JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Evaluation of the Stability and Antioxidant Activity of Nanoencapsulated Resveratrol during in Vitro Digestion

Mariarenata Sessa,[†,‡] Rong Tsao,[‡,∥] Ronghua Liu,[‡] Giovanna Ferrari,[†,§] and Francesco Donsì*[,†,∥]

[†]Department of Industrial Engineering, University of Salerno, via Ponte don Melillo, 84084 Fisciano (SA), Italy

[‡]Guelph Food Research Centre, Agriculture and Agri-Food Canada, 93 Stone Road West, Guelph, Ontario, Canada N1G 5C9

[§]ProdAl, Competence Center on Agro-Food Productions, University of Salerno, via Ponte don Melillo, 84084 Fisciano (SA), Italy

ABSTRACT: Resveratrol was encapsulated in oil-in-water food-grade nanoemulsions of subcellular size, produced by high-pressure homogenization. Physicochemical stability was evaluated under accelerated aging (high temperature and UV light exposure), as well as during simulated gastrointestinal digestion. Antioxidant activity was assessed at different stages of digestion by chemical assays and by an improved cellular assay, to measure exclusively the residual activity of resveratrol that penetrated inside Caco-2 cells. Results showed that the nanoemulsions based on soy lecithin/sugar esters and Tween 20/glycerol monooleate were the most physically and chemically stable, in terms of mean droplet size (always <180 nm) and resveratrol loading, during both accelerated aging and gastrointestinal digestion. These formulations also exhibited the highest chemical and cellular antioxidant activities, which was comparable to unencapsulated resveratrol dissolved in DMSO, suggesting that nanoencapsulated resveratrol, not being metabolized in the gastrointestinal tract, can be potentially absorbed through the intestinal wall in active form.

KEYWORDS: nanoencapsulation, nanoemulsion, resveratrol, in vitro digestion, antioxidant activity, Caco-2 cells

INTRODUCTION

Resveratrol, a polyphenolic compound that can be found in red grapes and peanuts, as well as a variety of other plant sources, is one of the natural compounds that have been intensively investigated in recent years for its health-beneficial properties and for potential applications in the fields of pharmaceutics, nutraceuticals, and functional foods.

Resveratrol is well-known for its high antioxidant activity and is considered to be a key component for the health benefits of red wine. Chemically, resveratrol can be one of the two geometrical isomers, *trans*-resveratrol and *cis*-resveratrol; however, *trans*-resveratrol is the natural isomer form and the more stable and the more active form.[1]

Clinical studies have demonstrated several health-promoting activities of *trans*-resveratrol, such as antioxidizing[2] and antiatherosclerotic effects, inhibition of platelet aggregation,[3] beneficial effects on the cardiovascular system, reducing lipid peroxidation, improving vasodilatation, and lowering blood pressure,[4] and chemoprotective advantages against cancer proliferation.[5]

However, the physicochemical properties of resveratrol, particularly its high reactivity and low solubility in aqueous and lipid phases, have been limiting factors for its bioavailability and the efficacies of the desired health-beneficial effects. Resveratrol is highly soluble in alcohols, but soluble in only trace amounts in aqueous or lipid phase. Its solubility was reported to be 0.023 mg/mL in drinking water[6] and about 0.18 mg/mL in coconut oil.[7] In addition, resveratrol is a very reactive molecule, very susceptible to reaction with dissolved oxygen, producing different degradation products, as well as very easily degraded by sunlight.[8]

In a biological system, resveratrol is rapidly and extensively metabolized, probably due to its low water solubility, which reduces the dissolution rate limited cell absorption,[9] thus reducing its oral bioavailability. For resveratrol to exert its beneficial effects, it must reach a concentration in the blood of at least 10 mg/L,[10] which, for an average weight person, translates to the absorption of 50 mg of resveratrol.

Therefore, exploitation of resveratrol as a functional food and nutraceutical ingredient or pharmaceutical compound is feasible only when encapsulated in a delivery system, which is capable of stabilizing and protecting it from degradation while preserving its biological activities and enhancing its bioavailability. Encapsulation is one such potential system. However, until now, only few studies have addressed the suitability of delivering encapsulated resveratrol to the site of action, and the main research focus has been its biological activity, especially in synergy with the consumption of other diet/beverage components.[11]

More specifically, encapsulation of resveratrol in delivery systems of nanometric size has been found to contribute even more significantly to the improvement of its cell uptake. For example, nanometric carriers for pharmaceutical applications were designed using lipospheres to efficiently transport resveratrol into the cardiovascular system,[12] whereas biodegradable polymeric nanoparticles[13] and liposomes were developed to enhance resveratrol chemopreventive efficacy.[11] More recently, polymeric micelles have been used to enhance the ability of resveratrol to protect cells from oxidative stress and apoptosis,[14] and solid lipid nanoparticles have been used to increase the uptake of resveratrol in keratinocytes cells.[15] In addition, cyclodextrins have been used as carrier molecules to increase both the bioavailability and stability of resveratrol through the formation of inclusion complexes.[16]

Received: August 4, 2011
Revised: October 25, 2011
Accepted: October 26, 2011
Published: October 26, 2011

ACS Publications © 2011 American Chemical Society

Most of the above nanoparticulate carriers are based on hydrophobic interactions of resveratrol either with functional groups of specific molecules, such as biopolymers or cyclodextrins, or with amphiphilic molecular assemblies, such as liposomes or polymeric micelles. In all of these cases, the ratio of the encapsulating functional molecules to resveratrol is very high, on the one hand strongly promoting its solubilization in aqueous phase but on the other hand severely limiting the application in food, due to taste, regulations, or cost constraints. Among the cited nanometric carriers, only solid lipid nanoparticles are suitable for food application, due to the high loading capacity and low emulsifier content. Nevertheless, the high cost of production, related to the high processing temperature and pressure required, is still a limit to their full exploitation.

In food applications, an optimal delivery system of resveratrol would include a formulation containing all natural ingredients, which has minimal impact on the organoleptic properties of the food product and is able to preserve resveratrol in its most active form during the different phases of food transformation and storage and to ensure high bioavailability upon ingestion.

Oil-in-water (O/W) nanoemulsions are ideal candidates for the encapsulation of resveratrol because of the easy fabrication by high-throughput processes (i.e., high-pressure homogenization), the possibility of using all natural ingredients with low concentrations of emulsifiers,[17−19] and the easy dispersibility in aqueous-based food matrices. Furthermore, nanoemulsions can significantly improve the bioavailability of the encapsulated compounds after ingestion, by favoring dissolution and solubilization in the gastrointestinal tract and increasing the permeability through the intestinal epithelium, as well as by accelerating the absorption process because the phase of enzymatic digestion of lipid droplets can be omitted.[20]

Nanoemulsions have never been reported before for the encapsulation of resveratrol because of the issues of formulation, stability, and cell absorption. Therefore this study aims at contributing to the development of efficient nanoemulsion-based delivery systems for resveratrol. In particular, the main goal of the investigation activity is the fabrication of delivery systems, with requisites of (a) formulation with natural ingredients, (b) high physical stability of the emulsions and chemical stability of the most active form of resveratrol (*trans*-resveratrol) under conditions simulating the transformation and storage of the final product, and (c) retention of the antioxidant activity of resveratrol, even after digestion, using an improved biological-based method.

