Asian Journal of Chemistry Vol. 21, No. 8 (2009), 6237-6246

In Vitro Skin Permeation Efficiency Study on Natural Flavornoid Extracts Incorporated into Nano-Emulsions

ING-MAY LING, WEN-HSIN LI and LAI-HAO WANG*
Department of Applied Chemistry, Chia Nan University of Pharmacy and Science, Tainan, 71743, Taiwan (Republic of China)
E-mail: e201466.wang@msa.hinet.net

Stable emulsions made with proper types and amounts of surfactants, fatty acids and water have been obtained. By varying the surfactant types (AOT, Brij 30 and HCO 040), concentrations of surfactants (2 to 10 %) and types of fatty acids (lauric acid C12, myristic acid C14, palmitic acid C16 and stearic acid C18), the best emulsions have been obtained by using AOT at 6 % and fatty acid C12 kept at 0.85 % (by weight) oil-to-water (O/W) ratio in a PIT (Phase Inversion Temperature) emulsification method. Emulsions obtained by using Brij 30 AOT at 6 % and fatty acid C12 kept at 0.85 % O/W ratio are also stable and satisfactory. The HLB (hydrophilic-lipophilic balance) temperature for the AOT at 6 % and C12 kept at 0.85 % O/W ratio is 38 ºC. This method produces emulsion droplet sizes ranging from 170 to 290 nm with the average at 230 nm, which has been verified by using a high resolution optical microscope. The droplets are near circular and the emulsification process is homogeneous. Therefore, this emulsion can be classified as a nano-emulsion. Natural, ultra-violet absorbing flavornoid extracts from herbal plants have been incorporated into the emulsion samples made by using AOT at 6 % and fatty acid C12 kept at 0.85 % O/W ratio. *In vitro* skin permeation experimental results by incorporating *Cinnamomum japonicum* Sieb and *Sophora japonica* L extracts indicate that the most effective flavornoid extract concentrations for both ingredients are around 2 %, with the first ingredient performing better than the latter.

Key Words: Nano-emulsions, Medical plants, *In vitro* release.

INTRODUCTION

Emulsions, a specific class of two-phase systems of matter called colloids, consist of liquid dispersed droplets (*e.g.* oil) immersed in another continuous liquid (*e.g.* water). Large droplets (> 10 microns in diameter) are relatively soft and deformable. Smaller droplets (< 3 microns) are more like hard spheres due to their Laplace pressure. In addition, emulsions have an oil-water interface with a surfactant layer to consider. In a real emulsion, the size of the droplets usually varies greatly. Emulsions prepared for an experiment usually contain droplets varying in diameter from *ca.* 0.05 to 5 μ. When the droplets are in the range of several hundreds of nanometers or smaller, they can be classified as nano-emulsions. Oil-in-water (O/W) nano-emulsions,

according to the average size of droplets, generally have a transparent (less than 100 nm) to bluish white (100-500 nm) appearance. Nano-emulsions can be prepared by a high-pressure homogenization method, a phase inversion temperature (PIT) method and a vortex stirring method[1-4].

Nano-emulsions represent an effective means for delivering medical and cosmetic active ingredients for diagnostic or therapeutic purposes. Cosmetic nano-emulsions can provide skin care by releasing active ingredients to the surface of the skin, the epidermis or the stratum corneum. For instance, nano-emulsions have been introduced as sunscreen materials[5-7].

In this work, nano-emulsions have been prepared with three surfactants (Brij 30, AOT and Emulgade SE, *i.e.* HCO 040) and various concentrations of C12-C18 fatty acids in water by using a PIT method. The sizes of droplets have been investigated with a high-resolution light microscope with sub-micron precision. Natural ultraviolet absorbing materials (flavornoids), extracted from herbal plants, have been incorporated into the nano-emulsions. Their ultraviolet absorbing capabilities and skin permeation efficiencies have been studied by using UV absorption spectroscopy.

