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Micelle-mediated extraction and preconcentration of ginsenosides from Chinese herbal medicine

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Abstract

The feasibility of employing micelle-mediated extraction as an alternative and effective method for the solubilization, purification and/or preconcentration of active ingredients from herbal products is demonstrated for the first time using the root of American ginseng as a model. When compared to methanol and water, an aqueous surfactant solution containing 10% Triton X-100 yielded faster kinetics and higher recovery for the extraction of various ginsenosides. An experimental design approach (uniform design) was demonstrated as a novel and useful method for the optimization of experimental factors involved in the micelle-mediated extraction process. For the preconcentration of ginsenosides prior to chromatographic determination, a salting-out agent (sodium sulfate) was employed to make the efficient cloud point extraction of both hydrophobic and hydrophilic ginsenosides into the surfactant-rich phase possible, as well as to increase the preconcentration factor by reducing the volume of the surfactant-rich phase.

Keywords: Micelle-mediated extraction; Cloud point extraction; Ginsenosides

1. Introduction

The special properties of polyoxyethylene-type non-ionic surfactants, such as solubilization power and mildness, have been demonstrated to be useful in the efficient extraction of enzyme proteins in their natural form from plant membranes [1]. Additionally, the unique cloud point behavior of surfactants (mostly non-ionic type of the Triton X-series) has recently received increasing attention for use in sample purification and/or preconcentration [2,3]. Although the majority of cloud point extraction (CPE) applications dealt with analytes present in aqueous samples, CPE can also be employed as an alternative method for the extraction of analytes present in solid materials [1,4].

The use of herbal products as alternative medicines is becoming more popular worldwide. However, the analysis of their active ingredients present in herbal products encounters major difficulties, in part due to the trace amounts of pharmacologically active compounds and to the complexity of the matrix [5]. In view of increasing demands for solving quality-related problems of herbal products, the development of simple and reliable methods for the purification and/or the sensitive and selective determination of active components in herbal products is essential [6].

The roots of *Panax quinquefolium* (American

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ginseng) and a related species, *Panax ginseng* (Asian ginseng), are widely used medicinal plants, and ginseng is the most popular medicinal herb used in traditional Chinese medicine [7]. The main active ingredients of ginseng are saponins (usually referred as ginsenosides), more than thirty of which have been identified. However, six of these (Rg1, Re, Rb1, Rc, Rb2 and Rd) have been reported to account for more than 90% of the saponin content of the root [8]. These saponins have been shown to have various therapeutic activities, including anti-cancer, anti-diabetic and anti-aging effects.

In this paper, the feasibility of employing micelle-mediated extraction as a simple and effective tool for the separation of the active ingredients from herbal products is demonstrated, using the root of American ginseng as a model. The micelle-mediated extraction process in the present study can be divided into two parts: (1) the solubilization and purification of active ingredients from the solid herbal material into the extractant (aqueous surfactant solution) and (2) the cloud point phase separation of the aqueous surfactant solution containing the active ingredients into a bulk aqueous phase and a smaller volume surfactant-rich phase.

To optimize the micelle-mediated solubilization and purification process, an experimental approach (uniform design) was used, for the first time, to characterize the various experimental factors that affect the extraction process. The major advantage of uniform design (UD) is that when compared to commonly known methods such as factorial design, the number of experiments can be significantly reduced to produce reliable results even when the number of levels for each experimental variable is large [9–13]. The main idea behind UD is to find a set of representative experimental combinations of conditions that can scatter uniformly and regularly in the domain to be investigated. The so-called good-lattice point set, generated with the help of the theoretic-number method, can be used to construct the experimental design table. Using optimized extraction conditions, comparisons of extraction recovery and kinetics were carried out between an aqueous surfactant solution (Triton X-100) and two commonly used solvents (water and methanol).

To induce CPE and the preconcentration of active ingredients (ginsenosides) with a wide range of polarity into the smaller volume surfactant rich phase, an appropriate salting out agent was used as an additive in the CPE process. The preconcentrated ginsenosides were separated by high-performance liquid chromatography (HPLC) and the ginsenoside profile of American ginseng was thus obtained.

