JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Particle-Stabilizing Effects of Flavonoids at the Oil−Water Interface

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ABSTRACT: It has been shown that some common food flavonoids can act as excellent stabilizers of oil-in-water emulsions through their adsorption as water-insoluble particles to the surface of the oil droplets, i.e., Pickering emulsions are formed. Flavonoids covering a wide range of octanol−water partition coefficients (P) were screened for emulsification behavior by low shear mixing of flavonoid + n-tetradecane in a vortex mixer. Most flavonoids with very high or very low P values were not good emulsifiers, although there were exceptions, such as tiliroside, which is very insoluble in water. When a high shear jet homogenizer was used with 20 vol % oil in the presence of 1 mM tiliroside, rutin, or naringin, much finer emulsions were produced: the average droplet sizes (d_{32}) were 16, 6, and 5 μm, respectively. These results may be highly significant with respect to the delivery of such insoluble compounds to the gut, as well as their digestion and absorption.

KEYWORDS: Flavonoids, Pickering emulsions, delivery, absorption, log P, partition coefficient

INTRODUCTION

Flavonoids are polyphenol plant secondary metabolites which may confer a number of benefits to the plant including attraction of pollinators, protection against predation, and protection against UV damage. Flavonoids include over 4000 compounds which can be divided into six subclasses and further identified by different substitution patterns and groups of the C6−C3−C6 basic backbone, including the presence of an oxo-group at position 4, and a 2−3 double bond (see Figure 1 and Table 1).

Flavonoids have generated considerable interest in recent years because of the significant association between their dietary consumption and protection against disease.[1] Evidence of potential health benefits of flavonoids comes from studies of their in vitro activity (antioxidant and anti-inflammatory[2] activity, etc.) plus in vivo animal studies.[3] The wide occurrence of considerable amounts of flavonoids in daily diets (the estimated mean daily flavonoid intake is 190 mg[4]) indicates that, if they do indeed have positive protective effects against such diseases, they might provide a better alternative to treatment with drugs since any deleterious side effects and intolerance are less likely to occur.

In order to fully understand the in vivo consequences of dietary flavonoid ingestion and to make sense of in vitro and animal studies, the processes of flavonoid absorption and metabolism need to be better understood. In other words, bioactivity cannot be fully understood unless bioavailability is addressed. Understanding of the physicochemical properties of flavonoids is probably the first step in trying to rationalize their bioavailability. The chemical structures of the flavonoids (Figure 1) have hydroxyl groups that will contribute to the hydrophilic nature of the molecules, while the ring structures will contribute hydrophobicity, such that flavonoids may be described as amphiphilic molecules. Experimentally, flavonoids have been shown to have very poor water solubility[5] (with the exceptions of flavan-3-ols and anthocyanins, since they may be present in ionized forms). Aggregation[6] of the flavonoids in water may also be partly responsible for the poor solubility.

The poor solubility of flavonoids has spurred on a large number of studies to try and encapsulate them with various structures that might aid their dispersibility, using similar strategies as for water-insoluble drugs. Thus, spray drying,[7] solvent evaporation from polymer solutions,[8] cyclodextrins,[9,10] vesicles and liposomes,[11,12] yeast cells,[13] and nanoemulsions[14] have been investigated, for example. The problem with all these techniques is that they add expense and other ingredients that may have to be approved for food usage.

Oil solubility is just as important as aqueous solubility. The preference of a compound to be present in a hydrophobic environment is an important factor in determining whether or not this compound can cross cell membranes by passive diffusion. The partition coefficient (P) of a compound between water and a nonpolar solvent is considered to be a useful measurement of hydrophobicity. Before 1971, a number of different solvent systems had been used to measure P of various solutes.[15−17] Not surprisingly, the results using different solvent systems were difficult to compare. The octanol−water system was eventually standardized as a reference system by Leo and Hansch[18] for a number of reasons: (a) at that time, the largest number of measured values was for this system and for solutes with the widest range of functional groups; (b) the usefulness of the octanol−water system as a model for describing the binding forces between small molecules and macromolecules had already been established;[19] (c) in comparison with many of the other solvents used, octanol has relatively little effect on the solubilizing properties of water itself;[10] (d) good correlations between $\log_{10}P$ for octanol−water and the biological activity of various compounds was evident.[20,21] Since this time $\log_{10}P$ for octanol−water has become firmly established as a standard measurement for indicating solute hydrophobicity.

In the present study, we have explored further the hydrophobic/hydrophilic nature of a range of flavonoids. The $\log_{10}P$ values measured (here or obtained by others) have been compared with

Received: October 29, 2010
Accepted: January 19, 2011
Revised: January 12, 2011
Published: February 18, 2011