Finally, this work is also intended to give a novel contribution to the investigation of the stability of nanoencapsulated resveratrol during gastrointestinal digestion, because previous studies mainly focused on in vitro digestion of crude polyphenols in food,[21,22] but only few authors have investigated the effects of the encapsulation on its gastrointestinal transit.[23]

MATERIALS AND METHODS

Chemicals. Resveratrol, extracted from grape skin (purity > 98%), was a kind gift from Organic Herb Inc., China. Emulsion formulation was based on peanut oil (Sagra, Lucca, Italy) as organic phase and the combination of lipophilic and hydrophilic emulsifiers. Lipophilic emulsifiers were soy lecithin Solec IP (a kind gift from Solae Italia s.r.l., Milan, Italy), soy lecithin Lecinova (Nutrition and Santè, Varese, Italy), and glycerol monooleate (Sigma-Aldrich s.r.l., Milan, Italy). Hydrophilic compounds were sugar ester P1670 (a kind gift from Prodotti Gianni, Milan, Italy), defatted soy lecithin Solec FS-B (a kind gift from Solae Italia s.r.l.), and polysorbate Tween 20 (Sigma-Aldrich s.r.l., Milan, Italy). Bidistilled water was used as continuous phase.

Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile salts, and phenylmethanesulfonyl fluoride (PMSF) were all purchased from Sigma-Aldrich (Oakville, ON, Canada).

Ascorbic acid, 2,4,6-tripyridyl-*s*-triazine (TPTZ), fluorescein, dimethyl sulfoxide (DMSO), and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were from Sigma Chemical Co. (St. Louis, MO), iron(III) chloride hexahydrate and hydrogen peroxide solution at 30% w/w were obtained from Sigma-Aldrich (Oakville, ON, Canada), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Caco-2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Dulbecco's modified Eagle medium (DMEM), Hanks' balanced salt solution (HBSS), and antibiotic−antimycotic (100×) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Thermo Scientific HyClone (Logan, UT).

Fabrication of Nanoemulsions Loaded with Resveratrol. Resveratrol (0.01 wt %) was encapsulated in peanut oil-based nanoemulsions, produced by high-pressure homogenization. A preliminary study was carried out to optimize the formulation by constructing a pseudoternary phase diagram of kinetic stability, which was used to determine the optimal fractions of the different emulsion ingredients, as previously described.[17]

The tested formulations had the following composition: 0.01 wt % resveratrol, 0.2 wt % ethanol, 1 wt % lipophilic soy lecithin Solec IP, 0.5 wt % defatted soy lecithin Solec FS-B, 6 wt % peanut oil, 92.29 wt % water (R/LSL-DSL); 0.01 wt % resveratrol, 0.2 wt % ethanol, 2.1 wt % soy lecithin Lecinova, 27.9 wt % peanut oil, 69.79 wt % water (R/LEC); 0.01 wt % resveratrol, 0.2 wt % ethanol, 1 wt % lipophilic soy lecithin Solec IP, 0.3 wt % sugar ester, 9 wt % peanut oil, 89.49 wt % water (R/LSL-SE); 0.01 wt % resveratrol, 0.2 wt % ethanol, 1.5 wt % polysorbate Tween 20, 1.5 wt % glycerol monooleate, 7 wt % peanut oil, 89.79 wt % water (R/T20-GMO).

Because the oil phase alone did not increase sufficiently the solubility of resveratrol, a small amount of ethanol (ethanol/resveratrol weight ratio was 20) was used to dissolve resveratrol crystals prior to mixing at room temperature with peanut oil and the eventual lipophilic emulsifier. The concentration of ethanol in oil was kept at 0.024 g/g, which is significantly lower than the equilibrium solubility of ethanol in different oils, which was reported to range at 25 °C from 0.125 g/g for canola oil to 0.147 g/g for corn oil.[24]

The lipid phase containing resveratrol was subsequently dispersed in the aqueous phase containing the hydrophilic emulsifier by high-speed homogenization using an Ultra Turrax T25 blender (IKA Labortechnik, Jahnke and Kunkel, Germany) at 24000 rpm for 4 min, to obtain a primary emulsion. The primary emulsion was then disrupted to the nanometric size in a Nano DeBEE electric bench-top laboratory high-pressure homogenizer (BEE International), by means of 10 passes at 300 MPa. The operating temperature was maintained at 10 °C, by initial conditioning of the primary emulsion in a thermostatic bath, and by rapidly cooling the heat generated by friction with a heat exchanger, fitted immediately downstream of the homogenization valve. All formulations tested were prepared in triplicate.

Physical Stability. The physical stability of nanoencapsulated resveratrol was evaluated in terms of evolution of the mean droplet size of the nanoemulsions and of the creaming volume over time under

accelerated aging conditions. For each formulation, three independent samples (15 mL) were stored vertically in plastic tubes at different temperatures (4, 30, and 55 °C) in dark condition, with observations being carried out for 4 weeks.

The droplet size distribution was determined by photon correlation spectroscopy (PCS) at 25 °C (HPPS, Malvern Instruments, U.K.). The mean droplet size (*Z*-diameter) was determined by cumulant analysis of the intensity−intensity autocorrelation function, $G(q,t)$.[25] Prior to measurements, the samples were diluted with bidistilled water to a suitable concentration.

The value of the creaming volume percentage C was computed for each nanoemulsion using eq 1[26]

$$C = 100\,\frac{(V_t - V_s)}{V_t} \tag{1}$$

where V_t (mL) is the total volume of the sample and V_s (mL) is the volume of the lower phase layer (serum). According to eq 1, the value of C tending to 100 is an indication of a stable emulsion. Physical stability tests were conducted in triplicate.

Chemical Stability. The chemical stability of nanoencapsulated resveratrol was also evaluated under accelerated conditions, by determining the kinetics of degradation upon UV light exposure and at three different storage temperatures (4, 30, and 55 °C).

For UV light stability, samples (20 mL) of each nanoemulsion containing resveratrol were exposed to a laboratory UV-C lamp (280−100 nm) at room temperature for 2 h. Aliquots were sampled at predetermined time points (10, 30, 60, and 120 min) for quantitative analysis.

For thermal stability, samples (15 mL) of resveratrol nanoemulsion were stored vertically in open test tubes in dark condition, and aliquots were sampled each week for 4 weeks for quantitative analysis.