2. Experimental

2.1. Apparatus

All extractions were performed in an ultrasonic bath (Model 2210 E-DTH, Branson Ultrasonics., Danbury, USA). To facilitate the phase separation process in CPE, the extracted samples were placed in a centrifuge (Model EBA 8, Hettich, Tuttlingen, Germany). For HPLC analyses, a BAS (Bioanalytical Systems, Lafayette, IN, USA) modular HPLC system consisting of a PM-80 solvent delivery system, LC-26A vacuum degasser, CC-5 liquid chromatograph (20-μl sample loop) and an UV-116A UV–Vis detector was employed. The separation column (4.6 mm×25 cm) packed with 5 μm C_{18} material was obtained from Beckman (Fullerton, CA, USA).

2.2. Chemicals

American ginseng samples were purchased from a local herbal shop in Hong Kong. The ginsenoside standards (Rg1, Re, Rb1, Rc, Rb2 and Rd) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The non-ionic surfactants, Triton X-100 and Triton X-114, were obtained from Fluka (Buchs, Switzerland) and used as received without further purification. Various concentrations (w/v) of aqueous surfactant solutions were prepared by weighing appropriate amounts of the surfactants and direct dissolution in water. HPLC-grade methanol and acetonitrile were purchased from Acros (Geel, Belgium). All other chemicals, including the salting out agent (sodium sulfate), were of analytical grade obtained from Aldrich (St. Louis, MO, USA). All solvents and non-preconcentrated sample solutions were filtered through 0.45-μm nylon membrane filters. All aqueous solutions (doubly de-ionized

distilled water) were prepared from a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Procedures for the extraction and preconcentration of ginsenosides

The roots of American ginseng were dried in an oven at 50°C for 6 h, then ground and sieved to produce samples with particle sizes in the range between 130 and 300 μm. For experiments involving the use of the UD method, to find the best set of parameters for extraction, a sample amount of 250 mg was used. Experimental conditions for the various extraction variables are listed in Table 1. After extraction, the extracts, which contained various ginsenosides in aqueous surfactant solutions, were filtered and then injected into the HPLC system. The data obtained were then interpreted using a UD program supplied by the Statistics Research and Consultancy Centre at Hong Kong Baptist University.

For experiments which compared the extraction kinetics and recovery of three different types of extractants (10% Triton X-100, methanol and water), identical experimental conditions were used: sample amount =250 mg, extractant volume =50 ml and extraction temperature =40°C. In studying the effect of salt on the extraction of ginsenosides into the surfactant-rich phase and on the preconcentration factor, the following conditions were employed: sample amount =1.5 g, extractant =10% Triton X-100, volume =300 ml, extraction temperature =40°C and extraction time =4 h.

To induce phase separation of the aqueous surfactant solution and the preconcentration of the extracted ginsenosides into the surfactant rich phase, an appropriate amount of the salt was added to the sample solution and was then vigorously shaken for 10 min to dissolve the salt. The sample solution was

Table 1
Uniform design table: experimental conditions and recovery values

Experiment no.	Surfactant (TX−)	Concentration (%)	Volume (ml)	Time (h)	Temperature (°C)	Recovery[a] (%)
1	114	0.5	30	8	20	88
2	100	0.5	5	5	50	75
3	114	0.5	40	3	20	88
4	114	0.5	10	2	20	85
5	100	1	40	1	60	90
6	114	1	20	0.5	20	77
7	100	1	50	8	30	95
8	100	1	20	5	20	97
9	114	5	5	3	20	84
10	100	5	30	2	50	94
11	100	5	5	1	40	87
12	114	5	40	0.5	20	88
13	100	10	10	8	65	96
14	114	10	50	5	20	95
15	114	10	20	3	20	91
16	100	10	50	2	20	96
17	114	20	30	1	20	88
18	114	20	5	0.5	20	87
19	100	20	30	8	40	96
20	114	20	10	5	20	95
21	100	30	40	3	65	97
22	100	30	10	2	60	97
23	114	30	50	1	20	90
24	100	30	20	0.5	30	89

[a] Recoveries were determined for each of the five ginsenosides (Rg1, Re, Rb1, Rc and Rd) for each experiment. These recoveries were summed and the average value for the five ginsenosides (*R*%) was calculated for each of the 24 experiments.