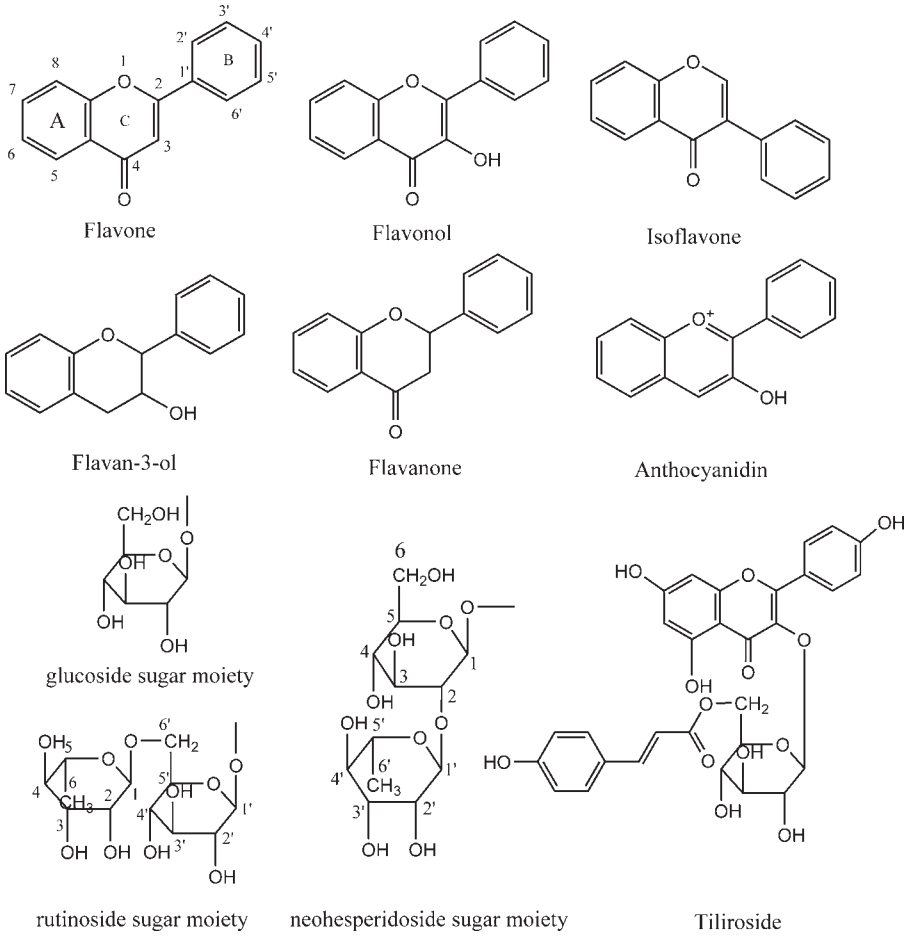

Figure 1. Structure of 6 flavonoid subclasses, tiliroside, and sugar moiety conjugates investigated in the present study. (See Table 1 for substitution patterns.)

the behavior of the flavonoids in the presence of water and the hydrocarbon oil *n*-tetradecane. This oil has proved to be a useful model of the oil phases typically present in real foods.[22] (Very few foods contain significant concentrations of nonpolar liquids of equivalent water solubility to *n*-octanol.) In certain circumstances, water-insoluble materials can show a strong preference for adsorption at the oil−water (O−W) interface and indeed can act as stabilizers of oil−water emulsions.[23] Such emulsions are termed Pickering emulsions, characterized by the accumulation of particles at the oil−water interface in the form of a densely packed layer that prevents droplet shrinkage and coalescence by a steric mechanism.[22−24] Unlike surfactant molecules, the particles involved in Pickering emulsions are usually much larger (tens of nm to μm) than individual molecules. The free energy of spontaneous desorption from the interface (ΔG) is proportional to $r^2(1 - \cos\theta)$, where r is the particle radius (for spherical particles), and θ is the contact angle of the particle at interface. As long as the contact angle is not too close to 0 or 180°, the value of ΔG can be 1000s of kT so that once the particles are adsorbed they are very difficult to remove. In other words, there is a huge energy barrier to droplet shrinkage or coalescence, and the emulsions are very stable.

In the following, we demonstrate that many flavonoids, present as water- and oil-insoluble particles, have a tendency for accumulation at the O−W interface and in some cases can provide very good stabilization of oil-in-water (O/W) emulsions.

MATERIALS AND METHODS

Materials. Water, purified by treatment with a Milli-Q apparatus (Millipore, Bedford, UK) with a resistivity not less than 18 MΩ cm, was used for the preparation of all solutions. Solvents 1-octanol (HPLC grade, ≥99%) and methanol (HPLC grade, ≥99.8%) were obtained from Sigma-Aldrich (Gillingham, UK). The hydrocarbon oil *n*-tetradecane (≥99%) was obtained from Alfa Aesar (Heysham, UK).

Unless otherwise stated, flavonoids were of HPLC grade (≥99%) and purchased from Extrasynthese (Genay, France). Tiliroside (HPLC grade) was kindly provided by Merck KGaA (Darmstadt, Germany). Rutin and naringin (HPLC grades) were purchased from Sigma-Aldrich.

Emulsion Preparation and Characterization. Emulsions were prepared in one of two ways. Coarse emulsions were prepared as follows. Flavonoid powder was weighed into a sterile polypropylene centrifuge tube. The appropriate volumes of *n*-tetradecane and water were then added to give a final flavonoid concentration ranging from 100 μM to 1 mM (based on the final volume) . The contents of the tube were mixed in a vortex mixer (Genie 2, Scientific Industries, USA) operating at full speed for 2.5 min. Fine emulsions were prepared by passing the aqueous flavonoid mixture (1 mM flavonoid concentration unless otherwise stated) and oil through a high pressure jet homogenizer[25] operating at 300 bar. Immediately after preparation, emulsions were sealed in either glass test tubes (height 75 mm, diameter 24 mm) or in sterile polypropylene centrifuge tubes (50 mL) at room temperature, in a dark place.

Emulsion droplet-size distributions were measured by static multiangle light scattering via a Mastersizer Hydro 2000 (Malvern

Table 1. Substitution Patterns of the Flavonoids Investigated in the Present Study[a]

flavonoids	3	5	7	3′	4′	5′	conjugates
			Flavonol				
quercetin	OH	OH	OH	OH	OH	H	
Q3R (rutin)	R	OH	OH	OH	OH	H	R
Q3,4′diG	G	OH	OH	OH	G	H	G, G
kaempferol	OH	OH	OH	H	OH	H	
K3G	G	OH	OH	H	OH	H	G
K3R	R	OH	OH	H	OH	H	R
K7Neo	OH	OH	Neo	H	OH	H	Neo
tiliroside	G+Cou	OH	OH	H	OH	H	G+Cou
			Flavanone				
naringenin	H	OH	OH	H	OH	H	
N7Neo (naringin)	H	OH	Neo	H	OH	H	Neo
N7R	H	OH	R	H	OH	H	R
hesperetin	H	OH	OH	OH	OCH_3	H	
H7R (hesperedin)	H	OH	R	OH	OCH_3	H	R
			Flavone				
apigenin	H	OH	OH	H	OH	H	
A7R	H	OH	R	H	OH	H	R
			Flavan-3-ols				
catechin	βOH	OH	OH	OH	OH	H	
epicathein	αOH	OH	OH	OH	OH	H	
EGCG	gallate	OH	OH	OH	OH	OH	gallate
			Isoflavone				
daidzein	phenol	H	OH	H	OH	H	
D7G (daidzin)	phenol	H	G	H	OH	H	G

[a] Abbreviations: G = glucoside moiety; R = rutinoside moiety; Neo = neohesperidoside moiety; G+Cou = glucoside + coumaroyl moiety; Q = quercetin; K = kaempferol; N = naringenin; H = hesperetin; A = apigenin; EGCG = epigallocathin-3-gallate; D = daidzein.

Instrument, Malvern, UK). Average droplet sizes were characterized in terms of the Sauter mean diameter d_{32} or volume mean diameter d_{43} defined by:

$$d_{ab} = \frac{\sum_i n_i d_i^a}{\sum_i n_i d_i^b} \quad (1)$$

where n_i is the number of the droplets of diameter d_i. All measurements were made at room temperature on at least three freshly prepared samples. The d_{43} parameter is particularly sensitive to the appearance of larger particles in a size distribution due to, for example, flocculation. The refractive indices of water and *n*-tetradecane were taken as 1.330 and 1.429, respectively. Attempts were also made to use the Mastersizer to size the flavonoid dispersions. Flavonoid powders were weighed and dissolved in Milli Q water, with a final concentration of 1 mM. The flavonoid suspension was then put through the jet homogenizer at 300 bar and a sample of the homogenized suspension added to the Mastersizer measurement cell. In the absence of further information, a refractive index of 1.429, i.e., the same as that of the tetradecane, was used.