Quantitative analysis was conducted by extracting resveratrol from the emulsion and by using HPLC. One milliliter of resveratrol nanoemulsion was mixed in a test tube with 400 μL of carbon tetrachloride and 400 μL of ethanol, by agitation on a vortex mixer for 15 s. The separation of the phases was improved by centrifugation at 6500 rpm for 5 min at 10 °C. The lighter phase, which contains resveratrol, was recovered with a Pasteur pipet, and the heavier phase was then extracted twice with 500 μL of ethanol. Anhydrous sodium sulfate was added to the organic phase to remove any traces of water, prior to concentration under reduced pressure (Rotavapor) at 36 °C, and the residue was redissolved in 1 mL of ethanol. The resveratrol extract was then injected in the HPLC system, consisting of a Waters 1525 binary HPLC pump and a UV−vis 2487 Waters dual absorbance detector. An RP C18 Spherisorb (Waters, 5 μm, 4.6 × 250 mm) column was used, and the separation was done using an isocratic mobile phase of 45% methanol in water (v/v), which was adjusted to pH 2.6 with acetic acid. The flow rate was 0.8 mL/min, and the injection volume was 10 μL. The UV−visible detector was set at 306 nm for *trans*-resveratrol and at 280 nm for *cis*-resveratrol.

Quantification of *trans*-resveratrol was based on a calibration curve generated with serial dilutions (0.1 and 100 mg/L) of the stock solution of 200 mg/L in ethanol. The calibration curve of *cis*-resveratrol was obtained using the standard solutions of *trans*-resveratrol exposed to daylight for 1 h, because of the unavailability of commercial standard. According to a previously reported method,[8] the concentrations of *cis*-resveratrol were assigned on the basis of the decreased levels of intensity of the *trans*-resveratrol peak, which were proportional to the area of the *cis*-resveratrol peak.

The effect of encapsulation on the kinetics of degradation under UV light and at different temperatures was evaluated in terms of retention percentage θ_{trans}, shown in eq 2, and of yield of *cis*-resveratrol with respect to the initial *trans*-resveratrol content, θ_{cis}, shown in eq 3.[27]

$$\theta_{trans} = \frac{c_{trans}(t)}{c_{trans}(t_0)} \tag{2}$$

$$\theta_{cis} = \frac{c_{cis}(t)}{c_{trans}(t_0)} \tag{3}$$

The chemical stability of encapsulated resveratrol was compared with that of unencapsulated resveratrol, which was dissolved in ethanol (ethanol/ resveratrol weight ratio was 20) and then in water until the same resveratrol concentration of nanoemulsions was reached. Chemical stability tests were conducted in triplicate.

In Vitro Gastrointestinal Digestion. The digestion process was conducted according to the method proposed by Boyer and coworkers[28] with some modifications. Briefly, for simulated gastric digestion, a sample of 6 mL of each nanoemulsion containing resveratrol was placed in a test tube with 3 mL of saliva and incubated in a water bath at 37 °C for 15 min. Saliva was collected in a sanitary manner to a sterile tube 5 min after a volunteer had consumed 250 mL of milk. Three milliliters of phosphate buffer saline was then added, the pH was adjusted to 2.0 by dropwise addition of 1 M HCl, and porcine pepsin was then added to a final concentration of 1.3 mg/mL. The sample was incubated in a water bath at 37 °C for 30 min. Aliquots of the digestion mixture were collected for HPLC analysis.

Then the pH was increased to 5.8 by dropwise addition of 1 M $NaHCO_3$, to prepare for the simulated intestinal digestion. Pancreatin and bile salts were added to final concentrations of 0.175 and 1.1 mg/mL, respectively. The pH was adjusted to 6.5 by dropwise addition of 1 M $NaHCO_3$, and the digestate was incubated in a water bath at 37 °C for 2 h, before sample collection. The two different stages of digestion (gastric and intestinal) were stopped by adding PMSF to a final concentration of 0.174 mg/mL.

For HPLC analysis, resveratrol was extracted from the digestion media using the same method described under Chemical Stability for resveratrol extraction from nanoemulsion.

Antioxidant Activity. The antioxidant activity of nanoencapsulated resveratrol was determined in comparison with that of resveratrol diluted in DMSO by using the ferric reducing antioxidant power assay (FRAP) and the oxygen radical absorbance capacity assay (ORAC).

The FRAP assay was conducted according to a previously reported procedure[29] with minor modifications. Reagents included 300 mM acetate buffer, pH 3.6 (3.1 g of $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL of $C_2H_4O_2$), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$. Working FRAP reagent was prepared by mixing the reagents in a ratio 10:1:1 (acetate buffer/TPTZ solution/$FeCl_3 \cdot 6H_2O$ solution). Aqueous solutions of known L-(+)-ascorbic acid concentration, in the range of 10−1000 μM, were used for generating the calibration curve. FRAP values were expressed as micromolar ascorbic acid equivalent by using the standard curve calculated for each assay.

Nanoencapsulated resveratrol (30 μL) was allowed to react with 900 μL of the working FRAP reagent for 30 min at room temperature. The sample was then centrifuged at 20000*g* for 3 min at 25 °C, and 300 μL of the supernatant was used for analysis. Absorbance readings were taken using a visible−UV microplate reader (Power Wave XS2, Bio-Tek Instruments Inc., Winooski, VT) set at 593 nm. A blank control was included at each time the assay also conducted for the samples and the standard. The blanks of the different delivery systems were the corresponding nanoemulsions without resveratrol. All of the reaction mixtures were prepared in triplicate.

The ORAC assay was conducted according to a previously reported method[30] with some modifications. Analyses were conducted in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 200 μL. Nanoemulsions (25 μL) containing resveratrol and fluorescein

