JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 9489–9495 9489
DOI:10.1021/jf902066g

Enhancement of the Encapsulation and Transmembrane Permeation of Isoflavone-Containing Red Clover Extracts in Phospholipid-Based Microemulsions Using Different Extraction Processes

Mei-Hwa Lee,[†] Ming-Wen Yu,[#] Li Kao,[‡] and Chuan-Chuan Lin*,[#]

[†]Department of Materials Science and Engineering, I-Shou University, Kaohsiung 840, Taiwan, [#]Department of Food Science, China University of Science and Technology, Taipei 115, Taiwan, and [‡]Department of Microbiology, College of Medicine, National Taiwan University, Taipei 100, Taiwan

Several isoflavone-enriched red clover extracts (RCE) prepared by using solid- or liquid-phase extraction or a combination of both were studied for their encapsulation into a phospholipid-based microemulsion system. The optimization process with regard to the bioactive ingredient-encapsulated amounts and transmembrane efficiency of various RCE micelles was demonstrated. The results indicated that the encapsulated amounts of isoflavones in RCE-encapsulated microemulsions (ME) of ME-RC5, ME-RC6, and ME-RC7 were inceased by >10-fold when compared with that of the raw red clover extracts. Comparison of the permeability coefficient K_p of the formononetin among the ME-RCs from the in vitro skin permeation study showed that ME-RC5 significantly exhibited the least permeability, whereas ME-RC6 exhibited enhanced permeability after two-stage solid-phase extraction, indicating the potential role of the matrix material as a barrier or enhancer in the transmembrane study.

KEYWORDS: Red clover extract; microemulsion; isoflavones; transmembrane permeation; encapsulation; extraction

INTRODUCTION

The phytoestrogen-containing natural sources as alternative hormone replacement therapy (HRT) have been suggested to prevent estrogen-related cardiovascular, menopausal symptoms, osteoporosis and to reduce the risk of breast cancer from both epidemiological data and experimental animal studies (*1*−*4*). Phytoestrogens are divided into four main groups: isoflavonoids, flavonoids, coumestans, and mammalian lignans (*1*−*3*). Among these, isoflavonoids are the most well-studied. According to information from the USDA-Iowa State University Database on the isoflavone content of foods, red clover contains the most abundant bioactive aglycone isoflavones, formononetin and biochanin A, among all natural sources (*5*). A clinical study indicated that treatment with 80 mg of isoflavones from red clover (Promensil) per day resulted in a significant reduction in hot flushes by 44% (*6*). Another study comparing different commercially available preparations intended as HRT derived from different plants showed higher estrogenic activity of red clover-derived products compared to those made from soy in a yeast model system (*7*). Red clover cultivars with high isoflavone content are selected and grown for nutraceutical use.

*Address correspondence to this author at the Department of Food Science, China University of Science and Technology, 245 Yen-Chiu-Yuan Rd., Sec. 3, Nankang, Taipei 115, Taiwan (telephone +886 2 2782 1862; fax +886 2 2786 4291; e-mail cclin@cc.cust.edu.tw.).

Isoflavone, which possesses a polyphenolic structure similar to that of curcumin, is water insoluble and scarcely dissolves in the organic phase, which might impede its application in the field of nutraceuticals and functional foods. There has been growing interest in the advantages of microemulsions (ME) for solving the problems of solubility as well as stability of nutraceuticals and food additives in aqueous solutions. Microemulsions are self-assembled mixtures of water, oil, and surfactants and have the advantages of being optically isotropic and thermodynamically stable. Microemulsions have attracted much interest over the past years as potential drug delivery systems because of their transparency, ease of preparation and long-term stability (*8*). Much attention has been given to the utilization of phospholipids in formulating pharmaceutically acceptable microemulsions (*9*, *10*). The techniques and applications of nanoparticles in nutraceutical delivery systems have been discussed and reviewed significantly over the past few years (*11*, *12*). Those studies in food microemulsions have focused on using food-grade, nonionic surfactants derived from natural products (*13*). The solubilization of lycopene and lutein derivatives in aqueous media was successfully improved by Amar et al. using microemulsion technology (*14*). Construction of lecithin-based microemulsions with a wide range of food-acceptable surfactants in food application has been studied (*15*). Recent study has indicated that phosphatidylcholine-embedded microemulsion systems improved the transmembrane bioavailability in both rat skins and Caco2 cells (*16*, *17*). In our previous paper, a curcumin-encapsulated

Published on Web 09/22/2009

oil/water microemulsion system using food-acceptable components, lecithin and Tween 80, as the surfactants and ethyl oleate as the oil phase was successfully constructed for the improvement of the transmembrane bioavailability of curcumin (*18*).

Most of the commercially available raw herbal extracts, if not further purified, have limits on innovation and patent protection in the development of related products. In addition, no matter what route it takes, either through oral or transdermal application, the bioavailability of raw herbal extracts is poor, and unavailable bioactive ingredients cannot reach the target tissues. The main purpose of this study was to enhance the bioavailability of isoflavone-containing red clover extracts. Several studies to improve the solubility and bioavailability of isoflavones by using β-cyclodextrin and self-emulsifying system have been reported (*19*, *20*). Due to the resemblance of the structural feature to curcumin, the isoflavone-enriched red clover crude extracts were studied for encapsulation into the previously published phospholipid-based microemulsion system (*18*). To enhance the contents of the estrogenic isoflavones in the extracts, preparation of several red clover extracts (RCEs) using solid- or liquid-phase extraction or a combination of both was conducted and compared. The estrogenic activities of the RCEs were also tested using a recombinant yeast system. On the other hand, by utilizing biocompatible materials, including ethyl oleate as oil phase and lecithin and Tween 80 as surfactants, the RCE can be readily encapsulated in a stable microemulsion system. The optimization process with regard to the bioactive ingredient-encapsulated amounts and transmembrance efficiency of the various RCE micelles was demonstrated.