Images of test tubes of the coarse emulsions and flavonoid dispersions were obtained with a Canon EOS 400D Digital SLR camera, operated without flash and in close up mode.

Confocal Microscopy. For microscopy of emulsions, a Leica TCS SP2 confocal laser scanning microscope (*CLSM*), mounted on a Leica Model DM RXE microscope base, was operated in fluorescence mode. Approximately 80 μL of sample were placed into a laboratory-made welled slide[26] filling it completely. A coverslip (0.17 mm thickness) was placed on top of the well, ensuring that there was no air gap (or bubbles) trapped between the sample and coverslip. The samples were scanned at 24 °C, using 10× or 40× oil-immersion objective lenses, of numerical apertures of 0.3 and 1.25, respectively, approximately 10 to 20 μm below the level of the coverslip, in order to minimize hydrodynamic (and other) interactions with the coverslip. Fluorescence from the sample was excited with the 488 nm Ar and 633 nm HeNe laser lines. Images were recorded at a resolution of 1024 × 1024 pixels and processed using the image analysis software Image J.

Measurement of Flavonoid Partition Coefficients. In order to obtain some measure of the hyrophobicity of the flavonoids, their partition coefficient between water and the standard solvent *n*-octanol was determined at room temperature (20 to 25 °C). The experimental method was based on that used earlier by Rothwell et al.[27] for this type of compound, with some slight modifications. Each polyphenol was dissolved in methanol to give a concentration of 200 μM and filtered through a 0.22 μm filter. Aliquots (100 μL) were placed in 1.5 mL Eppendorf tubes, and the methanol was evaporated away using an EZ-2 Genevac solvent evaporators (Genevac Ltd., Ipswich, U.K.) at 40 °C for 20 min, leaving a polyphenol residue. Filtered 1-octanol (200 μL) was added and vigorously mixed by vortexing for 1 min. Filtered Tris-HCL buffer (200 μL, pH 7.4) was added and then aqueous and octanol phases vigorously agitated by vortexing for 1 min. The tube contents were then centrifuged for 5 min at 2900*g* force to obtain clear separation of the phases. Of the upper octanol layer, 100 μL was removed via a micropipet and placed in a HPLC vial containing a small-volume insert for HPLC analysis. The remaining octanol phase was carefully removed and discarded. The lower buffer layer (100 μL) was removed and placed in a similar vial for HPLC analysis. The experiment was independently repeated at least 3 times for each compound. Recovery analysis was applied to check the reliability and consistency of the method, as follows. After all the methanol had been evaporated as above, further methanol (200 μL) was added to the Eppendorf tube. The contents were then mixed by vortexing for 1 min and then centrifuged at 2900*g* force for 5 min to remove any dust or insoluble matter before analyzing the solutions via HPLC to check the flavonoid concentration.

RESULTS AND DISCUSSION

Partition Coefficient Data and Coarse Emulsification Characteristics. As an example of the typical emulsifying behavior observed, Figure 2 shows digital images of coarse *n*-tetradecane emulsions prepared by vortexing in the presence of 1 mM tiliroside and an oil volume fraction (ϕ) ranging between 0.2 and 0.8. Also shown are the samples containing no oil and only oil after vortexing, i.e., $\phi = 0$ and 1, respectively. When no oil is present (Figure 2A), a turbid dispersion was formed, lightly colored yellow due to the natural color of tiliroside. At $\phi = 0.2$ (Figure 2B) an O/W emulsion was formed with the droplets creaming fairly rapidly. At $\phi = 0.5$ (Figure 2C) an O/W emulsion was again formed, that creamed a little more slowly than at $\phi = 0.2$, but at $\phi = 0.8$ (Figure 2D) a network of very large oil cells and droplets was formed with no obvious emulsion phase. Figure 2E ($\phi = 1$) clearly shows that the flavonoid did not disperse in oil but that it settled to the bottom of the oil phase. Thus, tiliroside apparently shows some capacity to stabilize O/W emulsions up to around $\phi = 0.5$. This behavior is typical of a water-soluble emulsifier, although it is clear from Figure 2A that the flavonoid is not completely soluble in water.

Figure 3 shows that the emulsifying capacity of tiliroside clearly increases with increasing flavonoid concentration. At

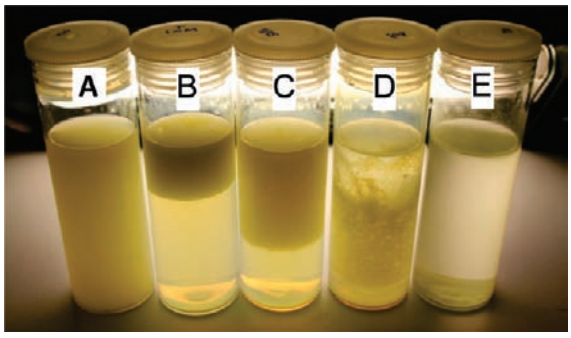

Figure 2. Coarse *n*-tetradecane emulsions and flavonoid dispersions prepared by vortexing in the presence of 1 mM tiliroside at different vol % of oil: (A) 0; (B) 20; (C) 50; (D) 80; and (E) 100 vol % oil.

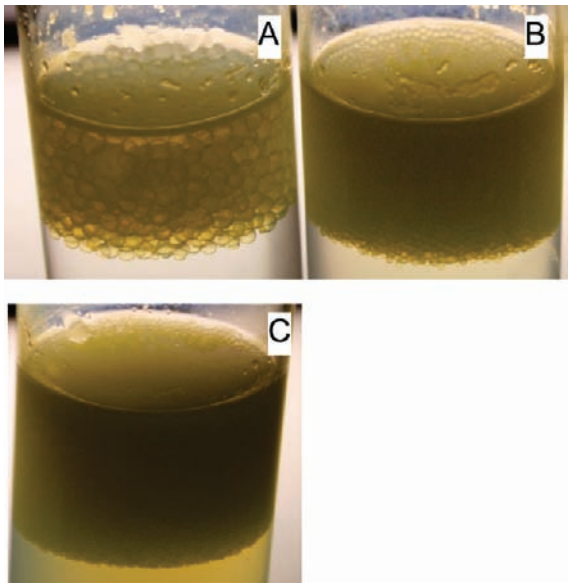

Figure 3. Coarse 20 vol % *n*-tetradecane emulsions prepared by vortexing in the presence of different concentrations of tiliroside: (A) 0.1; (B) 0.5; and (C) 1 mM.