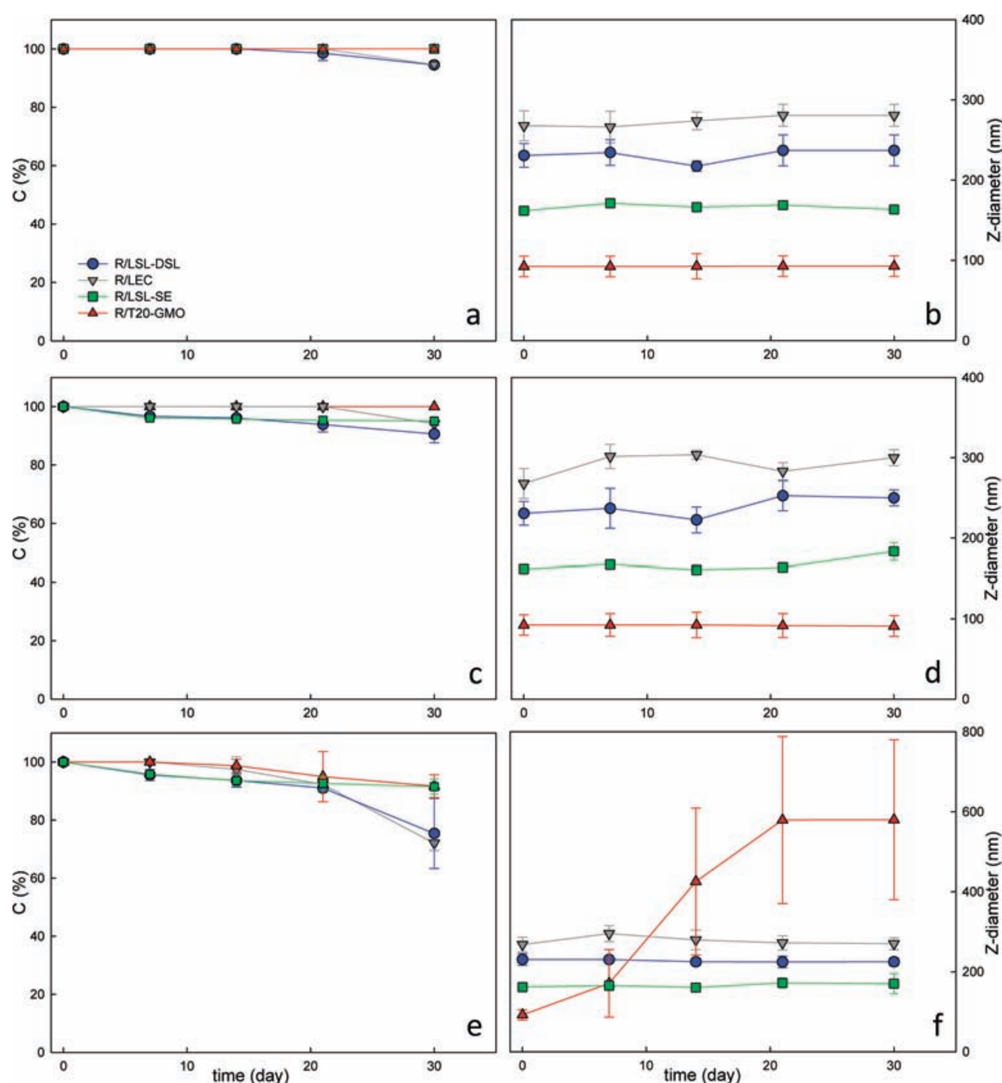

Figure 1. Evolution of the creaming volume percentage, C (a, c, and e), and of the mean droplet diameter, Z-diameter (b, d, and f), of the resveratrol-encapsulated nanoemulsions stored at 4 °C (a, b), 30 °C (c, d), and 55 °C (e, f) for 30 days.

(150 μL; 86.8 nM final concentration) solutions were placed in a 96-well plate and preincubated for 30 min at 37 °C in a fluorescence microplate reader (FLx800, Bio-Tek Instruments Inc.). The reaction was initiated by the addition of 25 μL of AAPH (153 mM final concentration) reagent followed by shaking at maximum intensity for 10 s. The fluorescence was then monitored kinetically with data taken every minute for 1 h. The 96-well plate was also loaded with the blank controls including phosphate buffer with DMSO and different nanoemulsions without resveratrol, as well as the standard solutions of Trolox (6.25, 12.5, 25, 50, and 100 μM). All of the reaction mixtures were prepared in triplicate. The area under the fluorescence decay curve corresponding to a sample (net AUC) was calculated by subtracting the AUC corresponding to a blank. ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay.

Cellular Antioxidant Activity (CAA). Caco-2 cells were grown in growth medium (DMEM supplemented with 10% FBS, 1% antibiotic−antimycotic) and were maintained at 37 °C in an incubator with 5% CO_2. The medium was changed every 2 days. Cells used in this study were at passage 25−30.

Caco-2 cells were seeded at a density of 4×10^4/well on a 96-well microplate (collagen-treated black plate) in 100 μL of growth medium/well and incubated for 15 days at 37 °C and 5% CO_2. The growth medium was then removed, and the cells were washed with 100 μL of treatment medium (HBSS + 10% FBS). Triplicate wells were treated with 100 μL of nanoencapsulated resveratrol suspended in 100 μL of treatment medium and incubated for 1 h at 37 °C. The wells were washed with 100 μL of treatment medium. Then the cells were treated with 200 μL of 100 μM DCFH-DA in treatment medium and incubated for 30 min at 37 °C. The wells were washed with 100 μL of treatment medium. Then 50 μM H_2O_2 was applied to the cells in 100 μL of treatment medium, and the 96-well microplate was placed into a fluorescence microplate reader (FLx800, Bio-Tek Instruments Inc.) at 37 °C. Emission at 528 nm was measured with excitation at 485 nm every minute for 1 h. Each plate included triplicate control, blank, and sample background wells: control wells contained cells treated with DCFH-DA and oxidant, blank wells contained cells treated with DCFH-DA without oxidant, and sample background wells contained cells treated with nanoemulsions and DCFH-DA without oxidant. The area under the curve of fluorescence versus time was integrated to calculate the CAA value, according to eq 4[31]

$$\text{CAA unit} = \left(100 - \frac{(\int \text{SA} - \int \text{BA})}{\int \text{CA}}\right) \times 100 \qquad (4)$$

dx.doi.org/10.1021/jf2031346 |J. Agric. Food Chem. 2011, 59, 12352–12360

where $\int$SA is the integrated area from the sample curve, $\int$BA is the integrated area from the blank curve or sample background curve, and $\int$CA is the integrated area from the control curve.

RESULTS AND DISCUSSION

Formulation of Stable Nanoemulsions. Resveratrol was encapsulated in peanut oil-based nanoemulsions at a final concentration of 0.01% by weight. The resveratrol concentration used was preliminarily chosen to be 10 times higher than the therapeutic blood concentration.

The percentage of each ingredient and the formulation of the nanoemulsion-based delivery systems were largely determined according to the use of the lipophilic and hydrophilic emulsifiers with the ultimate goal of improving resveratrol entrapment in the lipid phase by hydrophobic interactions, thus reducing the extent of localization of resveratrol at the oil/water interface.[12] The success of this approach can be inductively proved by the improvement in chemical stability of resveratrol of the tested delivery systems, in comparison with that of unencapsulated resveratrol, which is currently under investigation by fluorescence spectroscopy studies.

The physical stability of the resveratrol-encapsulating nanoemulsions was evaluated in terms of the evolution of their mean droplet diameter under accelerated aging conditions at different storage temperatures (4, 30, and 55 °C) for 30 days. Both lecithin-based nanoemulsions, R/LEC and R/LSL-DSL, characterized by initial mean droplet diameters of 270 and 230 nm, respectively, and sugar ester-based nanoemulsion R/LSL-SE, with an initial mean droplet diameter of 160 nm, exhibited no significant variations of the *Z*-diameters at all storage temperatures (Figure 1b,d,f). On the contrary, the nanoemulsion R/T20-GMO, which exhibited smaller initial mean droplet diameter (90 nm), was stable only at low temperatures (4 and 30 °C), whereas at 55 °C it underwent significant instability phenomena, highlighted by a drastic increase of *Z*-diameter already at day 14 (Figure 1f).