MATERIALS AND METHODS

Materials and Chemicals. Lecithin (L-α-phosphatidylcholine, purity > 60%), Tween 80, ethyl oleate (EO), and all other chemicals for yeast culture medium and liquid chromatography were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Yeast nitrogen base was purchased from DIFCO (Sparks, MD). Crude red clover extract was obtained from Wedar Nutrasource Inc. (Kaohsiung, Taiwan). Deionized water (DI water) with conductivity above 17 ΩM·cm was used for preparing microemulsions in this study.

Procedures for the Preparation of Red Clover Extracts. The commercially available crude red clover extract RC-1 (from Wedar Nutrasource Inc., appropriately 8% of isoflavones) was further extracted using a series of extraction methods and steps, as shown in **Table 1**. One hundred grams of RC-1 was extracted with 1 L of methanol by shaking at room temperature for 3 days. The RC-2 extract was obtained after filtration and evaporation under reduced pressure. RC-2 was further chromatographed on 20-fold of silica gel (70−230 mesh). The column was washed with hexane and then eluted with ethyl acetate. The eluent was collected until no more bright spots were detected under UV 254 nm on thin layer chromatography (TLC) analysis. The eluent was evaporated under reduced pressure to afford RC-3. RC-4 was prepared by extraction of RC-1 with 10-fold of methanol under reflux for 4 h. RC-4 was further chromatographed on 20-fold of Amberlite XAD-4 polyremic resin. The column was washed with water and 50% of methanol sequentially until no more color materials eluted. The isoflavone-containing fraction was then eluted with 2−3 bed volumes of methanol and then ethyl acetate to afford RC-5. RC-5 was then chromatographed on silica gel as described previously to afford RC-6. RC-7 was prepared from RC-4 following silica purification.

Plasmids, Yeast Strain, and Growth. The yeast reporter assay was conducted as previously described (*21*). Plasmids Yep90 and YepHEGO were kindly provided by Dr. P. Chambon (Strasbourg, France) (*22*). Briefly, the multicopy plasmid Yep90 is a 2 μ derived plasmid carrying an *HIS3* selectable marker and a yeast PGK promoter and terminator cassette containing a unique *Eco*RI cloning site. Plasmid Yep90HEGO contains the complete coding sequence of the hER gene cloned into the unique *Eco*RI site of YEp90. *Saccharomyces cerevisiae* strain DP160 strain was kindly provided by Dr. D. Picard (Geneva, Switzerland).

Table 1. Extraction Procedures and Yields for the Preparation of Different RCEs

RCE	extraction method	extraction yield (%)
RC-1	raw material	
RC-2	RC-1 → MeOH extract	52.7 ± 2.6
RC-3	RC-2 →[a] SPE (silica)	52.5 ± 4.8
RC-4	RC-1 → reflux with MeOH	37.1 ± 3.3
RC-5	RC-4 →[b] SPE (XAD-4)	39.7 ± 2.5
RC-6	RC-4 →[b] SPE (XAD-4) →[a] SPE (silica)	22.0 ± 3.7
RC-7	RC-4 →[a] SPE (silica)	45.4 ± 2.9

[a] Column was washed with *n*-hexane, and ethyl acetate was used as eluent for collection. [b] Column was washed with H_2O and then 1:1 H_2O/MeOH; MeOH was used as eluent for collection.

pU/ERE-Ade2 was linearized with *Bst*BI within the *URA3* gene and integrated into the *ura3* locus of YPH250 to yield strain DP160. Strain CLY-3 was derived from DP160 by transformation of the plasmid YepHEGO. General yeast operation was performed as described (*23*). CLY-3 was routinely cultured on synthetic complete medium supplemented with adenine (25 μg/mL) to avoid selection of suppressors of the endogenous mutant *ade2* locus. Yeast strain CLY-3 was grown for 3 days on 25 mL of culture media, consisting of synthetic complete medium without uracil and histidine, plus limiting amounts of adenine (5 μg/mL). The result of each red/white screening was expressed as R, red colonies (negative), and W, white colonies (equivalent to 100 pM estradiol) (*21*).

Preparation of Red Clover Extract-Encapsulated Microemulsions. The mixture was prepared by adding appropriate amounts of oil, lecithin, and Tween 80 of different mole ratios as surfactants and RCEs in a test tube and was kept at 50 °C and well mixed using a vortex mixer. Water was then added to the mixture in a bath sonicator at 50 °C until a transparent and isotropic microemulsion was obtained. The viscosities of microemulsion were obtained at different shear rates at different temperatures using a Brookfield type rotary viscometer. The viscosities of microemulsion ranged from 3 to 10 cP, depending on the component weight ratio of microemulsion. On the basis of the previously published result, a ternary phase diagram of phospholispid-based microemulsion was constructed in which the microemulsion region was identified by a clear and transparent appearance of the solution (*18*). To find a stable microemulsion for encapsulation of RC extracts in this study, a series of formulas within the microemulsion region, at specific surfactant/cosurfactant (lecithin/Tween80) mole ratio of 0.3, were prepared and re-evaluated in a total of 10 g. The weight ratios of oil/surfactant/water were varied as 2.5:7.5:90, 2.5:10:87.5, 2.5:12.5:85, 2.5:15:82.5, 5:15:80, 5:20:75, 5:25:70, and 7.5:22.5:70, as shown in **Figure 1**. The stability of microemulsions was evaluated by measuring the turbidity using a UV−vis (Thermo Spectronic, Madison, WI) at a wavelength of 502 nm.