EXPERIMENTAL

Aliphatic fatty acids: Lauric acid and palmitic acid obtained from Sigma, Nippon Shiyaku Koggo K.K, and Showa chemical Co., LTD, respectively. Myristic acid and stearic acid obtained from Top Rhyme Co., LTD.

Surfactants: A polyoxyethylene alkyl ether-type non-ionic surfactant (Brij 30), a sodium *bis*(2-ethylexyl) sulfosuccinate anionic surfactant (AOT) and an Emulgade SE (HCO 040) obtained from Acros, Fluka and Lockhouse (Bay, Auckland, New Zealand), respectively.

Ultraviolet absorbing materials: Natural ultraviolet absorbents purchased locally, which are extracts from natural flavonoid plants such as *Cinnamomum japonicum* Sieb and *Sophora japonica* L.

Dialysis membrane: Spectra/Por 4 dialysis membranes (molecular weight 12000-14000) purchased from Spectrum Medical Industries, Inc. in California.

Measurement equipment

Conductivity: Suntex SC-17A and Mettler Toledo 980-K19/120 Electrodes.

Droplet size: Optical microscope (Olympus MX40).

UV Absorbance: Cary UV/Visible Spectrophotometer (Varian, Australia); Quartz Cells of 1cm Path Length.

Experimental steps

Conductivity measurement: Samples of emulsions with a fixed oil/water weight ratio of 0.85/99.15 were prepared by shaking appropriate amounts of fatty acids and water containing 10^{-2} mol/L sodium chloride mixed with various concentrations of surfactants. The conductivity was measured as a function of temperature

for each sample. The HLB temperature or PIT was determined by the conductivity change at a specific temperature.

Emulsion sample preparation: Samples of emulsions were prepared by mixing various amounts of surfactants with water first. Then, different amounts of fatty acids were added to the surfactant/water solutions. A magnetic stirrer at approximately constant 700 rpm was used. The solutions were initially prepared at a temperature of 80 ºC and then rapidly cooled to go below 40 ºC (PIT) by immersing the samples in an icy water bath to form samples of emulsions.

Droplet size measurement: Light microscope method: For each emulsion sample, a drop of sample was placed on a glass slide. An ultra-thin glass cover slip was placed on top of the drop, needed for high-magnification microscope focusing. Once focused, the emulsion particles were observed and the droplets diameters were measured.

Incorporation of ultraviolet absorbing flavornoid extracts into emulsions: Extractions were performed by adding 0.25 g of pulverized flavornoid containing herbal plants to a mixture of 50 % aqueous methanol and water (20 mL), followed by stirring and refluxing at 70 ºC in a water bath for 0.5 h. The extracts were separated from the plant residues by centrifugation at 6000 rpm for 0.5 h. Each flavornoid extract was mixed thoroughly into the emulsion samples for further experiments.

Skin membrane permeation efficiency measurement: *In vitro* skin permeation experiments were performed by using horizontal side-by-side diffusion cells. The diffusion cells were constructed with cylindrical half-cells with a permeation area of 9.07 cm^2. Spectra/por 4 dialysis membranes were used. One of receptor compartments (11 mL) was filled with de-ionized water as media, another with emulsions containing flavornoid extracts.

The diffusion cells were kept at a constant temperature of 37 ºC. The receptor solutions were stirred during the experiments to ensure thorough mixing. At fixed time intervals, a portion (0.5 mL) of receptor samples was removed for analysis and replaced with fresh samples. The removed samples were diluted with de-ionized water and the skin membrane permeation efficiencies were measured by UV absorption data performed at 287 nm.

RESULTS AND DISCUSSION

Conductivity measurement: The conductivity for all emulsion samples can be measured. The typical conductivity data measured by using C12 fatty acid at 0.85 % O/W ratio and AOT surfactant at various concentrations as a function of temperature are depicted in Fig. 1.