The different physical stabilities of the nanoemulsions tested could be related to the different emulsifiers used, with the respective interfacial properties depending on the kinetics of adsorption at the O/W interface as well as on the subsequent molecular reorganization at the interface.[32] Molecules with high mobility and fast adsorption kinetics, such as polysorbate, significantly contribute to reduce recoalescence phenomena during high-pressure homogenization, because of the extremely short times of coverage of the newly formed droplet surfaces. On the other hand, at increasing temperature, the muted thermodynamic conditions may induce the displacement of emulsifier molecules from the O/W interface. Emulsifiers with higher molecular mobility will therefore exhibit higher instability and coalescence phenomena, as observed for polysorbate-based nanoemulsions (R/T20-GMO).

The evolution of the creaming volume percentage *C* over time was coherent with the droplet size measurements. *C* remained constant at about 100% for all nanoemulsions at 4 °C (Figure 1a) and decreased by 10% at most after 30 days for all of the formulations at 30 °C, except for R/T20-GMO, which remained completely stable (Figure 1c). This parameter was reduced by 30% for R/LEC and R/LSL-DSL and by 10% for R/T20-GMO and R/LSL-SE after 30 days at 55 °C (Figure 1e).

Chemical Stability. The chemical stability of the encapsulated resveratrol was evaluated for the retention percentage of

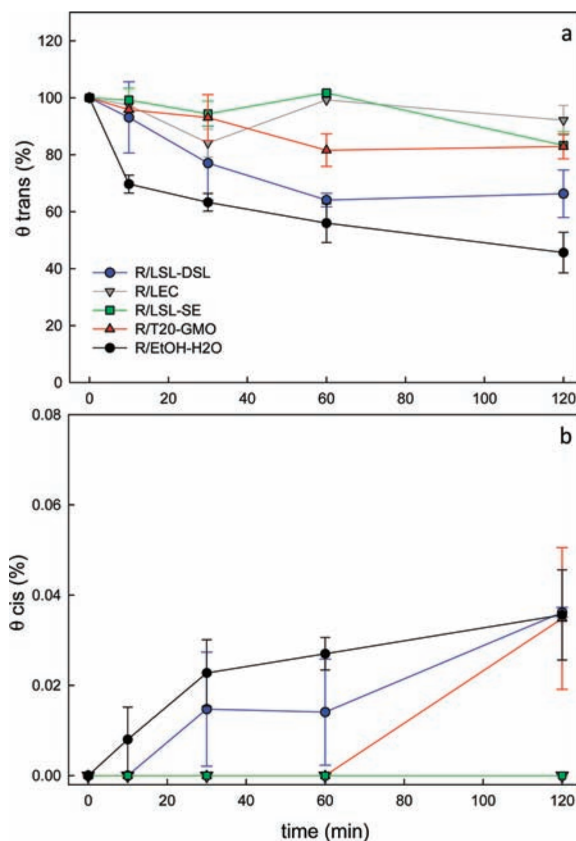

Figure 2. Retention percentage of *trans*-resveratrol, θ_{trans} (a), and *cis*-resveratrol yield, θ_{cis} (b), under UV-C light exposure.

trans-resveratrol, θ_{trans}, and the formation yield of *cis*-resveratrol, θ_{cis}, under different storage conditions.

Preliminary studies showed that *trans*-resveratrol, which is extremely photosensitive and susceptible to oxidative degradation, remained stable during the nanoencapsulation process. In fact, the typical HPLC chromatograms of unencapsulated and nanoencapsulated resveratrol had the same retention time at about 8 min under the same chromatographic conditions used in this study. In addition, the UV—vis spectra of the unencapsulated and nanoencapsulated resveratrol exhibited the same pattern, with the absorption maximum at 306 nm, in agreement with previous results.[8] The same HPLC retention time and UV—vis spectrum suggested that no chemical changes occurred during the nanoencapsulation process.

The exposure of unencapsulated resveratrol to UV-C light showed the formation of the cis-form of resveratrol. In fact, the area of *trans*-resveratrol decreased and a new peak appeared, with its area increasing with the exposure time. More specifically, the UV—vis spectrum of the new peak was consistent with that of *cis*-resveratrol, with the absorption maximum at 280 nm.[8] Therefore, the new peak can be ascribed to *cis*-resveratrol, formed from *trans*-resveratrol due to UV light exposure, as previously reported.[27] It must be pointed out that in this study the formation of *cis*-resveratrol was used as a qualitative instead of a quantitative indicator of *trans*-resveratrol oxidation.

Figure 2 reports the evolution of θ_{trans} (Figure 2a) and of θ_{cis} (Figure 2b) of unencapsulated and nanoencapsulated systems. *trans*-Resveratrol was significantly degraded ($\theta_{trans} \approx 45\%$) after a 2 h exposure to UV-C light. Concurrently, θ_{cis} increased up to

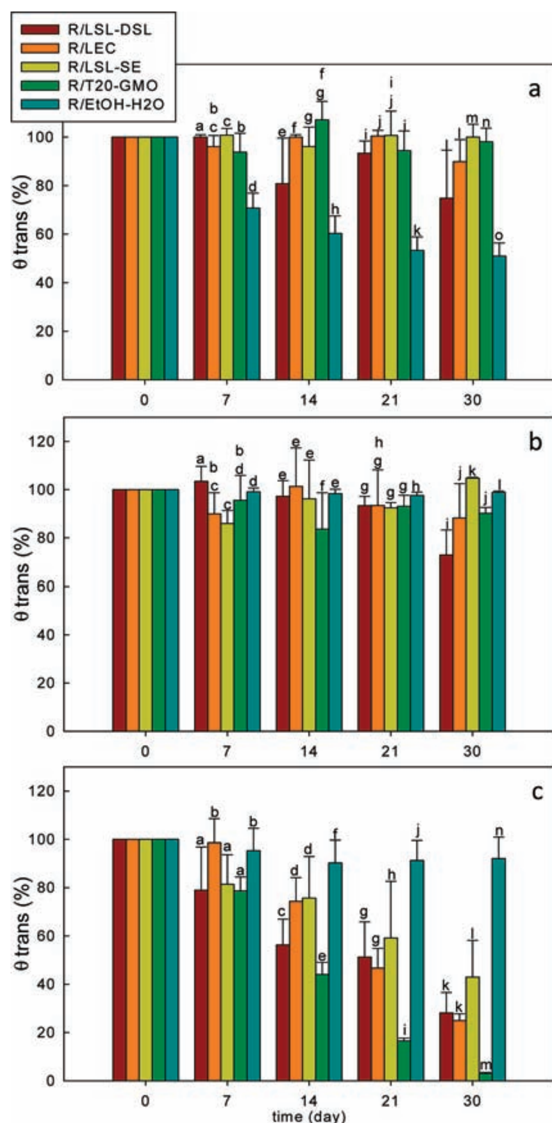

Figure 3. Retention percentage of *trans*-resveratrol, θ_{trans}, at 4 °C (a), 30 °C (b), and 55 °C (c) for 30 days. Different letters in the same day indicate statistically (Student *t* test) significant differences ($p < 0.05$).