Measurement of Diameters Distributions of Red Clover Extract-Encapsulated Microemulsions. The diameter distributions of various RCE-encapsulated microemulsions in different formulations were measured by a Zetasizer NANO-S (Malvern Instruments Ltd., Worcestershire, U.K.), equipped with a 632.8 nm 4 mW helium−neon laser light source. Diameter distributions were calculated using autocorrelation data analysis by NIBS (noninvasive back scatter) technology built in for increased particle sizing sensitivity, thus making it possible to characterize proteins and polymers of < 1 nm in diameter and with molecular masses as low as 1000 Da. For fitting, 70 bins distributed logarithmically between 0.4 and 10000 nm were chosen. The collection times for the autocorrelation function were 1−3 min.

Encapsulation Capacities of Red Clover Extracts in Microemulsions. Various amounts of RCEs (ranging from 2 to 60 mg) were added to a 10 g microemulsion formulated as described above. The resultant microemulsions were passed through a 0.45 μm filter to remove excess curcumin and then subjected to high-performance liquid chromatography (HPLC) to analyze the loading capacity. The separation was performed on a Cosmosil 5C 18 MS column (5 μm, 25 cm × 4.6 mm i.d., Nacalai Tesque, Kyoto, Japan). The sample (20 μL) was eluted with the mobile phase composed of 0.1% H_3PO_4 (40%) and acetonitrile (60%). The flow rate and detection wavelength were set to be 1.0 mL/min and 254 nm, respectively. Standard curves of formononetin and biochanin

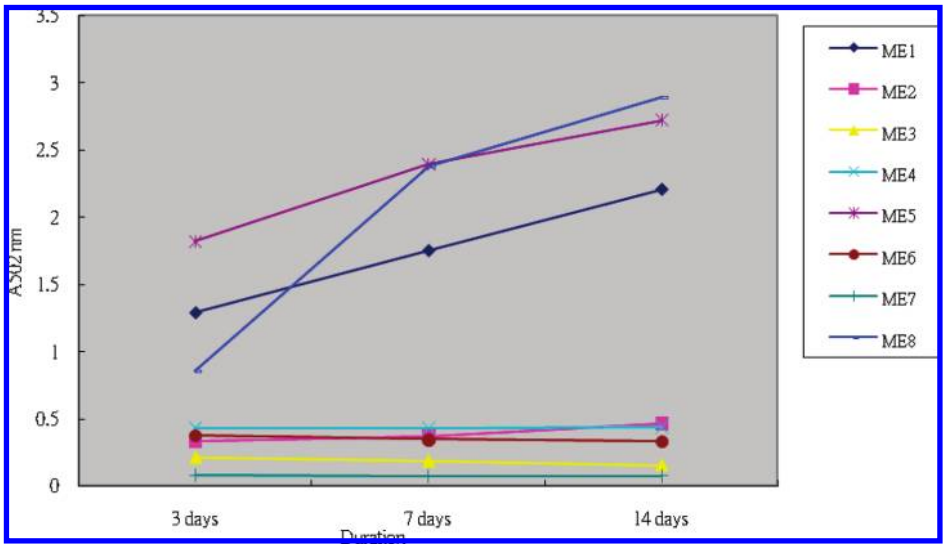


Figure 1. Measurements of the turbidity and stability of the selected microemulsions during 2 weeks.

A in 50% ethanol ranging from 0.1 to 0.001 mg/mL were used for calculation.

Skin Permeation of Red Clover Extract-Encapsulated Microemulsions. The permeability of formononetin through BALB/c mouse skin was investigated using Franz diffusion cells with an effective diffusional area of 0.785 cm^2. The hair of mice was removed. The skins were excised and then clamped between the donor and the receptor chamber with a 5.6 mL cell volume. The receptor chamber was filled with 50% ethanol to ensure sink conditions. The receptor chamber was thermostated at 37 °C, and the solution was stirred continuously at 300 rpm. One milliliter of formulation was pipetted into each donor compartment and sealed with paraffin to prevent evaporation. At time intervals of 2, 4, and 6 h, 500 μL of the receptor medium was taken for determining the permeated amount of formononetin using HPLC analysis at 254 nm as described previously. The cumulative amount of formononetin in RC extracts permeated through mouse skins was plotted as a function of time. The permeation rate of isoflavone (J_s) through mouse skin was calculated from the slope of the linear portion of the cumulative amount per unit area versus time plot ($\mu g/cm^2/h$). The permeability coefficient K_p ($\times 10^3 cm/h$) was calculated from the equation $K_p = J_s/C_0$, where C_0 represents the concentration of isoflavone in the microemulsion solution.

