity of QC in aqueous system. The use of sonication was able to enhance QC association with PG particulate, possibly due to a more intense mixing at micro-scale. X-ray powder diffraction showed the absence crystallinity with PG-QC solid, and FTIR revealed the hydrogen bonds formed between QC molecules and the hydroxyl groups of PG. The steric hindrance caused by the highly branched structure of PG may reduce the mobility of QC molecules and thus retard their recrystallization in the re-hydrated system. Importantly, such a steric hindrance did not seem to compromise the release of QC molecules in the Caco-2 permeation model, and increased QC dispersity due to PG-QC association led to increased permeation with Caco-2 epithelial membrane. PG is a native food-based carbohydrate polymer extracted from sweet corn, thus offering a practical opportunity for the formulation of food active ingredients and nutraceuticals that are poorly water-soluble.

555 **Acknowledgement**

556 The National Science Foundation grant No. 1006301 to Yuan Yao and Ganesan
557 Narsimhan provided the financial support for this study. We also appreciate the assistance by
558 Jingfan Chen in the manuscript preparation.

559

560 **References**

- 561 Aditya, N., Macedo A.S., Doktorovova S., Souto E.B., Kim S., Chang P.-S. & Ko S. (2014).
562 Development and evaluation of lipid nanocarriers for quercetin delivery: A comparative
563 study of solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and lipid
564 nanoemulsions (LNE). *LWT-Food Science and Technology*, 59, 115-121.
- 565 Althans, D., Schrader P. & Enders S. (2014). Solubilisation of quercetin: Comparison of
566 hyperbranched polymer and hydrogel. *Journal of Molecular Liquids*, 196, 86–93.
- 567 Araujo, J. & Araujo D. (2014). Micellar systems of poloxamer 407 and 403 for quercetin
568 delivery. BMC Proceedings, BioMed Central Ltd.
- 569 Boyer, J., Brown D. & Liu R.H. (2005). In vitro digestion and lactase treatment influence uptake
570 of quercetin and quercetin glucoside by the Caco-2 cell monolayer. *Nutrition Journal*, 4, 1–
571 15.
- 572 Chabane, M.N., Ahmad A.A., Peluso J., Muller C.D. & Ubeaud-Séquier G. (2009). Quercetin
573 and naringenin transport across human intestinal Caco-2 cells. *Journal of Pharmacy and*
574 *Pharmacology*, 61, 1473–1483.
- 575 Chen, H., Narsimhan G. & Yao Y. (2015). Particulate structure of phytyglycogen studied using β -
576 amylolysis. *Carbohydrate Polymers*, 132, 582–588.

577 Dalvi, S.V. & Dave R.N. (2009). Controlling particle size of a poorly water-soluble drug using
 578 ultrasound and stabilizers in antisolvent precipitation. *Industrial & Engineering Chemistry*
 579 *Research*, 48, 7581–7593.

580 Date, A.A., Nagarsenker M.S., Patere S., Dhawan V., Gude R., Hassan P., Aswal V., Steiniger F.,
 581 Thamm J. & Fahr A. (2011). Lecithin-based novel cationic nanocarriers (leciplex) II:
 582 improving therapeutic efficacy of quercetin on oral administration. *Molecular*
 583 *Pharmaceutics*, 8, 716–726.

584 Dora, C.L., Silva L.F., Putaux J.-L., Nishiyama Y., Pignot-Paintrand I., Borsali R. & Lemos-
 585 Senna E. (2012). Poly (ethylene glycol) hydroxystearate-based nanosized emulsions: Effect
 586 of surfactant concentration on their formation and ability to solubilize quercetin. *Journal of*
 587 *Biomedical Nanotechnology*, 8, 202–210.

588 Freitas, S., Hielscher G., Merkle H.P. & Gander B. (2006). Continuous contact-and
 589 contamination-free ultrasonic emulsification—a useful tool for pharmaceutical development
 590 and production. *Ultrasonics Sonochemistry*, 13, 76–85.

591 Ghows, N. & Entezari M.H. (2011). Fast and easy synthesis of core–shell nanocrystal (CdS/TiO
 592 2) at low temperature by micro-emulsion under ultrasound. *Ultrasonics Sonochemistry*, 18,
 593 629–634.

594 Gormaz, J., Quintremil S. & Rodrigo R. (2015). Cardiovascular disease: a target for the
 595 pharmacological effects of quercetin. *Current Topics in Medicinal Chemistry*, 15, 1735–
 596 1742.

597 Guo, Y. & Bruno R.S. (2015). Endogenous and exogenous mediators of quercetin bioavailability.
 598 *The Journal of nutritional biochemistry*, 26, 201–210.

599 Hecq, J., Deleers M., Fanara D., Vranckx H. & Amighi K. (2005). Preparation and
 600 characterization of nanocrystals for solubility and dissolution rate enhancement of nifedipine.
 601 *International Journal of Pharmaceutics*, 299, 167–177.

602 Huang, L. & Yao Y. (2011). Particulate structure of phytoglycogen nanoparticles probed using
 603 amyloglucosidase. *Carbohydrate Polymers*, 83, 1665-1671.