0.1 mM tiliroside (Figure 3A), the droplets were visible to the naked eye, with a size of approximately 1 mm diameter. At 0.5 mM tiliroside (Figure 3B), the droplets were still visible but with a diameter approximately 1/2 to 1/3 smaller than those formed at 0.1 mM tiliroside. At 1 mM tiliroside (Figure 3C), it was impossible to distinguish most of the individual droplets with the naked eye. (Although by magnifying the digital images, it can be seen that the size was around 0.1 mm.) It was interesting that the emulsifying capacity increased above 0.1 mM tiliroside because even at this concentration, the solubility limit is apparently reached since the solution/dispersion of tiliroside on its own was not clear. Also, although the size of the oil droplets was rather large compared to that of conventional surfactant-stabilized emulsions, all of the emulsions appeared very stable (for at least 3 months), which is again unusual for oil droplets stabilized by low molecular weight surfactants (LMWS).

The behavior of tiliroside shown in Figures 2 and 3 was representative of many of the other flavonoids studied, although the degree of emulsifying capacity and the stability of the emulsions once formed varied greatly. Figure 4 illustrates this

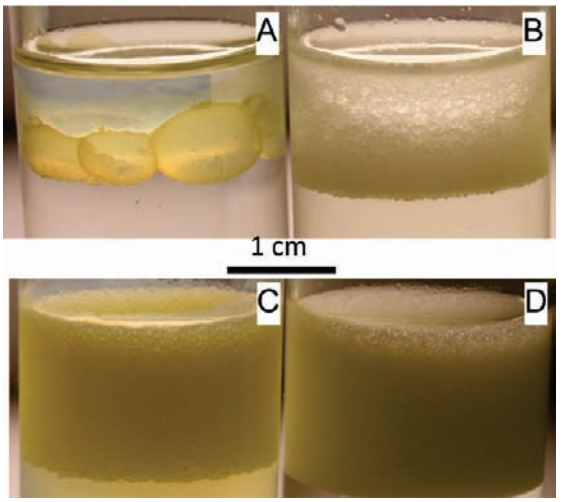

Figure 4. Coarse 20 vol % *n*-tetradecane emulsions prepared by vortexing in the presence of different flavonoids at concentration of 1 mM: (A) kaempferol; (B) kaempferol-3-glucoside; (C) tiliroside; and (D) rutin. The sequence A to D represents "no", "weak", "medium", and "good" emulsifying activity, respectively, as described in the text.

type of variation, by comparing 20 vol % *n*-tetradecane emulsions prepared by vortexing in the presence of different flavonoids at a concentration of 1 mM. The different flavonoids have been chosen to illustrate the qualitative descriptors that are subsequently used in this work to distinguish the different behavior of the wide range of compounds screened. Thus, kaempferol (Figure 4A) is described as having "no" emulsifying activity; kaempferol-3-glucoside (Figure 4B) is described as having "weak" emulsifying activity; tiliroside (Figure 4C) is described as having "medium" emulsifying activity; and rutin (Figure 4D) is described as having "good" emulsifying activity. It is seen that at the same observation time the better emulsifiers give a thicker apparent cream layer, due to smaller droplets and therefore slower creaming. Table 2 gives a more exact definition of the qualitative descriptors, and Table 3 summarizes the emulsifying behavior of all the flavonoids using these descriptors, grouped according to their structural class. This qualitative approach may be defended on the basis that not many of the flavonoids are good emulsifiers and therefore it is probably not relevant to characterize all the emulsions in detail, but also that it is difficult to characterize very unstable emulsions anyway.

Table 3 lists the octanol−water partition coefficients (P) determined experimentally either in this study or elsewhere.[27−32] Also shown are theoretical partition coefficients derived from solubility parameters, taken from various sources. At first sight, there is no obvious relationship between the $\log_{10}P$ values and the emulsifying behavior. In order to highlight any trends more easily, emulsifying capacity has been plotted against $\log_{10}P$ in Figure 5 in the form of a bar chart. The qualitative descriptors were assigned the arbitrary numerical values shown in Table 2. Average values of the experimentally determined $\log_{10}P$ values were used or where the experimental values had not been determined, the average values of the theoretically determined $\log_{10}P$ were used instead. This substitution seems justified since in most cases, there was reasonably good agreement between the experimental and theoretical values where both existed.

Following this procedure, it is seen in Figure 5 that, with the exception of tiliroside, all of the flavonoids fall into two main

Table 2. Descriptors of Flavonoid Emulsification Activity and Emulsion Stability[a]

descriptor	more detailed description of emulsification behavior observed	arbitrary numerical value assigned (see Figure 5)
"no"	No emulsion formed, clear oil and water phase separation within 1 h.	−2
"weak"	Emulsion formed with clearly visible droplets, but oiling off was clearly observable after 1 week storage.	−1
"medium"	Emulsion formed with clearly visible droplets, but little oiling off was observable after 1 week storage.	+1
"good"	Emulsion formed but individual droplets too small to distinguish and no observable oiling off after 1 week storage.	+2

[a] Note: oiling off refers to the formation of a distinct, clear layer of oil on top of the sample due to extensive coalescence of the oil droplets.

Table 3. Comparison of Theoretical $\log_{10}P$, Experimental $\log_{10}P$, and Emulsification Activity of Flavonoids Tested[a]

			activity			
flavonoids	theoretical $\log_{10}P$	experimental $\log_{10}P$	no	weak	medium	good
		Flavonol				
kaempferol	2.46	3.25[b], 3.11[c]	+			
K7Neo	−0.53	n/a				+
K3R	−0.57	n/a	+			
K3G	0.16	0.83[b]		+		
tiliroside	2.89	2.71[b]			+	
quercetin	2.16	1.82[c]	+			
Q3R (rutin)	−0.87	−0.27[b]				+
Q3, 4′diG	−2.41	−1.30[d]	+			
		Flavanone				
naringenin	2.84	2.70[b], 2.60[c]	+			
N7Neo(naringin)	−0.16	−0.13[b]				+
N7R	−0.16	n/a				+
hesperetin	2.68	2.60[e]	+			
H7R(hesperedin)	−0.31	n/a		+		
		Flavone				
apigenin	2.71	2.87[f], 3.02[e]	+			
A7R	−0.29	n/a			+	
		Flavan-3-ol				
catechin	1.80	0.32[g], 0.53[h]	+			
epicatechin	1.80	0.13[g], 0.6[h]	+			
EGCG	3.08	0.39[g], 0.26[h]	+			
		Isoflavone				
daidzein	2.73	2.51[c]	+			
D7G (daidzin)	0.46	0.32[c]	+			