RESULTS AND DISCUSSION

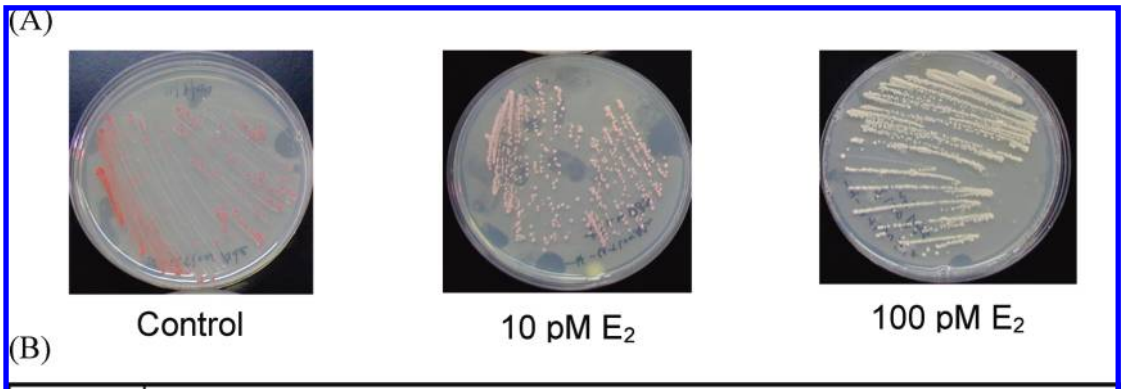
Characterization and Selection of a Stable Microemulsion Composition. In our previously published study, a ternary phase diagram of an oil/water (O/W) microemulsion system using food-acceptable components, lecithin and Tween 80, as the surfactants and ethyl oleate as the oil phase was successfully constructed for the encapsulation of curcumin (*18*). An area in the phase diagram was defined as microemulsion when a transparent and isotropic solution was formed from visual observation. The result showed that the largest stable O/W microemulsion region was obtained with the lecithin/Tween 80 molar ratio of 0.3. The range of oil-phase weight percent in the formulation of microemulsion varied from 2.1 to 10.3 wt %, whereas the surfactant/oil ratio was within the range of 3:1 to 6:1. However, the weight percent of water should not be <70% in order to form a nonviscous solution. On the basis of this principle, a series of formulations were re-evaluated from turbidity measurement to select the most stable one for the encapsulation study of red clover extracts.

The eight formulas of microemulsion (ME1−ME8) inside the enclosed curve of the lecithin/Tween 80 molar ratio of 0.3 were prepared (**Table 2**). The weight ratios of oil/surfactant/water were varied as 2.5:7.5:90, 2.5:10:87.5, 2.5:12.5:85, 2.5:15:82.5, 5:15:80, 5:20:75, 5:25:70, and 7.5:22.5:70 for formulas ME1−ME8, respectively. It is supposed that the turbidity is proportional to the particle diameter and the sample solution becomes opaque when the absorption value is >1. The results showed that ME1, ME6, and ME8 were unstable after 2 weeks of storage, as indicated from both visual observation and turbidity measurement (**Figure 1**). The order of the turbidity of the other five formulas was ME2, ME4 > ME6 > ME3 > ME7. The mean diameters of ME3 and ME7 were further examined by diameter analysis after 14 days of storage in the dark at room temperature. It is surprising to discover that ME7, constructed by using a higher percentage of surfactants, formed a liquid crystal clear solution with no particle size detected, whereas ME3 remained transparent with an average size diameter of 60−80 nm during 2 weeks of storage (**Table 2**). It is expected that the microemulsion droplets with more oil weight percent (>5%) or a lower surfactant/oil ratio (lower than 4:1) aggregated readily after 14 days. Therefore, ME3 was selected as the most suitable microemulsion in this study.

Comparison of the Contents of Isoflavones and Phytoestrogenic Activity of the Different Red Clover Extracts by a Recombinant Yeast Assay. To understand the effects of different extraction techniques on the contents and bioactivity of the RC extracts, liquid extraction with methanol followed by solid-phase extraction, including using silica gel and polymeric resins (Amberlite XAD) as adsorption materials or combination of both, was conducted and compared. The nonionic polymeric resins XAD have been widely used for the recovery of flavonoids from plant extracts or elimination of the water-soluble contaminants in the past few years (*24*). The preparation procedures and the contents of the two major components, formononetin and biochanin A, of the seven RC extracts are shown in **Table 1**. First, the commercially available RC1 was extracted with methanol at room temperature or under reflux to afford RC2 and RC4, respectively. It is obvious that RC4 contained more isoflavones than RC2, and the amount of formononetin was increased much more in RC7 than in RC3, due to the enhanced extraction temperature. Further solid-phase extraction from RC4 resulted in remarkable increases of the two isoflavones from 8.86 to 20.6, 21.55, and 19.37% for RC5, RC6, and RC7, respectively. Next, we used a recombinant yeast-based assay, which was constructed with a classical consensus palindromic ERE (estrogen response element) incorporated and an *ADE2* reporter system to test the estrogenicity of these seven RC extracts (*21*). In our experiment, the activation of

Table 2. Compositions and Characteristics of the Selected Microemulsions

	ethyl oleate (%)	lecithin/Tween 80 (mole ratio 1:3) (%)	water (%)	characteristics	state
ME1	2.5	7.5	90	opaque	macroemulsion
ME2	2.5	10	87.5	transparent	microemulsion
ME3	2.5	12.5	85	transparent with 60−80 nm size diameter	microemulsion
ME4	2.5	15	82.5	transparent	microemulsion
ME5	5	15	80	opaque	macromulsion
ME6	5	20	75	transparent	microemulsion
ME7	5	25	70	transparent (liquid crystalline with no particle size detected)	microemulsion
ME8	7.5	22.5	70	opaque	macroemulsion