In Fig. 1 for each surfactant concentration, the conductivity initially increases with temperature from 25 ºC. When the temperature reaches the HLB temperature (or PIT), the conductivity starts to decrease with temperature. According to Fig. 1, the HLB temperature ranges from 35 to 48.5 ºC. Furthermore, the HLB temperature increases while the surfactant concentration decreases.

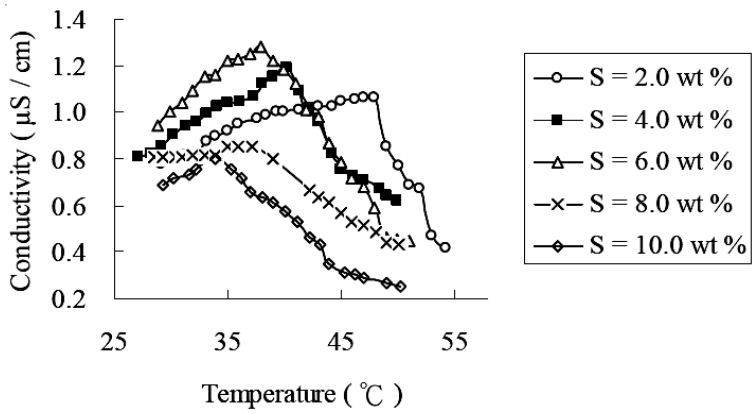


Fig. 1. Conductivity *vs.* temperature for emulsions using C12 lauric acid at 0.85 % O/W ratio and AOT surfactant at various concentrations, containing 10^{-2} mol/L NaCl

The preliminary emulsions obtained from the conductivity measurement experiment, the samples made by using C12 at 0.85 % O/W ratio and AOT at 6 % or 4 % concentrations seem to be the most stable, (*i.e.* without de-layering (creaming) caused by coalescence after one week of storage time), with the corresponding HLB temperatures at 38 and 40 ºC, respectively.

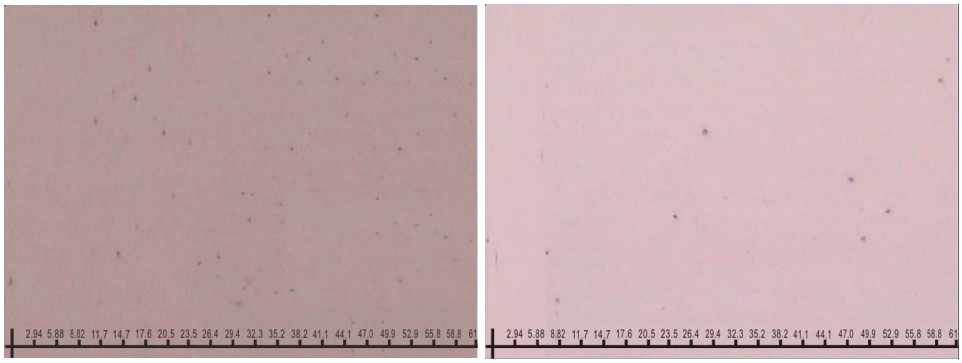
To study the differences between various fatty acids, the emulsion HLB temperatures for using various fatty acids at 0.85 % O/W ratio and surfactant AOT at 2 % are tabulated in Table-1. According to Table-1, the HLB temperature tends to decrease when the fatty acid hydrocarbon alkyl chain length increases if keeping the oil-to-water ratio and the surfactant concentration constant.