604 Kakran, M., Sahoo N. & Li L. (2011). Dissolution enhancement of quercetin through
 605 nanofabrication, complexation, and solid dispersion. *Colloids and Surfaces B: Biointerfaces*,
 606 88, 121–130.

607 Khaled, K.A., El-Sayed Y.M. & Al-Hadiya B.M. (2003). Disposition of the flavonoid quercetin
 608 in rats after single intravenous and oral doses. *Drug Development and Industrial Pharmacy*,
 609 29, 397–403.

610 Kim, K., Vance T.M. & Chun O.K. (2015). Estimated intake and major food sources of
 611 flavonoids among US adults: changes between 1999–2002 and 2007–2010 in NHANES.
 612 *European Journal of Nutrition*, 1–11.

613 Koontz, J.L., Marcy J.E., O'Keefe S.F. & Duncan S.E. (2009). Cyclodextrin inclusion complex
 614 formation and solid-state characterization of the natural antioxidants α -tocopherol and
 615 quercetin. *Journal of Agricultural and Food Chemistry*, 57, 1162–1171.

616 Kumari, A., Yadav S.K., Pakade Y.B., Singh B. & Yadav S.C. (2010). Development of
 617 biodegradable nanoparticles for delivery of quercetin. *Colloids and Surfaces B:*
 618 *Biointerfaces*, 80, 184–192.

619 Li, B., Konecke S., Harich K., Wegiel L., Taylor L.S. & Edgar K.J. (2013). Solid dispersion of
 620 quercetin in cellulose derivative matrices influences both solubility and stability.
 621 *Carbohydrate Polymers*, 92, 2033–2040.

622 Mukai, R., Fujikura Y., Murota K., Uehara M., Minekawa S., Matsui N., Kawamura T., Nemoto
 623 H. & Terao J. (2013). Prenylation enhances quercetin uptake and reduces efflux in Caco-2
 624 cells and enhances tissue accumulation in mice fed long-term. *The Journal of Nutrition*, 143,
 625 1558–1564.

626 Mullen, W., Edwards C.A. & Crozier A. (2006). Absorption, excretion and metabolite profiling
 627 of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and
 628 urine after ingestion of onions. *British Journal of Nutrition*, 96, 107–116.

629 Murota, K., Shimizu S., Chujo H., Moon J.-H. & Terao J. (2000). Efficiency of absorption and
 630 metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2.
 631 *Archives of Biochemistry and Biophysics*, 384, 391–397.

632 Pool, H., Mendoza S., Xiao H. & McClements D.J. (2013). Encapsulation and release of
 633 hydrophobic bioactive components in nanoemulsion-based delivery systems: impact of
 634 physical form on quercetin bioaccessibility. *Food & function*, 4, 162–174.

635 Ratnam, D.V., Ankola D.D., Bhardwaj V., Sahana D.K. & Kumar M.N.V.R. (2006). Role of
 636 antioxidants in prophylaxis and therapy: a pharmaceutical perspective. *Journal of Controlled*
 637 *Release*, 113, 189–207.

638 Rezaei-Sadabady, R., Eidi A., Zarghami N. & Barzegar A. (2014). Intracellular ROS protection
 639 efficiency and free radical-scavenging activity of quercetin and quercetin-encapsulated
 640 liposomes. *Artificial Cells, Nanomedicine, and Biotechnology*, 1–7.

641 Sampson, L., Rimm E., Hollman P.C., de VRIES J.H. & Katan M.B. (2002). Flavonol and
 642 flavone intakes in US health professionals. *Journal of the American Dietetic Association*,
 643 102, 1414–1420.

644 Scalia, S. & Mezzena M. (2009). Incorporation of quercetin in lipid microparticles: effect on
 645 photo-and chemical-stability. *Journal of Pharmaceutical and Biomedical Analysis*, 49, 90–
 646 94.

647 Scarfato, P., Avallone E., Iannelli P., Aquino R.P., Lauro M.R., Rossi A. & Acierno D. (2008).
 648 Quercetin microspheres by solvent evaporation: preparation, characterization and release
 649 behavior. *Journal of Applied Polymer Science*, 109, 2994–3001.

650 Spencer, J., Kuhnle G., Williams R. & Rice-Evans C. (2003). Intracellular metabolism and
 651 bioactivity of quercetin and its in vivo metabolites. *Biochemical Journal*, 372, 173–181.

652 Sun, M., Wang S., Nie S. & Zhang J. (2014). Enhanced oral bioavailability of quercetin by
 653 nanostructured lipid carriers *The FASEB Journal*, 28, 1044.1024.

654 Tian, X.-J., Yang X.-W., Yang X. & Wang K. (2009). Studies of intestinal permeability of 36
 655 flavonoids using Caco-2 cell monolayer model. *International Journal of Pharmaceutics*, 367,
 656 58–64.

657 Tran, T.H., Guo Y., Song D., Bruno R.S. & Lu X. (2014). Quercetin-containing self-
 658 nanoemulsifying drug delivery system for improving oral bioavailability. *Journal of*
 659 *Pharmaceutical Sciences*, 103, 840–852.