	0.5ppm	1ppm	2ppm	3ppm	4ppm	5ppm
RC-1	R	R	R	P	W	W
RC-2	R	R	R	P	W	W
RC-3	R	R	P	W	W	W
RC-4	R	R	R	P	W	W
RC-5	R	R	P	W	W	W
RC-6	R	P	W	W	W	W
RC-7	R	P	P	w	W	W

Figure 2. Comparison of the estrogenicity of the RCEs in the recombinant yeast-based assay (**A**) estradiol; (**B**) various concentrations of isoflavonoids were added to the synthetic complete media without uracil and histidine, plus limiting amounts of adenine (5 μg/mL) for 3 days. R, red colonies (negative); W, white colonies (equivalent to 100 pM of estradiol). Each experiment was performed three times for confirmation.

the reporter gene, *ADE2*, is dependent on the amounts of estrogenic components in the medium. The *ADE2* gene encodes an enzyme of the adenine biosynthesis pathway. When the reporter gene is off, colonies will turn red, the color of metabolic intermediate from adenine metabolism. The estrogenic compounds will activate the expression of the reporter gene through the binding of estrogen receptor to ERE, resulting in white colonies. The quantitative estrogenic activity of test sample was converted to the relative potency compared to estradiol (E_2). The result in **Figure 2** indicates that the estrogenic activity of all the RC extracts was detected at a concentration >4 ppm, as indicated by the white colonies observed. The order of the estrogenic activity among these extracts was RC6 > RC7 > RC3, RC5 > RC1, RC2, RC4, by comparison of the relative potencies of each RC extract within a concentration range between 0.5 − 3 ppm. It is interesting to find that the relative estrogenic potencies among these extracts were not correlated exactly with the contents of the total two major isoflavones, formononetin and biochanin A. The extracts that resulted from the column chromatography with silica gel, that is, RC6, RC7, and RC3, showed higher estrogenic activities than those prepared without silica extraction. It was also noted that even though the contents of the major isoflavones in RC5 were comparable to those in RC6 and RC7, lower estrogenic activity was observed in RC5. In our previous study, prominent estrogenic activity was observed in five aglucones: biochanin A, daidzein, formononetin, genistein, and glycitein (*25*). The result indicated that the rank order of potency among the five isoflavonoids was genistein (1 μM) > biochanin A, formononetin (5 μM) > daidzein (10 μM) > glycitein (> 10 μM), as indicated by the concentrations required to active the *ADE2* reporter as 100 pM E_2. It is expected