0.04%, with trace amounts of *cis*-resveratrol appearing already after 10 min of UV-C light exposition.

Nanoencapsulation of resveratrol was more stable under the UV-C light compared to the unencapsulated compound, with a slower rate of degradation of *trans*-resveratrol and less formation of *cis*-resveratrol (Figure 2). In particular, for R/LSL-SE and R/LEC only a limited degradation of *trans*-resveratrol was observed, with θ_{trans} being reduced to 83 and 92%, respectively, and no formation of *cis*-resveratrol being detected over the exposure time. R/T20-GMO system exhibited a comparable *trans*-resveratrol degradation (≈82%), but detectable *cis*-resveratrol ($\theta_{cis} \approx 0.03\%$), after 2 h of light exposure. R/LSL-DSL was the least stable system, which exhibited a reduction of the θ_{trans} value of ≈65% and a sustained increase of the θ_{cis} value, which was, however, slower than that of the unencapsulated resveratrol.

Figure 3 shows the kinetics of degradation of unencapsulated and nanoencapsulated resveratrol at three different storage temperatures (4, 30, and 55 °C). In all cases *cis*-resveratrol was never detected.

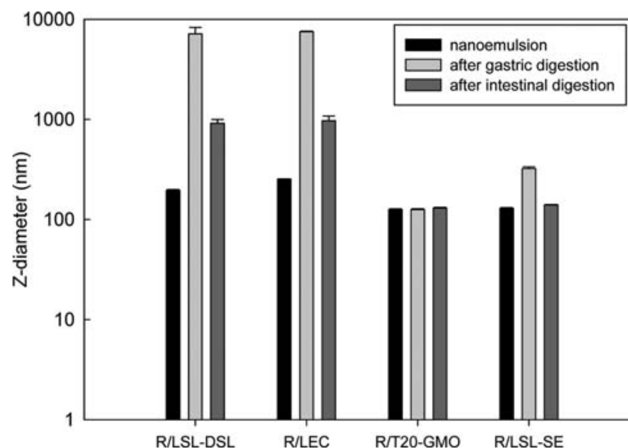

Figure 4. Mean droplet diameter, *Z*-diameter, of the nanoemulsions encapsulating resveratrol before and after the in vitro digestion process.

Under refrigerated conditions (4 °C) and at 30 °C nanoencapsulated resveratrol remained extremely stable for all of the formulations, with a reduction of θ_{trans} value of ≈70% being observed only for R/LSL-DSL after 30 days (Figure 3a,b).

On the contrary, the unencapsulated resveratrol exhibited a different behavior, with active resveratrol being better preserved at higher temperatures. In particular, a severe chemical degradation occurred at 4 °C, which reduced the θ_{trans} value to ≈50% after 30 days (Figure 3a), whereas the θ_{trans} value was substantially stable at 30 °C (Figure 3b) and at 55 °C (Figure 3c). This can be explained by increased oxidative degradation due to the higher solubility of oxygen in water at lower temperatures. Therefore, the observed stable behavior of the encapsulated compound at lower temperature means that the nanoencapsulation process prevented the chemical degradation of resveratrol, effectively protecting it from contact with oxygen molecules dissolved in the water.

trans-Resveratrol in all nanoemulsion formulations degraded significantly after 30 days at 55 °C (Figure 3c), which can be explained by its interaction with the emulsion ingredients and the destabilization of the nanoemulsion as shown in Figure 1f.

In Vitro Digestion. The chemical and physical stability of the nanoencapsulated resveratrol during the digestion process was evaluated under conditions simulating cumulatively the digestion activities of the stomach and the small intestine.

During the gastric and intestinal digestions, resveratrol remained chemically stable, with no significant changes in the quantity and quality of the encapsulated resveratrol. The HPLC analysis has confirmed the stability of the nanoencapsulated resveratrol with no additional peaks detected after digestion. It can be then speculated that resveratrol remains encapsulated in the lipid phase, is not metabolized in the gastrointestinal tract, and can therefore reach the colon in active form to be absorbed through the intestinal wall. Nevertheless, final proof of resveratrol being still encapsulated requires further experimental investigations.

However, several studies based on animal and human models have demonstrated that for unencapsulated resveratrol, only traces of its active form actually circulate in the plasma; resveratrol was found to be extensively glucuronidated and sulfated in the gut and liver.[33] Biliary excretion has been found to be an important elimination pathway for resveratrol, and previous studies have shown that in rats at least 30% of the administered

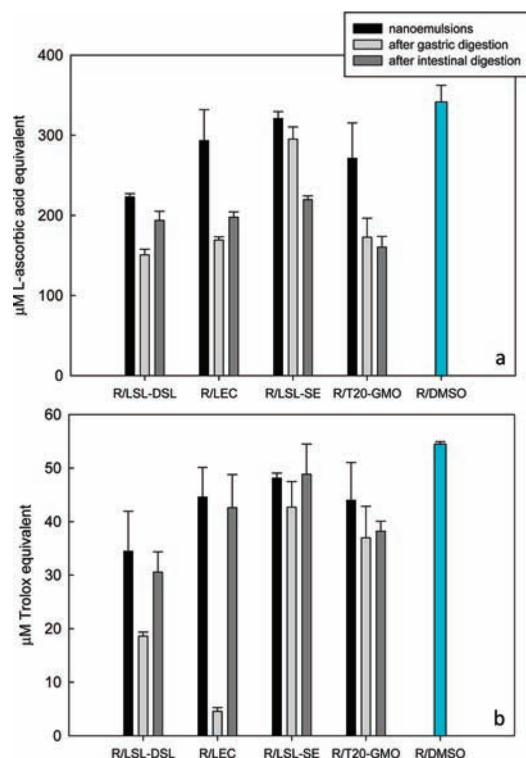

Figure 5. Antioxidant activity of nanoemulsions containing resveratrol by FRAP assay (a) and ORAC assay (b) before and after the in vitro digestion process, in comparison with resveratrol dissolved in DMSO (R/DMSO).

dose was eliminated via feces in the form of conjugated metabolites.[34]

The integrity of the nanocapsules during digestion was assessed by measuring the variation of the mean droplet diameters after the different digestion phases (Figure 4). The R/T20-GMO and R/LSL-SE nanoemulsions were physically stable during gastric and intestinal digestion, without any variation of the *Z*-diameter being observed. The lecithin-based nanoemulsions, R/LEC and R/LSL-DSL, characterized by a higher initial mean droplet diameter in comparison to the previous formulations, appeared to be not as stable during gastric digestion, with a significant increase of the *Z*-diameter. Nevertheless, the following step of intestinal digestion determined the reduction of the mean droplet size due to the action of the bile salts, which emulsified the fat aggregates produced during gastric digestion.

Chemical Antioxidant Activity. The retention of the antioxidant activity of nanoencapsulated resveratrol during in vitro digestion was preliminarily assessed by two chemical assays, namely, FRAP and ORAC, in comparison with the unencapsulated resveratrol diluted in DMSO (Figure 5).