TABLE-1
HLB TEMPERATURES USING VARIOUS FATTY ACIDS AT 0.85 % O/W RATIO AND SURFACTANT AOT CONCENTRATION AT 2 %

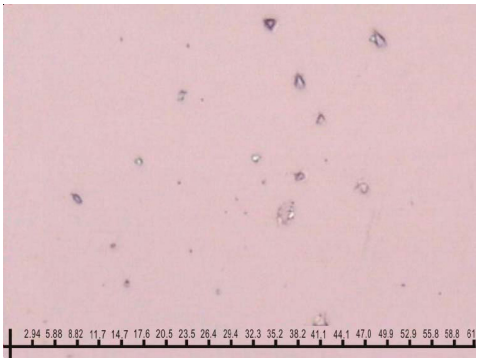
Oils	T_{HLB} (ºC)
Lauric acid (C12)	48.5
Myristic acid (C14)	38.5
Palmitic acid (C16)	32.0
Stearic acid (C18)	30.8

Emulsification experiments

Surfactant variation: The emulsification experimental results by varying the types of surfactants 6 % while using C12 fatty acid fixed at oil/water weight ratio of 0.85 % are depicted in Fig. 2. The photomicrographs in Fig. 2a, 2b, 2c are referring to AOT, Brij 30 and HCO 040, respectively, which have been obtained by using a high-resolution light microscope and stored electronically. The stored electronic image data have been processed by using computer graphics tools to measure the oil droplet sizes. The results are tabulated in Table-2.



(a) (b)



(c)

Fig. 2. Light microscope emulsion images for surfactants (a) AOT; (b)Brij 30; (c)HCO 040 all at 6 % while Fixing C12 O/W at 0.85 %

TABLE-2
DROPLET SIZE DATA FOR SURFACTANTS FROM (Fig. 2a) AOT, (Fig. 2b) BRIJ 30, (Fig. 2c) HCO 040 ALL AT 6 % WHILE FIXING C12 O/W AT 0.85 %

Surfactant (6 %)	Droplet size distribution (nm)	Droplet average size (nm)
Fig. 2a) AOT	170-289	227
Fig. 2b) Brij 30	223-405	309
Fig. 2c) HCO 040	215-1552	542

In Fig. 2, micro ruler markers are provided for easy viewing of the emulsion droplet dimensions. Each ruler marker division is *ca.* 3000 nm wide. Visually, the droplets in Fig. 2a and 2b are tiny dots which can be classified as nano-particles. Their size measurement results are contained in Table-2. For AOT, the droplet size ranges from 170 to 289 nm with the average at 227 nm. For Brij 30, the size ranges from 223 to 405 nm with the average at 309 nm. For AOT and Brij 30, the droplets are more circular and homogeneous. For HCO 040, even though the average size is only at 542 nm, the droplet size has a wide size distribution rage from 215 to 1552

nm. Many droplets are not circular in shape and the size distribution is wide ranged and non homogeneous. Therefore, it cannot be classified as a nano-emulsion, which can also be visually verified by looking at Fig. 2c.

Moreover, many large droplets in Fig. 2c for HCO 040 appear irregular which may be formed by smaller droplets aggregating together or through an incomplete emulsification process. Clearly among the three types of surfactants, AOT perf-orms the best and Brij 30 a close second, while HCO 040 is a distant third.

Fatty acid variation: The experimental results by varying the types of fatty acids (all at 0.85 %) while using AOT surfactant fixed at 6 % are depicted in Fig. 3. Fig. 3a, 3b, 3c and 3d are referring to C12, C14, C16, and C18, respectively. The corresponding droplet sizes are tabulated in Table-3. Fig. 3a is actually the same as Fig. 2a and it's placed here for clear comparisons between C12 and other fatty acids, *i.e.* C14, C16, and C18. By visually examining Fig. 3b, 3c, and 3d, none of these can be classified as nano-emulsions. The data in Table-3b, 3c and 3d indicate that they are over 1 μm for the large droplets.