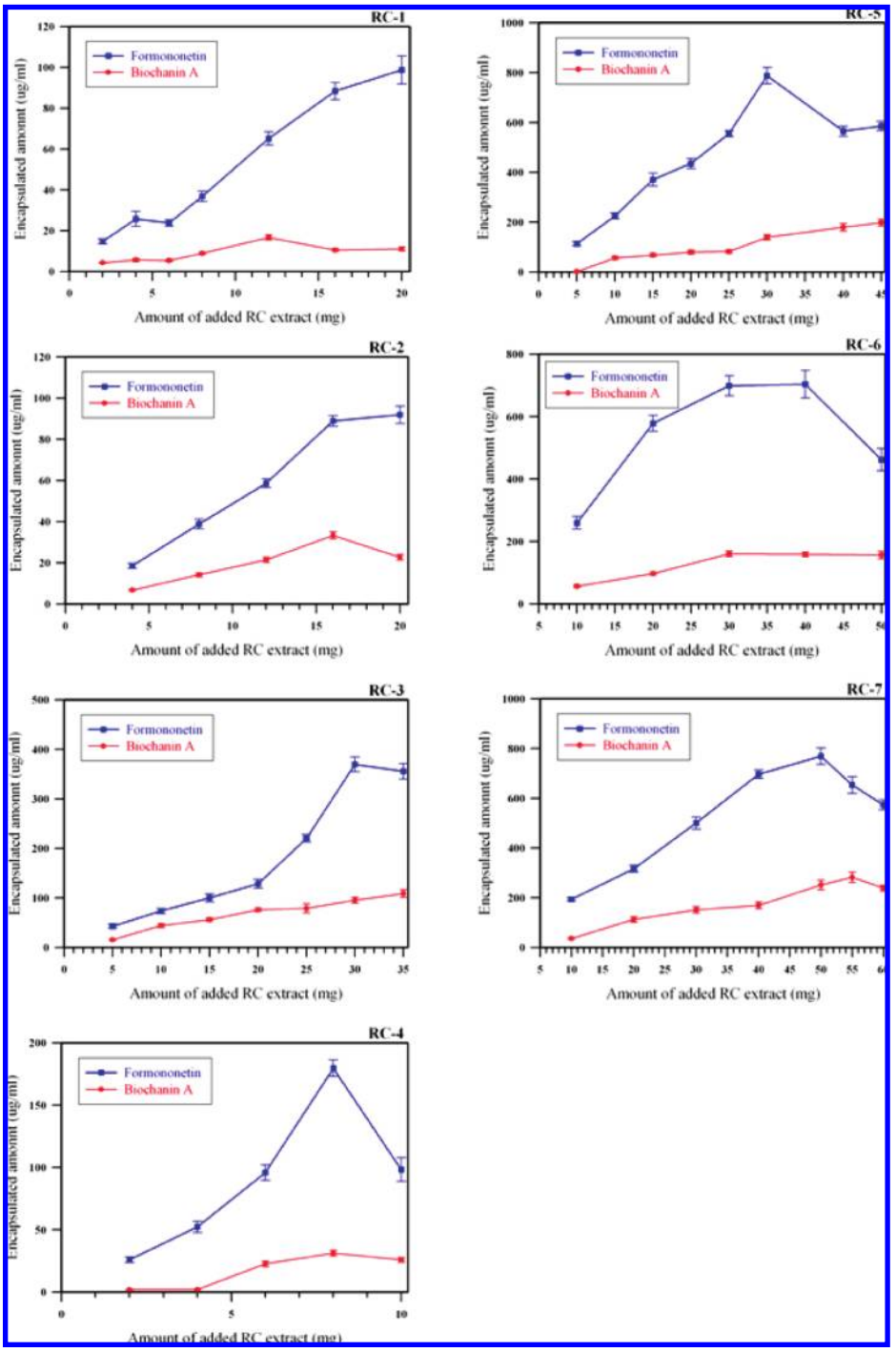


Figure 3. Relationship of added doses versus encapsulated amounts of formononetin and biochanin A in different RCE-encapsulated microemulsions.

that other minor isoflavones, including daidzein and genistein, which have been reported in raw RCE, with strong phytoestrogenic activity might be extracted more readily by using silica gel than XAD.

Determination of the Loading Capacities of Red Clover Extracts in the Microemulsion. To determine the maximum loading capacities of RCEs in the microemulsion, a series of increasing amounts of RCEs were loaded to the previous selected formulation. The results shown in **Figure 3** indicate that as the added amount of RCEs increased, the encapsulated concentration was increased in a dose-dependent manner. It is also worth noting that the maximum capacity was obtained and then dropped immediately. This phenomenon is probably due to the sudden rupture of the thermodynamically stable microemulsion. In **Table 3**, the maximum encapsulated amounts of formononetin in RC1−RC7 were calculated as 65.15 ± 3.68, 86.01 ± 2.63, 369.73 ± 7.54, 179.75 ± 6.63, 787.83 ± 12.25, 703.75 ± 35.74, 769.12 ± 16.53, respectively. It was interesting to find that in the case of RC1 and RC2, as the encapsulated amounts of biochanin A reached saturation, the encapsulation of formononetin showed a slow increase. It is possible that a trace amount of formononetin might dissolve in the aqueous phase by its concomitant inclusion with the aqueous soluble matrix materials. The phenomenon did not occur in the cases of RC3−RC7, in which matrix materials of high polarity were removed through the solid-phase extraction with silica and XAD. In the case of biochanin A, the maximum encapsulated amounts of each RCE were 16.65 ± 0.73, 33.27 ± 1.67, 95.28 ± 8.91, 30.43 ± 2.30, 139.15 ± 12.28, 156.29 ± 11.96,

Table 3. Comparison of the Contents of the Two Major Isoflavones in Different RCEs and the Maximum Encapsulated Amounts in Microemulsions

	contents of isoflavone (%)		maximum encapsulated amounts (μg/mL)	
	formononetin	biochanin A	formononetin	biochanin A
RC-1	5.75 ± 0.31	1.43 ± 0.30	65.15 ± 3.68	16.65 ± 0.73
RC-2	4.09 ± 0.49	1.63 ± 0.28	86.01 ± 2.63	33.27 ± 1.67
RC-3	5.91 ± 0.48	3.01 ± 0.61	369.73 ± 7.54	95.28 ± 8.91
RC-4	7.09 ± 0.12	1.77 ± 0.36	179.75 ± 6.63	30.43 ± 2.30
RC-5	17.30 ± 0.55	3.30 ± 0.64	787.83 ± 12.25	139.15 ± 12.28
RC-6	17.84 ± 0.20	3.71 ± 0.05	703.75 ± 35.74	156.29 ± 11.96
RC-7	15.20 ± 0.78	4.17 ± 0.18	769.12 ± 16.53	251.18 ± 13.61

and 251.18 ± 13.61, respectively (**Table 3**). The result indicated that the enhanced encapsulation effect was observed in the RCE-encapsulated microemulsions of ME-RC5, ME-RC6, and ME-RC7. In comparison with ME-RC1 prepared from the raw red clover extract, the encapsulated amounts of isoflavones were increased by > 10-fold.

In Vitro Skin Permeation Studies. The microemulsion formulations (ME-RCs) prepared from loading approximately 10 mg of RC extract into the previously described formula were tested for their transmembrane permeation into the mouse skin. Owing to the poor solubility of isoflavones in water, 50% of ethanol was used as the donor medium to provide the sink condition. The amounts of isoflavones permeated were determined at 2-h time intervals of permeation for the seven formulations. The time-dependent increase in permeated formononetin was observed in all seven of the microemulsions. On the contrary, no permeation of biochanin A was detected in any of the microemulsions (data not shown). Next, the transmembrane rate of formononetin in each ME-RC was examined by calculating the slope of the linear portion of the cumulative amount versus time. To examine the effect of matrix materials on the release of the isoflavone from the microemulsion system among the ME-RCs, the permeability coefficient K_p was further calculated, and the results are shown in **Table 4**. The order of the K_p ($\times 10^3$cm/h) value was ME-RC5 (1.95) < ME-RC4 (2.90) < ME-RC3 (3.68) < ME-RC2 (4.50) < ME-RC7 (4.70) < ME-RC1 (5.07) < ME-Form (5.13) < ME-RC6 (6.79). It is interesting to note that ME-RC5 exhibited significantly the least permeability among the ME-RCs, implying the important role of the matrix materials removed from XAD extraction on the control release of the formononetin. In the case of ME-RC6, the permeability was enhanced after removal of the matrix materials using silica gel extraction. This is the first report discussing the effect of the partially purified natural products extract on the controlled release in the microemulsion system.