It is important to investigate the antioxidant activity of resveratrol also in nondigested delivery systems, because one of the advantages of the nanometric delivery systems is the acceleration of the transport to the intestinal epithelial cells, due to the high dispersibility and hence diffusivity through the intestinal aqueous boundary layer,[35] making unnecessary the stage of bile emulsification.

The results of both FRAP and ORAC analyses, which were very consistent with each other, showed that the nanoencapsulated resveratrol controls retained an antioxidant activity

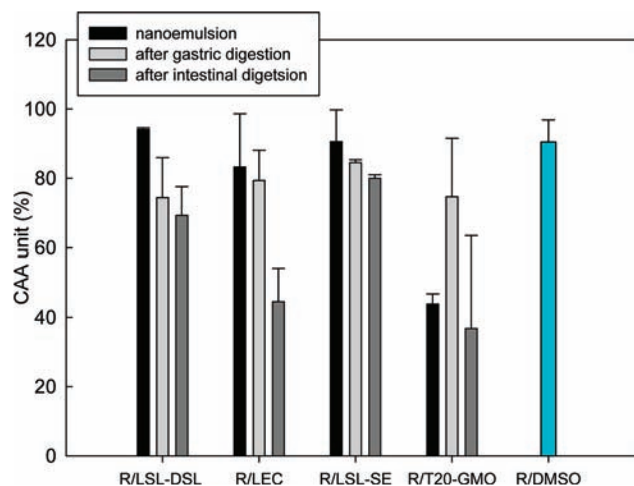

Figure 6. Cellular antioxidant activity, CAA unit, of resveratrol-encapsulated nanoemulsions before and after in vitro digestion.

comparable to that of the unencapsulated form, with higher values being observed for R/T20-GMO and R/LSL-SE.

In vitro digestion caused for all formulations a reduction of the antioxidant activity, which was particularly evident in the gastric step for R/LSL-DSL and R/LEC formulations.

This result can be explained with the increase of the mean droplet sizes observed after gastric digestion (Figure 4), which was responsible for a significant reduction of the surface area (of 2 orders of magnitude) and, consequently, making resveratrol unavailable through the oil/water interfaces.

Cellular Antioxidant Activity. The antioxidant activity of the control (undigested nanocapsules) and digested samples was also assessed using a cell-based antioxidant assay. Caco-2 cells were seeded on a 96-well microplate, and the CAA assay was performed 15 days after seeding, to measure the antioxidant activity on mature differentiated intestinal cells with fully developed brush border enzymes and transporters. Caco-2 cells were pretreated with nanoencapsulated resveratrol, which, due to the nanometric size of the capsules, passed through the membrane to enter the cell. In this study Caco-2 cells were first pretreated with the nanoencapsulated resveratrol, followed by DCFH-DA treatment, rather than pretreating the cells with the DCFH-DA and the antioxidant together, as suggested in a previous work.[31] Preliminary results (not reported) showed that this modification eliminates the possibility of having the antioxidant effect outside the cells in the medium and restricts the measured CAA solely to the interaction of intracellular resveratrol with DCFH. The cells were subsequently treated with H_2O_2, instead of a synthetic radical.[31] H_2O_2 is a biologically relevant reactive oxygen species and is readily diffused into the cells. Once inside the cells, H_2O_2 oxidizes the intracellular DCFH to the fluorescent DCF. The nanoencapsulated resveratrol exerts its cellular antioxidant activity by preventing the oxidation of DCFH and reducing the formation of DCF.

Figure 6 shows the cellular antioxidant activity of the nanoemulsions containing resveratrol in comparison with the unencapsulated compound and the variation of the CAA during the in vitro digestion process. All formulations exhibited excellent antioxidant activity on Caco-2 cells, which, for R/LSL-SE, R/LEC, and R/LSL-DSL, was >80% and comparable to the CAA of resveratrol dissolved in DMSO, suggesting that the nanometric

size of the nanocapsules may improve the uptake of the antioxidant compound into the cells. Moreover, R/LEC and R/LSL-DSL showed a cellular antioxidant activity higher than expected considering the FRAP and ORAC results (Figure 5), which reported lower antioxidant activity for these formulations in comparison to the others due to a better resveratrol entrapment in the lipid phase and consequent reduction of the extent of its localization at the oil/water interface, as well as of the interaction with the oxidant reagents. The better entrapment of resveratrol can be likely attributed to the formation of reversed micelles of the more lipophilic emulsifier (i.e., glycerol monooleate, soy lecithin) within the lipid droplets stabilized by the more hydrophilic emulsifier (i.e., Tween 20, defatted soy lecithin, sugar ester).

The digestion process did not significantly affect the cellular antioxidant activity of encapsulated resveratrol, suggesting its being stable after gastric and intestinal digestion and available to be absorbed by the cells in its active antioxidant form.

It must be highlighted that, despite very surface active bile salts perhaps having displaced some emulsifier molecules at the O/W interface, no significant changes were observed in the efficiency in delivering resveratrol to the cells in antioxidant activity measurements conducted before and after the gastric and intestinal digestion processes, probably thanks to the dual-emulsifier formulation that improved protection of resveratrol and its transport through the biological membranes.

Moreover, the most physically and chemically stable formulations also exhibited the highest chemical and cellular antioxidant activity, which was comparable to that of the undigested and unencapsulated resveratrol dissolved in DMSO. The present study serves as a useful model for developing delivery systems of nutraceutical and functional food ingredients that are stable, efficient, and highly bioavailable.

AUTHOR INFORMATION

Corresponding Author

*Phone: (0039) 089 96-3466. Fax: (0039) 089 96-4168. E-mail: fdonsi@unisa.it.

Author Contributions

‖These authors contributed equally.

Funding Sources

M.S. acknowledges financial support from the Doctorate Program of the University of Salerno during her stay at the Guelph Food Research Centre. This project was supported by the A-Base research (RBPI 109) of Agriculture and Agri-Food Canada (AAFC) and the AAFC-EU Twinning Program.

REFERENCES

(1) Filip, V.; Plockova, M.; Smidrkal, J.; Spickova, Z.; Melzoch, K.; Schmidt, S. Resveratrol and its antioxidant and antimicrobial effectiveness. *Food Chem.* **2003**, *83*, 585–593.

(2) Orallo, F. Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol. *Curr. Med. Chem.* **2006**, *13*, 87–98.

(3) Olas, B.; Wachowicz, B. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. *Platelets* **2005**, *16*, 251–260.

(4) Bradamante, S.; Barenghi, L.; Villa, A. Cardiovascular protective effects of resveratrol. *Cardiovasc. Drug Rev.* **2004**, *22*, 169–188.

(5) Jang, M. S.; Cai, E. N.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218–220.

(6) Asensi, M.; Medina, I.; Ortega, A.; Carretero, J.; Bano, M. C.; Obrador, E.; Estrela, J. M. Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radical Biol. Med.* **2002**, *33*, 387–398.