[a] Key: + indicates emulsifying activity observed, as defined in Figure 4 and Table 2. Abbreviations: G, glucoside sugar moiety; R, rutinoside sugar moiety; Neo, neohesperidoside sugar moiety; Q quercetin; K, kaempferol; N, naringenin; H, hesperetin; A, apigenin; EGCG, epigallocathin-3-gallate; D, daidzein. [b] Experimental $\log_{10}P$ from present studies. [c] Experimental $\log_{10}P$ from Rothwell et al.[27]. [d] Experimental $\log_{10}P$ from Murota et al.[28]. [e] Experimental $\log_{10}P$ from Cooper et al.[29]. [f] Experimental $\log_{10}P$ from Li et al.[30]. [g] Experimental $\log_{10}P$ from Shibusawa et al.[31]. [h] Experimental $\log_{10}P$ from Lin et al.[32]. Theoretical $\log_{10}P$ predicted values were obtained via Java based online chemical software "Marvin and Calculator Plugin Demo", on Web site: http://www.chemaxon.com/demosite/marvin/index.html (accessed in March 2010).

camps: for $-0.6 < \log_{10}P < 0$, they are either active (K7Neo, A7R, rutin, N7R and naringin) or not active as emulsifiers, whilst outside this range they have no activity. [There is only one compound studied with very high water solubility, Q3, 4′diG ($\log_{10}P = -1.30$), which is inactive.] Between ca. −0.6 and 0 is just the region of $\log_{10}P$ where O/W emulsifying activity and surface active behavior might be expected since the compounds have some hydrophobic character but are tending to be more water-soluble (i.e., $\log_{10}P < 0$), whereas when compounds are very hydrophilic (low P) or very hydrophobic (high P), they tend to remain largely in the aqueous or oil phase, respectively. This highlights even more clearly the unique position of tiliroside, which is very insoluble in water but acts as a moderately good emulsifier. It is proposed that this is due to the tiliroside acting as a particulate emulsifier, i.e., the emulsions stabilized by tiliroside are O/W Pickering emulsions. Further evidence for this is discussed below.

Confocal Microscopy of Emulsions. Figure 6 shows representative *CLSM* images of coarse emulsions (formed via the vortex mixer) stabilized by 1 mM tiliroside or rutin, at high and low

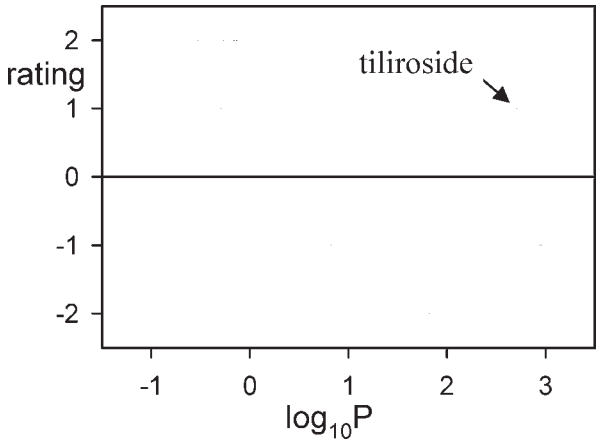

Figure 5. Qualitative rating of emulsion stabilizing activity versus logarithm of the mean experimental or theoretical octanol−water partition coefficient ($\log_{10}P$) of the various flavonoids studied.

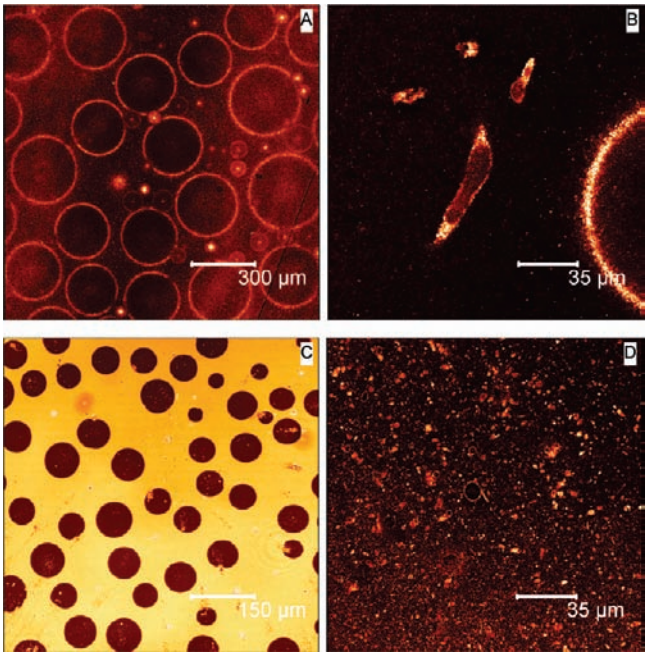

Figure 6. CLSM images of coarse 20 vol % *n*-tetradecane emulsions prepared by vortexing in the presence of 1 mM tiliroside or rutin: (A) tiliroside via 10× lens; (B) tiliroside via 40× lens (C); rutin via 10× lens; and (D) rutin via 40× lens. The brightness in the images is caused by the autofluorescence of the flavonoid particles themselves.

magnification. In these images, no dye was added, and the brightness is due to the natural fluorescence of the flavonoids themselves.[33,34] With a distinctly lower experimental $\log_{10}P$ value of −0.27, rutin is considerably more water-soluble than tiliroside, which has an experimental $\log_{10}P$ value of 2.71 (i.e., by at least 2 orders of magnitude). Nevertheless, it is obvious that for both tiliroside and rutin, most of the flavonoid is present as insoluble particles in the aqueous phase. Furthermore, there is a distinct preponderance for these particles to be associated with the surface of the round objects visible, which are the oil droplets. It was noticeable that there were more large particles, presumably crystals, of flavonoid present in the tiliroside system than in the rutin system. This might be expected if tiliroside is less water-soluble and