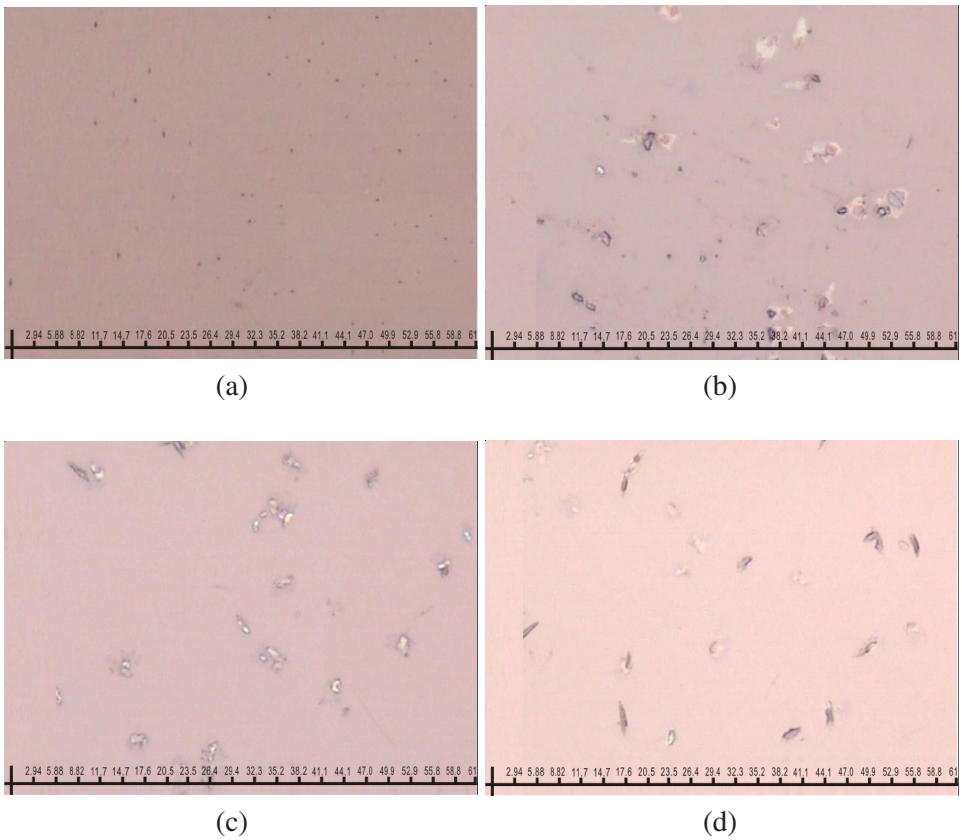


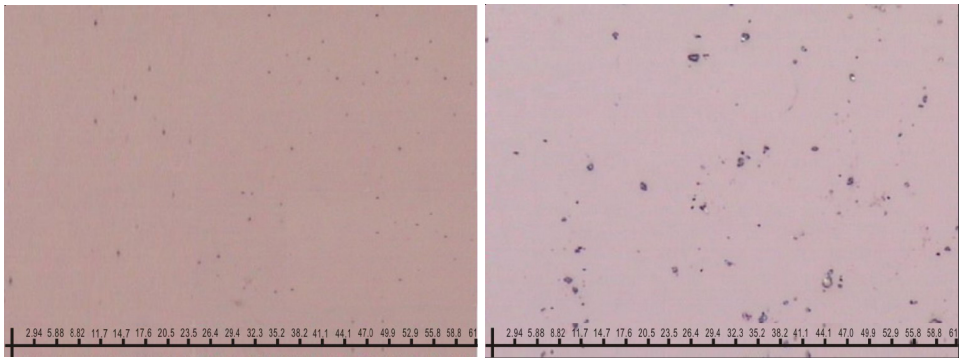
Fig. 3. Light microscope emulsion images for fatty acids (a) C12; (b) C14; (c) C16 and (d) C18 all at 0.85% O/W ratio while using surfactant AOT at 6 %

TABLE-3
DROPLET SIZE DATA FOR ACIDS FROM (Fig. 3a) C12, (Fig. 3b) C14, (Fig. 3c) C16, (Fig. 3d) C18 ALL AT 0.85% O/W RATIO WHILE USING SURFACTANT AOT AT 6 %