660 Walgren, R.A., Karnaky Jr K.J., Lindenmayer G.E. & Walle T. (2000). Efflux of dietary
 661 flavonoid quercetin 4'- β -glucoside across human intestinal Caco-2 cell monolayers by apical

662 multidrug resistance-associated protein-2. *Journal of Pharmacology and Experimental*
663 *Therapeutics*, 294, 830–836.

664 Walgren, R.A., Lin J.T., Kinne R.K.H. & Walle T. (2000). Cellular uptake of dietary flavonoid
665 quercetin 4'- β -glucoside by sodium-dependent glucose transporter SGLT1. *Journal of*
666 *Pharmacology and Experimental Therapeutics*, 294, 837–843.

667 Walgren, R.A., Walle U.K. & Walle T. (1998). Transport of quercetin and its glucosides across
668 human intestinal epithelial Caco-2 cells. *Biochemical Pharmacology*, 55, 1721–1727.

669 Wu, T.H., Yen F.L., Lin L.T., Tsai T.R., Lin C.C. & Cham T.M. (2008). Preparation,
670 physicochemical characterization, and antioxidant effects of quercetin nanoparticles.
671 *International Journal of Pharmaceutics*, 346, 160–168.

672 Zhang, L., Yang X., Li S. & Gao W. (2011). Preparation, physicochemical characterization and in
673 vitro digestibility on solid complex of maize starches with quercetin. *LWT-Food Science and*
674 *Technology*, 44, 787–792.

675 Zhang, Y., Yang Y., Tang K., Hu X. & Zou G. (2008). Physicochemical characterization and
676 antioxidant activity of quercetin-loaded chitosan nanoparticles. *Journal of Applied Polymer*
677 *Science*, 107, 891–897.

678

679

680

681

682 **Figure Captions**

683 **Figure 1.** Procedure of PG-QC formulations preparation. QC: quercetin; PG: phytoglycogen

684 **Figure 2.** Influence of the initial mass ratios between phytoglycogen and QC hydrate (PG/QC)
685 (2/1, 5/1, 10/1, 20/1, 30/1 and 40/1) on the solubility of QC in PG-QC formulations (PG-QC-#1S
686 and PG-QC-#1N) and physical mixtures (PM). Data are expressed as mean $\pm$ SD (n=3)

687 **Figure 3.** Schematic of the partitioning of quercetin (QC) molecules in the water-ethanol
688 dispersion that contains phytoglycogen (PG). The scenarios for low (top) and high PG/QC ratio
689 (bottom) are depicted. The stages of “mixing of QC and PG” and “QC distribution” are described,
690 showing a partitioning process of QC molecules in the presence of QC crystallites and PG
691 particulates. The symbols that represent various types of unit in the dispersion system are
692 provided at the top-right of the scheme. The cutaway structure of a PG particulate with QC
693 adsorption is presented at the bottom-right. In the two presenting for “mixing of QC and PG”, the
694 crystallites are highlighted using black, dotted circles.

695 **Figure 4.** X-ray diffraction spectra of quercetin hydrate (QC), processed QC without PG (QC
696 solid prepared through dispersing QC with 80vol% aqueous ethanol with sonication and
697 thereafter solvent removal), phytoglycogen (PG), physical mixture with PG and QC hydrate ratio
698 of 2/1 and 5/1 (Physical mixture: PG/QC 2/1 and Physical mixture: PG/QC 5/1, respectively),
699 and PG-QC complex with PG and QC hydrate ratio of 2/1 and 5/1 (PG-QC-#1S: PG/QC 2/1 and
700 PG-QC-#1S: PG/QC 5/1, respectively). For clarity of comparison, results are vertically displaced
701 by arbitrary amounts.

Figure 5. FTIR spectra of quercetin hydrate (QC), phytyglycogen (PG), physical mixtures of PG and QC hydrate at ratios of 2/1 and 20/1, and PG-QC complexes at ratios of 2/1 and 20/1. For clarity of comparison, results are vertically displaced by arbitrary amounts.

Figure 6. Distribution of quercetin (QC) in the apical compartment, basolateral compartment, and cellular layer after subjecting the QC-containing dispersions to Caco-2 cell monolayer permeation test. QC 25 and QC 50 indicate the apical loading of dispersions of 25 and 50 nmol/well QC hydrate (with QC alone), respectively. PG-QC 25, PG-QC 50, PG-QC 250, and PG-QC 465.5 indicate the apical loading of dispersions of PG-QC formulations (PG-QC-#2) that contained 25, 50, 250, and 465.5 nmol/well QC hydrate, respectively. The QC amounts are shown with mean values with error bars of standard deviations (n=3). On the top of each group, the percentage of total recovered QC from apical, basolateral, and cellular portions, based on initial apical loading, are indicated. The smaller chart in the center magnifies the bars for QC 25, QC 50, PG-QC 25, and PG-QC-50 for better view.

1 **Figures**

3 **Figure 1**

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6