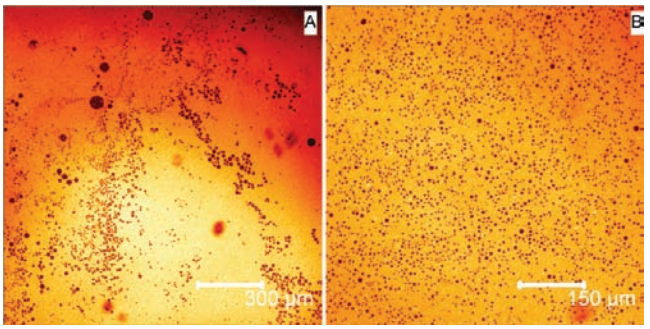

Figure 7. CLSM images (via the 10× lens) of fine 20 vol % *n*-tetradecane emulsions prepared via the jet homogenizer in the presence of (A) 1 mM tiliroside and (B) 1 mM rutin. The brightness in the images is caused by autofluorescence of the flavonoid particles themselves.

therefore grows to form larger crystals than rutin at the same concentration of added flavonoid. Consequently, it is suspected that all the other flavonoids that showed some emulsifying capacity may have done so as a result of adsorption of flavonoid particles to the O/W interface. This is because even those flavonoids that had low $\log_{10}P$ were still largely insoluble in water at a concentration of 1 mM, at which concentration excellent emulsion stabilization was observed for some flavonoids. Mauludin et al.,[35] for example, have determined the solubility limit of rutin as ca. 0.1 mM. These authors also went to considerable lengths to try and improve the solubility of rutin via application of high pressure homogenization and achieved a slight increase in the solubility (by about 20%) to ca. 0.12 mM. This higher solubility is still much less than 1 mM and actually means that really there was improved *dispersal* of rutin by the procedures applied. Finally, confocal microscopy images collected on the other flavonoid emulsion systems were qualitatively similar to those shown for tiliroside and rutin in Figure 6. There is no space here to show all these images. Instead, attention is focused further on the behavior of tiliroside and rutin since they both seemed to suggest Pickering emulsification, while their $\log_{10}P$ values are close to the extremes of values for many other flavonoids (see Table 3).

Figure 7 shows representative CLSM images for the fine emulsions prepared using the jet homogenizer, again using 1 mM tiliroside or rutin as the emulsifier. The emulsion droplets were obviously a lot smaller than those prepared using the vortex mixer (Figure 6), as expected due to the higher shear forces applied in the jet homogenizer. Also, it is apparent that there are fewer large flavonoid particles present in both systems. This suggests that the jet homogenizer may also help to break up the flavonoid crystals or break up aggregates of flavonoid crystals into smaller entities. Furthermore, as also suggested in Figure 6, the droplets tended to be smaller when rutin was the emulsifier rather than tiliroside. This agrees with the qualitative observations of emulsion stability. If both systems are indeed stabilized by flavonoid particles, the smaller particles present in the rutin system would allow greater coverage of the O−W interface and therefore a smaller average droplet size, leading to slower creaming.

To further substantiate the claim that the systems were Pickering emulsions stabilized by flavonoid particles, images were collected at different depths of focus on single large droplets and at the highest possible magnification. Examples are shown in Figure 8 for an emulsion prepared via vortexing in the presence of 1 mM tiliroside. The presence of a continuous bright ring at the O−W interface at various sections through a single oil droplet

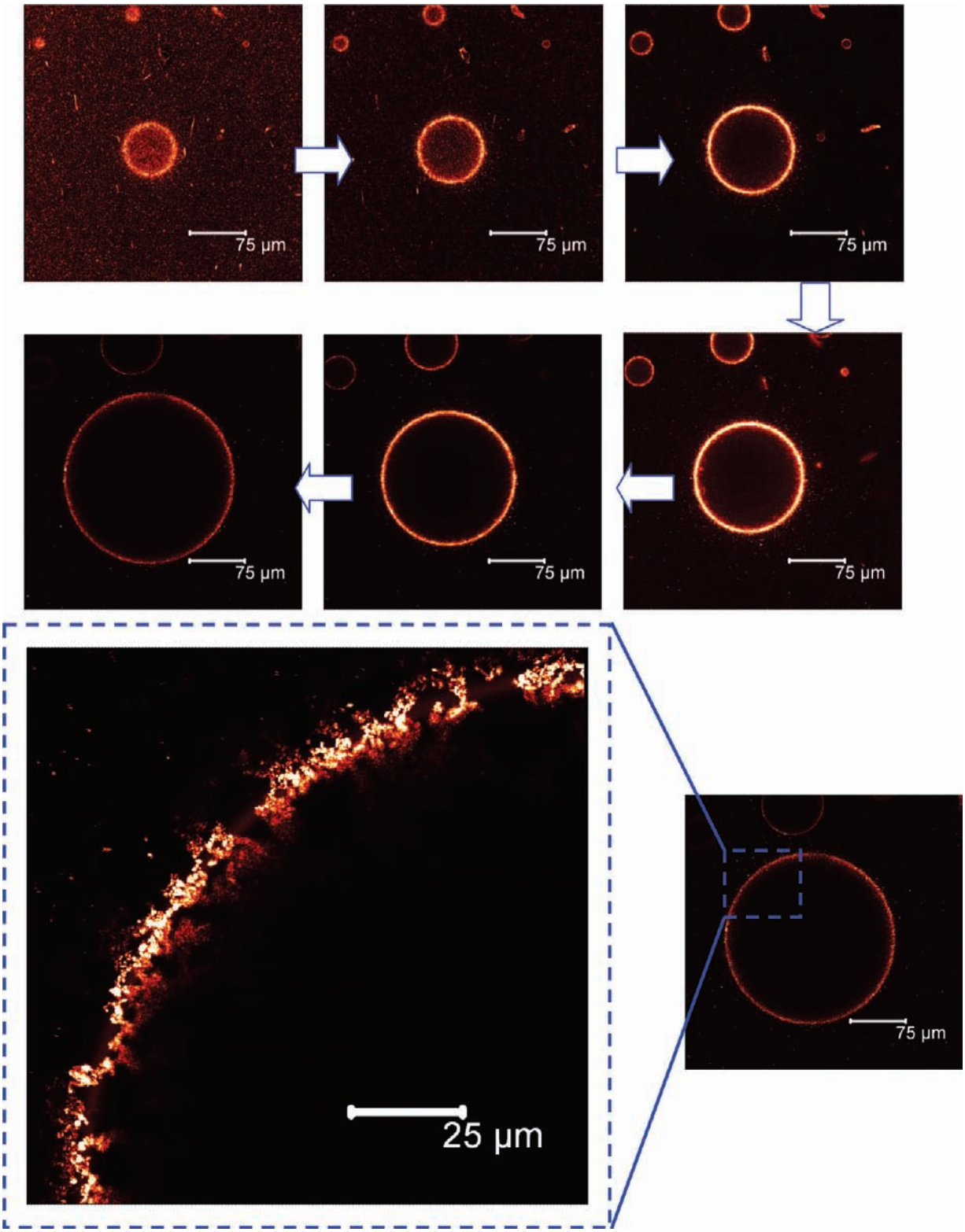

Figure 8. CLSM images (via the 40× lens) of fine 20 vol % *n*-tetradecane emulsions prepared via vortexing in the presence of 1 mM tiliroside. The arrows indicate the sequence of images collected at depths of focus differing by approximately 2 μm. The brightness in the images is caused by autofluorescence of the flavonoid particles themselves. The enlarged image highlights the presence of small tiliroside particles and their aggregates at the surface of the oil droplets.

and images of zoomed-in regions of the interface such as that shown make it hard to refute the assertion that the oil droplet surface is covered in a layer of small tiliroside particles.