To further characterize the physical properties of these formulations, the averaged diameters were determined, and the result is shown in **Table 4**. The diameters of ME-RCs were distributed within the range of 70−95 nm. The order of the averaged diameter was ME-Form < ME-RC5 < ME-RC6 < ME-RC7 < ME-RC3 < ME-RC2 < ME-RC4 < ME-RC1. The result indicated that ME-RCs prepared from partially purified RCEs by solid-phase extraction with either XAD or silica gel resulted in smaller diameters. However, in comparison with the result from the transdermal permeation study, it is concluded that the particle sizes of ME-RCs that are small enough to cross the barrier are not the factor that contributes to the differential transmembrane permeability among ME-RCs, as indicated by the result from ME-RC5, which possesses the smallest mean diameter but the lowest permeability coefficient. Several studies on the mechanism of microemulsion have concluded that there are three key factors that may contribute to the enhancement of skin permeation, that is, the mobility of the bioactive ingredient in the designed formulation, the concentration gradient, and the particle diameter (*26*−*29*). The choice of oil components and surfactant/cosurfactant in the formulation should influence the ease of releasing the ingredient across the barrier. Lecithin, a naturally occurring biocompatible surfactant, should contribute to the enhanced permeability in this study (*16*, *17*). In our study, the results indicated that the matrix associated with the main ingredients in red clover extracts might influence the permeability coefficients of isoflavones into the mouse skin. It was also noted that the matrix resulted from different solid-phase extractions performed differentially, indicating the potential role of the matrix as a barrier or enhancer in the transmembrane study.

Table 4. Size Diameters and in Vitro Skin Permeation Parameters of the Different RCE-Encapsulated Microemulsions

vehicle	diameter (nm)	J_s (μg/cm^2/h)	K_p ($\times 10^3$cm/h)
ME-RC1	94.6 ± 1.9	0.56 ± 0.07	5.07 ± 0.63
ME-RC2	88.7 ± 7.9	0.50 ± 0.12	4.50 ± 1.10
ME-RC3	85.7 ± 1.3	0.62 ± 0.05	3.68 ± 0.28
ME-RC4	89.0 ± 11.9	0.44 ± 0.11	2.90 ± 0.75[a]
ME-RC5	71.5 ± 1.0	0.40 ± 0.06	1.95 ± 0.31[a]
ME-RC6	72.3 ± 2.8	1.78 ± 0.19	6.79 ± 0.73
ME-RC7	80.2 ± 0.8	0.92 ± 0.46	4.70 ± 2.34
ME-Form	68.2 ± 1.4	1.10 ± 0.24	5.13 ± 1.13

[a] Significantly different from ME-Form ($P < 0.05$) by Student's t test.

In conclusion, the enhancement of the encapsulation of the isoflavone-containing red clover extracts in phospholipid-based microemulsions using different extraction processes was conducted and compared. The results indicated that the encapsulated amounts of isoflavoens in RCE-encapsulated microemulsions of ME-RC5, ME-RC6, and ME-RC7 were increased by > 10-fold, compared with that of ME-RC1 prepared from the raw red clover extract. Comparison of the permeability coefficients K_p of the formononetin in the in vitro skin permeation study among the ME-RCs showed that ME-RC5 significantly exhibited the least permeability, whereas ME-RC6 exhibited enhanced permeability after two-stage solid-phase extraction, indicating the potential role of the matrix as a barrier or enhancer in the transmembrane study. Furthermore, the particle sizes of ME-RCs that are small enough to cross the barrier are not the factor that contributes to the differential transmembrane permeability among ME-RCs, as indicated by the result from ME-RC5 that possesses the smallest mean diameter but the lowest permeability coefficient. Further study on the controlled release of this established microemulsion system for other polyphenols and their natural extracts is underway.

ABBREVIATIONS USED

RCE, red clover extact; ME, microemulsion; ME-RC, red clover extract-encapsulated microemulsion; HRT, hormone replacement therapy; ERE, estrogen response element; EO, ethyl oleate; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; K_p, permeability coefficient; J_s, permeation rate.

LITERATURE CITED

(1) Magee, P. J.; Rowland, I. R. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *Br. J. Nutr.* **2004**, *91*, 513–531.

(2) Cornwell, T.; Cohich, W.; Raskin, I. Dietary phytoestrogens and health. *Phytochemistry* **2004**, *65*, 995–1016.

(3) Albertazzi, P.; Purdie, D. W. The nature and utility of the phytoestrogens: a review of the evidence. *Maturitas* **2002**, *42*, 173–185.