Fatty acid (0.85 % O/W)	Droplet size distribution (nm)	Droplet average (nm)
(Fig. 3a) C12	178-289	227
(Fig. 3b) C14	240-1862	517
(Fig. 3c) C16	281-2234	798
(Fig. 3d) C18	497-2623	1290

The large droplets for C14, C16, and C18 are highly irregular. The size data in Table-3 are obtained by taking the averages of the x-dimensions and y-dimensions. The large dimensions in Table-3b, 3c and 3d indicate that the droplets produced using C12 are far superior to those by C14, C16, and C18.

Surfactant concentration variation: The experimental results by varying the surfactant (AOT) concentration level while using fatty acid C12 fixed at a fixed O/W ratio of 0.85% are depicted in Fig. 4. Fig. 4a and 4b are for AOT of 4 and 6 %, respectively. The corresponding droplet sizes are tabulated in Table-4. Fig. 4a is actually the same as Fig. 2a and 3a and it's placed here for clear comparisons between AOT of 6 % and AOT of 4 %.



(a) (b)

Fig. 4. Light microscope emulsion images for (a) AOT at 6 % and (b) AOT at 4 % while keeping fatty acid C12 at 0.85 % O/W ratio

TABLE-4
DROPLET SIZE DATA FOR AOT AT 6 % AND AOT AT 4 % WHILE KEEPING FATTY ACID C12 AT 0.85 % O/W RATIO

Surfactant concentration	Droplet size distribution (nm)	Droplet average size (nm)
(Fig. 4a) 6 % AOT	178-289	227
(Fig. 4b) 4 % AOT	190-1117	322

Both light microscopic images and droplet size data suggest that using AOT at 6 % produces better results than using AOT at 4 %. The droplet sizes for AOT at 4 % range from 190 nm to 1117 nm, which is slightly higher than 1000 nm.

Summing up the results given in emulsification experiments, the emulsion samples processed with the surfactant AOT at 6 % concentration level and the fatty acid C12 at 0.85 % O/W ratio contain the best and the finest droplets. These samples are further used for flavornoid extract incorporations and UV absorption experiments.

UV-A and UV-B absorption results: Various natural flavornoid extracts incorporated into the C12 (0.85% O/W ratio) and AOT (6 %) emulsion samples have been tested for UV absorption capabilities. The results are listed in Table-5. According to Table-5, three extracts, *i.e. Sophora japonica* L, *Nelumbo nucifera* Gaertn, and *Languas galanga* (L.) Stuntz, show UV-A absorption capability. Four other extracts, *i.e. Cinnamomum haponicum* Sieb, *Ginko biloba* L, *Calendula arvensis* L and *Glycyrrhiza uralensis* Fisch exhibit UV-B absorption capability. Finally, one extract, i.e. *Alpinia ifficinarum* Hance, exhibits both UV-A and UV-B absorption capabilities.

TABLE-5
UV-A AND UV-B ABSORPTION CAPABILITIES FOR FLAVORNOID EXTRACTS

Flavornoid extract	Absorption spectra	Maximum wavelength absorption in water (nm)
Sophora japonica L.	UVA	255, 348
Nelumbo nucifera Gaertn.	UVA	273, 343
Languas galanga (L.) stuntz	UVA	258, 331
Alpinia officinarum Hance	UVA, UVB	265, 286, 351
Cinnamomum japonicum Sieb.	UVB	284
Ginkgo biloba L.	UVB	270, 315
Calendula arvensis L.	UVB	253, 289
Glycyrrhiza uralensis Fisch	UVB	268, 311

Further investigations show that the active components of the flavornoid extracts are identified to kaempferol, quercetin and naringenin by comparisons to the HPLC data of generic samples[8]. These ingredients have been demonstrated to be effective when used as sunscreen materials[8].