Emulsion Droplet Size Distributions. Further information on selected emulsions is given in Figure 9. Droplet size distributions as determined by the Mastersizer are given for both the fine

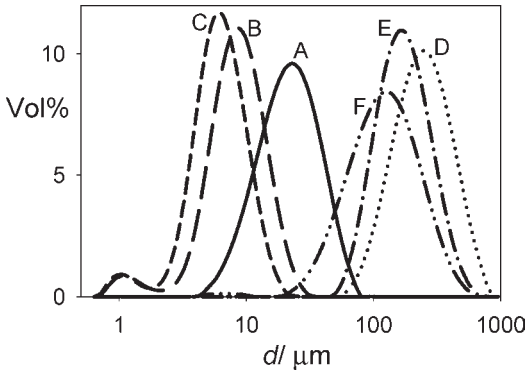

Figure 9. Droplet size distributions of 20 vol % *n*-tetradecane emulsions. Volume % (Vol %) is plotted against droplet diameter (d). Fine emulsions prepared via the jet homogenizer in the presence of 1 mM tiliroside (solid line A); 1 mM rutin (long dashed line B); 1 mM naringin (short dashed line C). Coarse emulsions prepared by vortexing in the presence of 0.5 mM tiliroside (dotted line D); 0.5 mM rutin (dashed-dotted line E); and 0.5 mM naringin (dashed-double dotted line F).

Table 4. Comparsion of Droplet Size of the 20 vol % *n*-Tetradecane Emulsions Prepared by Using Jet Homogenizer, Stabilized by 1 mM Flavonoid[a]

flavonoid	d_{32}/μm	d_{43}/μm
tiliroside	15.6 ± 3.4	25.3 ± 3.0
rutin	5.8 ± 0.1	9.2 ± 0.5
naringin	4.9 ± 0.4	7.5 ± 1.1

[a] Errors given are the standard deviations of 6 measurements on independently prepared emulsions.

and coarse emulsions stabilized by tiliroside, rutin, and naringin. As expected, the fine emulsions produced via the jet homogenizer have a much lower mean droplet size than those produced via vortexing, which agrees with their observed slower rate of creaming and serum separation on storage in the sample tubes. Table 4 gives the mean values of d_{32} and d_{43} determined for the 3 systems. Naringin was included as one of the best emulsifiers that also has a low (−0.13) $\log_{10}P$ value, although it again must be emphasized that at 1 mM, it is still largely insoluble in water, like rutin. In terms of producing the smallest droplet sizes, the order of best emulsification is naringin > rutin > tiliroside, for both the fine and coarse emulsions.

Because the majority of the flavonoid appeared to be particulate in nature, it is possible that the particle size distributions of the emulsions as determined by the Mastersizer are in error because the Mastersizer cannot distinguish between emulsion droplets and flavonoid particles as scattering centers. Attempts were therefore made to size the tiliroside, rutin, and naringin suspensions after the passage of 1 mM suspensions through the homogenizer at the same pressure as that used to make the emulsions. However, sufficient light scattering intensity could only be obtained with the tiliroside suspension. The average of 3 independent measurements on tiliroside gave values of d_{32} and d_{43} as 0.144 ± 0.001 μm and 0.312 ± 0.002 μm, respectively. It is seen that the measured tiliroside particle size is considerably smaller than that measured for the corresponding emulsions stabilized by tiliroside. (This is in general agreement with the confocal microscopy observations; see Figure 6.) Consequently, at least

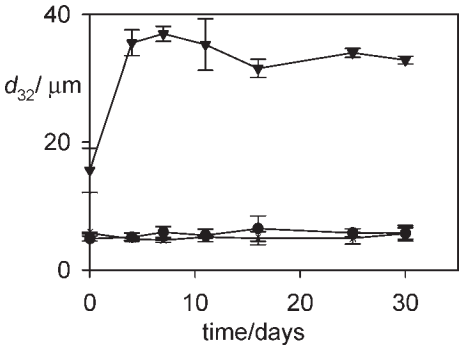

Figure 10. Mean droplet size (d_{32}) versus storage time for fine 20 vol % *n*-tetradecane emulsions prepared via the jet homogenizer in the presence of 1 mM tiliroside (▼); 1 mM rutin (×); and 1 mM naringin (●). The error bar indicates the standard deviation ($N \geq 4$).

for tiliroside, the emulsion droplet size distributions are probably quite reliable.

Emulsion Stability. Even the coarse emulsions stabilized by the "good" flavonoid emulsifiers, such as tiliroside, rutin, and naringin, showed no obvious visual changes over several weeks of storage, despite the large size of the droplets (see Figures 4 and 9). This in itself is a good indication that the systems are probably particle-stabilized, i.e., Pickering emulsions, since such large droplets stabilized by molecular surfactants are usually unstable to coalescence, whereas very large Pickering emulsion droplets can be indefinitely stable.[36,37] However, it is more difficult to obtain reliable droplet size distributions for such coarse emulsions in the Mastersizer because of their tendency for either rapid creaming or coalescence on dilution into the Mastersizer cell. Therefore, detailed measurements were only made for the fine emulsions. Figure 10 shows d_{32} determined over 1 month of storage for the fine emulsions stabilized by 1 mM tiliroside, rutin, and naringin, i.e., the same samples as referred to in Figure 9. For rutin and naringin, there is virtually no change in d_{32}, confirming the high stability of the emulsions stabilized by particles of these flavonoids. For tiliroside, there is an increase in d_{32} by a factor of 2 to 3 over the first week, but thereafter, d_{32} is stable. Whether this indicates some initial coalescence or some sort of sampling problem it is not clear, but the continued stability at $d_{32} \approx 35$ μm for several weeks is still impressive.