(7) Hung, C. F.; Chen, J. K.; Liao, M. H.; Lo, H. M.; Fang, J. Y. Development and evaluation of emulsion-liposome blends for resveratrol delivery. *J. Nanosci. Nanotechnol.* **2006**, *6*, 2950–2958.

(8) Vian, M. A.; Tomao, V.; Gallet, S.; Coulomb, P. O.; Lacombe, J. M. Simple and rapid method for *cis*- and *trans*-resveratrol and piceid isomers determination in wine by high-performance liquid chromatography using Chromolith columns. *J. Chromatogr., A* **2005**, *1085*, 224–229.

(9) Wenzel, E.; Somoza, V. Metabolism and bioavailability of *trans*-resveratrol. *Mol. Nutr. Food Res.* **2005**, *49*, 472–481.

(10) Yu, L.; Sun, Z. J.; Wu, S. L.; Pan, C. E. Effect of resveratrol on cell cycle proteins in murine transplantable liver cancer. *World J. Gastroenterol.* **2003**, *9*, 2341–2343.

(11) Narayanan, N. K.; Nargi, D.; Randolph, C.; Narayanan, B. A. Liposome encapsulation of curcumin and resveratrol in combination reduces prostate cancer incidence in PTEN knockout mice. *Int. J. Cancer* **2009**, *125*, 1–8.

(12) Fang, J. Y.; Hung, C. F.; Liao, M. H.; Chien, C. C. A study of the formulation design of acoustically active lipospheres as carriers for drug delivery. *Eur. J. Pharm. Biopharm.* **2007**, *67*, 67–75.

(13) Shao, J. F.; Li, X. L.; Lu, X. W.; Jiang, C.; Hu, Y.; Li, Q. P.; You, Y. P.; Fu, Z. Enhanced growth inhibition effect of resveratrol incorporated into biodegradable nanoparticles against glioma cells is mediated by the induction of intracellular reactive oxygen species levels. *Colloid Surf. B* **2009**, *72*, 40–47.

(14) Lu, X. W.; Ji, C. B.; Xu, H. E.; Li, X. L.; Ding, H. X.; Ye, M.; Zhu, Z. S.; Ding, D.; Jiang, X. Q.; Ding, X. S.; Guo, X. R. Resveratrol-loaded polymeric micelles protect cells from A β-induced oxidative stress. *Int. J. Pharm.* **2009**, *375*, 89–96.

(15) Teskac, K.; Kristl, J. The evidence for solid lipid nanoparticles mediated cell uptake of resveratrol. *Int. J. Pharm.* **2010**, *390*, 61–69.

(16) Lucas-Abellan, C.; Fortea, I.; Lopez-Nicolas, J. M.; Nunez-Delicado, E. Cyclodextrins as resveratrol carrier system. *Food Chem.* **2007**, *104*, 39–44.

(17) Donsì, F.; Senatore, B.; Huang, Q.; Ferrari, G. Development of novel pea protein-based nanoemulsions for delivery of nutraceuticals. *J. Agric. Food Chem.* **2010**, *58*, 10653–10660.

(18) Donsì, F.; Wang, Y.; Huang, Q. Freeze-thaw stability of lecithin and modified starch-based nanoemulsions. *Food Hydrocolloids* **2011**, *25*, 1327–1336.

(19) Donsì, F.; Annunziata, M.; Sessa, M.; Ferrari, G. Nanoencapsulation of essential oils to enhance their antimicrobial activity in foods. *LWT—Food Sci. Technol.* **2011**, *44*, 1908–1914.

(20) McClements, D. J.; Li, Y. Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food Funct.* **2010**, *1*, 32–59.

(21) Tagliazucchi, D.; Verzelloni, E.; Bertolini, D.; Conte, A. In vitro bio-accessibility and antioxidant activity of grape polyphenols. *Food Chem.* **2010**, *120*, 599–606.

(22) Gumienna, M.; Lasik, M.; Czarnecki, Z. Bioconversion of grape and chokeberry wine polyphenols during simulated gastrointestinal in vitro digestion. *Int. J. Food Sci. Nutr.* **2011**, *62*, 226–233.

(23) Augustin, M. A.; Abeywardena, M. Y.; Patten, G.; Head, R.; Lockett, T.; De Luca, A.; Sanguansri, L. Effects of microencapsulation on the gastrointestinal transit and tissue distribution of a bioactive mixture of fish oil, tributyrin and resveratrol. *J. Funct. Foods* **2011**, 3, 25–37.

(24) da Silva, C. A. S.; Sanaiotti, G.; Lanza, M.; Follegatti-Romero, L. A.; Meirelles, A. J. A.; Batista, E. A. C. Mutual solubility for systems composed of vegetable oil plus ethanol plus water at different temperatures. *J. Chem. Eng. Data* **2010**, *55*, 440–447.

(25) Stepanek, P. Static and dynamic properties of multiple light-scattering. *J. Chem. Phys.* **1993**, *99*, 6384–6393.

(26) Roland, I.; Piel, G.; Delattre, L.; Evrard, B. Systematic characterization of oil-in-water emulsions for formulation design. *Int. J. Pharm.* **2003**, *263*, 85–94.

(27) Shi, G. R.; Rao, L. Q.; Yu, H. Z.; Xiang, H.; Yang, H.; Ji, R. Stabilization and encapsulation of photosensitive resveratrol within yeast cell. *Int. J. Pharm.* **2008**, *349*, 83–93.

(28) Boyer, J.; Brown, D.; Liu, R. H. In vitro digestion and lactase treatment influence uptake of quercetin and quercetin glucoside by the Caco-2 cell monolayer. *Nutr. J.* **2005**, *4*, 1.

(29) Tsao, R.; Yang, R.; Xie, S.; Sockovie, E.; Khanizadeh, S. Which polyphenolic compounds contribute to the total antioxidant activities of apple?. *J. Agric. Food Chem.* **2005**, *53*, 4989–4995.

(30) Davalos, A.; Gomez-Cordoves, C.; Bartolome, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *J. Agric. Food Chem.* **2004**, *52*, 48–54.

(31) Wolfe, K. L.; Liu, R. H. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* **2007**, *55*, 8896–8907.

(32) Donsì, F.; Sessa, M.; Ferrari, F. Effect of emulsifier type and disruption chamber geometry on the fabrication of food nanoemulsions by high pressure homogenization. *Ind. Eng. Chem. Res.* **2011**, DOI: doi: 10.1021/ie2017898.

(33) Walle, T.; Hsieh, F.; DeLegge, M. H.; Oatis, J. E.; Walle, U. K. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos.* **2004**, *32*, 1377–1382.

(34) Wenzel, E.; Soldo, T.; Erbersdobler, H.; Somoza, V. Bioactivity and metabolism of *trans*-resveratrol orally administered to Wistar rats. *Mol. Nutr. Food Res.* **2005**, *49*, 482–494.

(35) Acosta, E. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. *Curr. Opin. Colloid Interface Sci.* **2009**, *14*, 3–15.