(4) Adlercreutz, H. (**1995**) Phytoestrogens: epidemiology and a possible role in cancer protection. *Environ. Health Perspect*. **1995**, *103* (Suppl. 7), 103-112.
(5) Beck, V.; Rohr, U.; Jungbauer, A. Phytoestrogens derived from red clover: an alternative to estrogen replacement therapy? *J. Steroid Biochem. Mol. Biol*. **2005**, *94*, 499–518.
(6) Van De Weijer, P. H. M.; Barentsen, R. Isoflavones from red clover (Promensil) significantly reduce menopausal hot flush symptoms compared with placebo. *Maturitas* **2002**, *42*, 187–193.
(7) Jungbauer, A.; Beck, V. Yeast reporter system for rapid determination of estrogenic activity. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*. **2002**, *777*, 167–178.
(8) Kreilgaard, M. Influence of microemulsions on cutaneous drug delivery. *Adv. Drug Delivery Rev*. **2002**, *54* (Suppl. 1), S77−S98.
(9) Lundberg, B. Preparation of drug-carrier emulsions stabilized with phosphatidylcholine-surfactant mixtures. *J. Pharm. Sci*. **1994**, *83*, 72–75.
(10) Magdassi, S.; Siman-Tov, A. Formation and stabilization of perfluorocarbon emulsions. *Int. J. Pharm*. **1990**, *59*, 69–72.
(11) Acosta, E. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. *Curr. Opin. Colloid Interface Sci*. **2009**, *14*, 3–15.
(12) Flanagan, J.; Singh, H. Microemulsions: a potential delivery system for bioactives in food. *Crit. Rev. Food Sci. Nutr*. **2006**, *46*, 221–237.
(13) Garti, N.; Yaghmur, A.; Leser, M. E.; Clement, V.; Watzke, H. J. Improved oil solubilization in oil/water food grade microemulsions in the presence of polyols and ethanol. *J. Agric. Food Chem*. **2001**, *49*, 2552–2562.
(14) Amar, I.; Aserin, A.; Garti, N. Solubilization patterns of lutein and lutein esters in food grade nonionic microemulsions. *J. Agric. Food Chem*. **2003**, *51*, 4775–4781.
(15) Patel, N.; Schmid, U.; Lawrence, M. J. Phospholipid-based microemulsions suitable for use in foods. *J. Agric. Food Chem*. **2006**, *54*, 7817–7824.
(16) Spernath, A.; Aserin, A.; Sintov, A. C.; Garti, N. Phosphatidylcholine embedded micellar systems: enhanced permeability through rat skin. *J. Colloid Interface Sci*. **2008**, *318*, 421–429.
(17) Spernath, A.; Aserin, A.; Ziserman, L.; Danino, D.; Garti, N. Phosphatidylcholine embedded microemulsions: physical properties and improved Caco-2 cell permeability. *J. Controlled Release* **2007**, *119*, 279–290.
(18) Lin, C. C.; Lin, H. Y.; Chen, H. C.; Yu, M. W.; Lee, M. H. Stability and characterisation of phospholipid-based curcumin-encapsulated microemulsions. *Food Chem*. **2009**, *116*, 923–928.
(19) Lee, S. H.; Kim, Y. H.; Yu, H. J.; Cho, N. S.; Kim, T. H.; Kim, D. C.; Chung, C. B.; Hwang, Y. I.; Kim, K. H. Enhanced bioavailability of soy isoflavones by complexation with β-cyclodextrin in rats. *Biosci., Biotechnol., Biochem*. **2007**, *71*, 2927–2933.
(20) Quan, D. Q.; Xu, G. X.; Wu, X. G. Studies on preparation and absolute bioavailability of a self-emulsifying system containing puerarin. *Chem. Pharm. Bull*. **2007**, *55*, 800–803.
(21) Balmelli-Gallacchi, P.; Schoumacher, F.; Liu, J. W.; Epenberger, U.; Mueller, H.; Picard, D. A yeast-based bioassay for the determination of functional and non-functional estrogen receptors. *Nucleic Acids Res*. **1999**, *27*, 1875–1881.
(22) Metzger, D.; White, J. H.; Chambon, P. The human oestrogen receptor functions in yeast. *Nature* **1988**, *334*, 31–36.
(23) Guthrie, C.; Fink, G. R. *Guide to Yeast Genetics and Molecular and Cell Biology, Methods in Enzymology*; Academic Press: New York. 1991; Vol. *194*.
(24) Tomas-Barberan, F. A.; Blazquez, M. A.; Garcia-Viguera, C.; Ferreres, F.; Tomas-Lorente, F. A comparative study of different Amberlite XAD resins in flavonoid analysis. *Phytochem. Anal*. **1992**, *3*, 178–181.
(25) Lin, C. C.; Tsai, Y. L.; Ho, C. T.; Teng, S. C. Determination of the differential estrogenicity of isoflavonoids by E_2-ER-ERE-dependent gene expression in recombinant yeast and MCF-7 human breast cancer cells. *Food Chem*. **2008**, *108*, 719–726.
(26) Chen, H.; Chang, X.; Weng, T.; Zhao, X.; Gao, Z.; Yang, Y.; Xu, H.; Yang, X. A study of microemulsion systems for transdermal delivery of triptolide. *J. Controlled Release* **2004**, *98*, 427–436.
(27) Escribano, E.; Calpena, A. C.; Queralt, J.; Obach, R.; Domenech, J. Assessment of diclofenac permeation with different formulations: anti-inflammatory study of a selected formula. *Eur. J. Pharm. Sci*. **2003**, *19*, 203–210.
(28) Peltola, S.; Saarinen-Savolainen, P.; Kiesvaara, J.; Suhonen, T. M.; Urtti, A. Microemulsions for topical delivery of estradiol. *Int. J. Pharm*. **2003**, *254*, 99–107.
(29) Sintov, A. C.; Shapiro, L. New microemulsion vehicle facilitates percutaneous penetration in vitro and cutaneous drug bioavailability in vivo. *J. Controlled Release* **2004**, *95*, 173–183.

Received June 17, 2009. Revised manuscript received August 30, 2009. Accepted September 04, 2009.