Estimated Minimum Particle Size for Surface Coverage. To test further the hypothesis that the emulsions could be stabilized solely by particles of flavonoid, it is instructive to estimate the mean particle size required to achieve this, as we have done previously for starch-particle stabilized emulsions.[38] However, one has to make certain assumptions about the size and shape of the flavonoid particles; the contact angle (θ) of the particles at the interface; and the area packing fraction of particles at the interface (ϕ_i). Unfortunately, neither θ nor ϕ_i is known, and the particles are certainly of different shapes and sizes. However, by assuming spherical flavonoid particles of all the same size, of radius r, and physically reasonable values of θ or ϕ_i, one can calculate the maximum surface area that a certain type of particle could stabilize. We will make the assumption that θ = 90°, which means that the particle will be strongly adsorbed and that it will occupy the maximum area of the actual interface = πr^2.

Making the above assumptions one can write:

$$N_p \pi r^2 / \phi_i = N_d 4\pi R^2 \quad (2)$$

dx.doi.org/10.1021/jf1041855 |J. Agric. Food Chem. 2011, 59, 2636–2645

where N_p = the number of flavonoid particles, and N_d = the number of droplets of radius R. N_p and N_d are given by

$$N_p = V_p/(4/3\pi r^3) \quad (3)$$

and

$$N_d = V_d/(4/3\pi R^3) \quad (4)$$

where V_p = the volume of particles, and V_d = the volume of oil droplets in the system. Substituting for N_p and N_d from eqs 3 and 4 into eq 2 and rearranging, it is easy to show that

$$r = (V_p R)/(4V_d\phi_i) \quad (5)$$

Thus, for an emulsion of given oil volume fraction, the minimum particle size required to cover all of the oil droplets is directly proportional to the mean droplet size and inversely proportional to ϕ_i. Unfortunately, ϕ_i is perhaps the most difficult parameter to estimate. We will assume a value of $\phi_i = 0.5$ so that at least half of the surface of the droplets must be covered with particles. The actual value of ϕ_i may be much greater or much lower than this since it is known[23,39] that coverage by surface active particles does not have to be complete to produce stable emulsions (and foams), as long as the adsorbed particle layer forms a strong enough network around the droplets. This can take place at much lower ϕ_i if the particles are asymmetric rather than spherical,[40] but in any case, we wish to estimate the *minimum* particle size required to see if this value turns out to be physically reasonable.

It is noted that this calculation also assumes that all the flavonoid adsorbs to the droplet surfaces. This is probably not the case either since the *CLSM* images suggest that at least some of the flavonoid remains as aggregates in the aqueous phase. This will have the effect of pushing down further the minimum size of r. From the material density of tiliroside (=1.68 g cm^{-3}), V_p can be calculated, and using the stable mean droplet size of d_{32} = 35 μm for the 20 vol % tetradecane emulsion stabilized by 1 mM tiliroside (see Figure 10), a value of the minimum required size of of the tiliroside particles is calculated as $2r$ = 0.1 μm via eq 5. Given the gross nature of some of the above assumptions, this is surprisingly close to the value of 0.144 μm estimated in the Mastersizer. Possibly, the crudest assumption is that the tiliroside crystals are spherical, when in actual fact they appear to have a reasonably high aspect ratio. For the same contact angle, an aspect ratio $\neq$1 will tend to increase considerably the minimum particle size (length) that is necessary to stabilize the droplets, due to the greater surface area to volume ratio of the particles and also lower ϕ_i required, as noted above.

The above calculation therefore illustrates that there could be easily enough water-insoluble, particulate flavonoid to stabilize the emulsions via the Pickering mechanism. The reasons why flavonoids of similar structure can apparently have very different emulsion stabilizing properties will therefore be more complex than simply depending upon their relative $\log_{10}P$ values, i.e., molecular solubility or molecular surface activity. It will depend also on the particle size and shape, i.e., crystal habit of the particles if they are single crystals, or the size and shape of the aggregates, as well as their surface charges and contact angle properties at the interface, which may well be different for different crystal planes. The surface charge and contact angle properties may depend upon the pH if some of the functional groups are weakly ionizing. Also of importance will be the ionic composition of the aqueous phase, which will determine the ions bound to the particles and the screening of electrostatic repulsion between the particles and between the particles and O−W interface.

From a biochemical point of view, the particulate stabilizer activity of the flavonoids may have significant effects on their absorption, as well as effects on the absorption of other food components, such as fat and fat-soluble vitamins, since they require emulsification before absorption. Several studies hint that absorption of some flavonoids is influenced by the presence of O/W emulsions. For example, it has been reported that absorption of quercetin in rats was increased by coadministration with lipids and emulsifiers.[41] Another study[42] reported a significant association between flavonoids and lipid micelles, while oral administration of tiliroside has been shown to exert potent antiobesity effects in mice.[3] None of the mechanisms of these effects is known. Treating flavonoids as surface active particles may provide a new approach to increasing our understanding of such effects and how to better control the delivery, bioavailability, and absorption of flavonoids.

Conclusions. It has been shown that some common flavonoids can act as stabilizers of oil-in-water emulsions through their adsorption as water-insoluble particles to the surface of oil droplets, i.e., Pickering emulsions are formed. From the molecular structure of an individual flavonoid or its partition coefficient (P) between octanol and water, it is difficult to predict how good an emulsifier the flavonoid will be, although most flavonoids with very high or very low P are not good emulsifiers. There are exceptions to even this rule, however, as exemplified by the example of tiliroside, which is almost 1000× more soluble in octanol than water (but still not very soluble in oil). The reasons for this unpredictability are probably due to the different size, shape, and surface charge properties of the flavonoid crystals or their aggregates, which will affect their contact angle and adsorption energy with the oil−water interface. Such factors are currently being studied in more detail in our group.

The practical significance of these findings remains to be seen. The emulsion droplets formed can be quite large, due to the large size of the flavonoid particles, and although the emulsions can be quite stable, better practical food oil emulsifiers undoubtedly exist. However, the results may have significance with respect to (a) the location of flavonoid compounds in real food systems, i.e., at oil−water interfaces, since foods frequently contain emulsified oil; (b) transport and delivery of these rather insoluble materials to the gut, avoiding the use of much more complex encapsulation techniques; (c) digestion and absorption of flavonoids within the alimentary canal of humans.

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ACKNOWLEDGMENT

We thank Dr. Rammile Ettelaie (School of Food Science & Nutrition, Leeds) for useful initial discussions and Merck KGaA for sponsorship and materials.

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