In vitro skin permeation experiments have been performed by using horizontal side-by-side diffusion cells. Spectra/por 4 dialysis membranes are used to mimic human skins. The membrane permeation efficiency data for the flavornoid extracts have been obtained by measuring the UV absorption data at the beginning of each experiment and then at regular time intervals up to 24 h, assuming the extracts will diffuse through the membranes given enough time. The membrane permeation efficiency data, *i.e.* release percentage data, for *Cimmamomum japonicum* Sieb (abbreviated as C) and *Sophora japonica* L (abbreviated as L) are shown in Fig. 5a, 5b, and 5c at various extract concentration levels.

According to Fig. 5a, 5b and 5c, the release rates increase with time indicating that membrane permeations are taking place as time progresses. The data show that excessive concentrations, *e.g.* higher than 2 %, may decrease the permeation efficiencies, indicating saturations of flavornoid extracts on membranes surface may

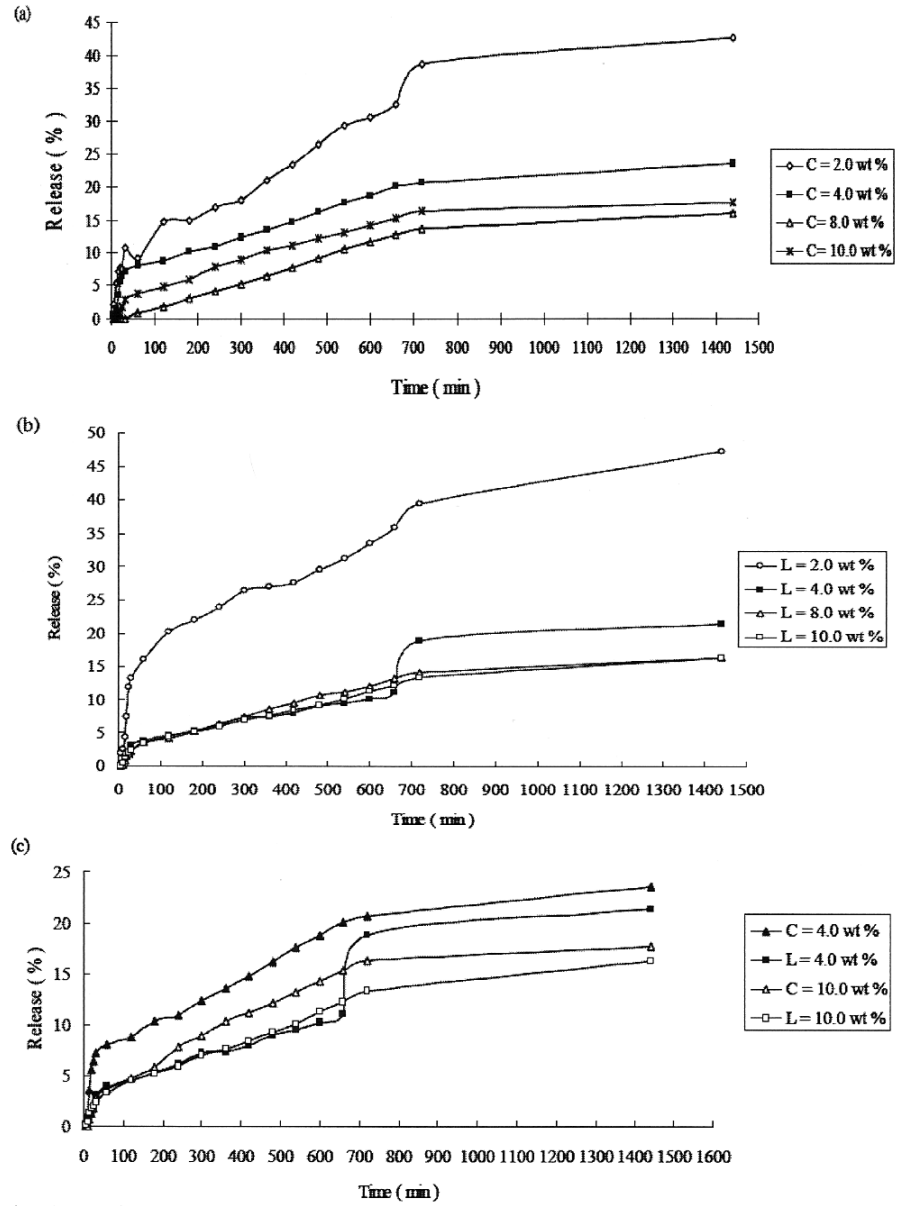


Fig. 5. Release percentage data *vs.* time for nano-emulsions containing (a) *Cinnamomum japonicum* Sieb (C); (b) *Sophora japonica* L (L) and (c) both (C and L)

occur if the concentration is higher than 2 %. Therefore, *Cinnamomum japonicum* Sieb at 2 %, *Sophora japonica* L at 2 %, or the combination of both actually produces the best membrane permeation results. Between the 2 % *Cinnamomum japonicum*

Sieb and 2 % *Sophora japonica* L membrane permeation curves in Fig. 5a and 5b, *Cinnamomum japonicum* Sieb performs better than *Sophora japonica* L as an UV sun screen agent when incorporated into 6 % AOT, 0.85 % C12 nano-emulsions.

Conclusion

The typical HLB temperature ranges from 35 to 48.5 ºC for emulsions using C12 lauric acid at 0.85 % oil-to-water ratio and AOT surfactant at various concentrations. The HLB temperature for the AOT at 6 % is 38 ºC.

By varying the types and concentrations of surfactants and types of fatty acids, the best emulsions have been obtained by using AOT at 6 % and fatty acid C12 kept at 0.85 % oil-to-water ratio in a PIT emulsification method. This best method produces emulsion droplet sizes range from 170 to 290 nm with the average at 230 nm, which has been verified by using a high resolution optical microscope. The droplets are near circular and the emulsification process has been homogeneous. Therefore, this emulsion can be classified as a nano-emulsion.

Ultra-violet absorbing natural flavornoid extracts from herbal plants have been incorporated into the emulsion samples made by using AOT at 6 % and fatty acid C12 kept at 0.85 % oil-to-water ratio. *In vitro* skin permeation experimental results by incorporating *Cinnamomum japonicum* Sieb and *Sophora japonica* L indicate that the most effective flavornoid extract concentrations for both ingredients are around 2 %, with the first ingredient performing better than the latter.

REFERENCES

1. P. Izquierdo, J. Esquena, T.F. Tadros, C. Dederen, M.J. Garcia, N. Azemar and C. Solans, *Langmuir*, **18**, 26 (2002).
2. A. Forgiarini, J. Esquena, C. Gonzalez and C. Solans, *Langmuir*, **17**, 2076 (2001).
3. P. Izquierdo, J. Esquena, T.F. Tadros, C. Dederen, M.J. Garcia, N. Azemar and C. Solans, *Comun. J. Com. Esp. Deterg.*, **31**, 359 (2001).
4. D. Morales, J.M. Gutierrez, M.J. Garcia-Celma and C. Solans, *Langmuir*, **19**, 7196 (2003).
5. F. Thomas, H. Soraya and H. Barbara, Ger. Offen DE 19532543 A, 16 March (1997).
6. G.R. Tu, W.S. Wang, L.X. Zhang, G.X. Du, H.Z. Zhao and X.F. Liu, *Jingxi Huagong*, **18**, 379 (2001).
7. S.A. Wissing and R.H. Muller, *J. Control. Rel.*, **81**, 225 (2002).
8. L.H. Wang and W.H. Li, *Pharm. Chem. J.*, **41**, 225 (2007).

(*Received*: 19 November 2008; *Accepted*: 26 May 2009) AJC-7603