Table 2
Mobile phase gradient program for ginsenoside determination

Time (min)	A (19.5:80.5 (v/v) ACN–H_2O)	B (95:5 (v/v) ACN–H_2O)
0	100	0
40	100	0
80	72.3	27.7
80.1	0	100
100	0	100
100.1	100	0
130	100	0

then kept in a constant temperature bath at 60°C for 5 min. Separation of the cloudy solution into two distinct phases was then achieved via centrifugation for 10 min at 3500 rpm. The preconcentrated ginsenosides were then analyzed by injecting a portion of the surfactant-rich phase directly into the HPLC system.

2.4. Analysis of the extracts by HPLC and UV absorbance detection

The HPLC mobile phase consisted of solvent A: acetonitrile–water (19.5:80.5, v/v) and solvent B: acetonitrile–water (95:5, v/v) mixed according to a linear gradient program as shown in Table 2. From 80 to 100 min of the program, solvent B was used to elute any surfactant remaining in the column. The flow-rate and the detection wavelength were set at 1.3 ml/min and 202 nm, respectively. Peaks in the chromatograms were identified by comparison with retention times of the six ginsenoside standards (Rg1, Re, Rb1, Rc, Rb2 and Rd).

Percentage of recovery (*R*%) as indicated in the UD (Table 1 and Fig. 1) and extraction kinetics (Fig. 2) experiments were determined by comparing the amounts of the various ginsenosides obtained from a single extraction using either aqueous surfactant solution, methanol or water as the extractant to those

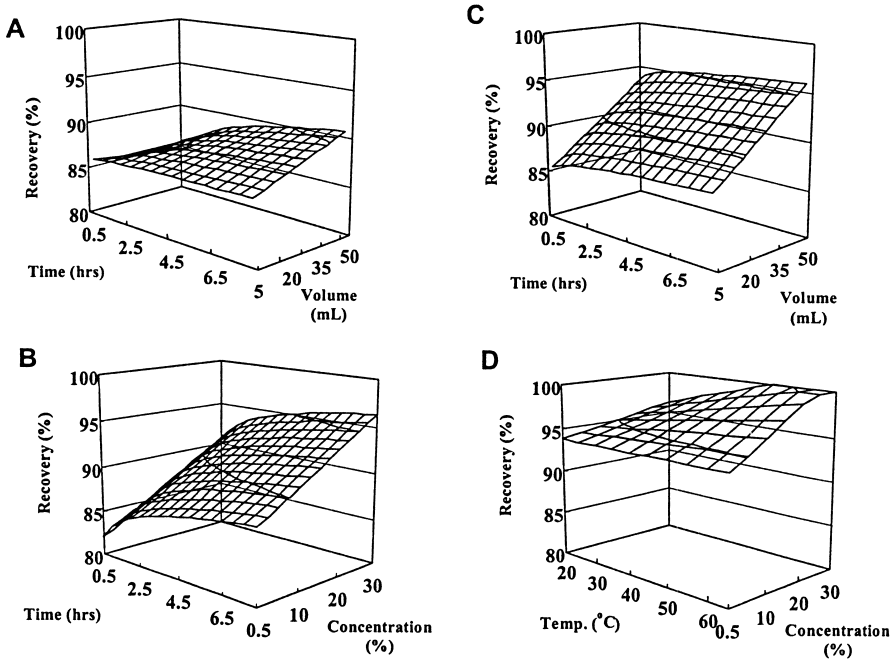

Fig. 1. Plots of percentage of recovery as a function of various experimental variables (extraction time, temperature, extractant concentration and extractant volume) using (A, B) aqueous Triton X-114 and (C, D) Triton X–100 solution as the extraction solvent. (A) Triton X-114 concentration = 10% and extraction temperature = 20°C; (B) volume of aqueous Triton X-114 solution = 50 ml and extraction temperature = 20°C; (C) Triton X-100 concentration = 10% and extraction temperature = 40°C; (D) volume of aqueous Triton X-100 solution = 50 ml and extraction time = 4 h.

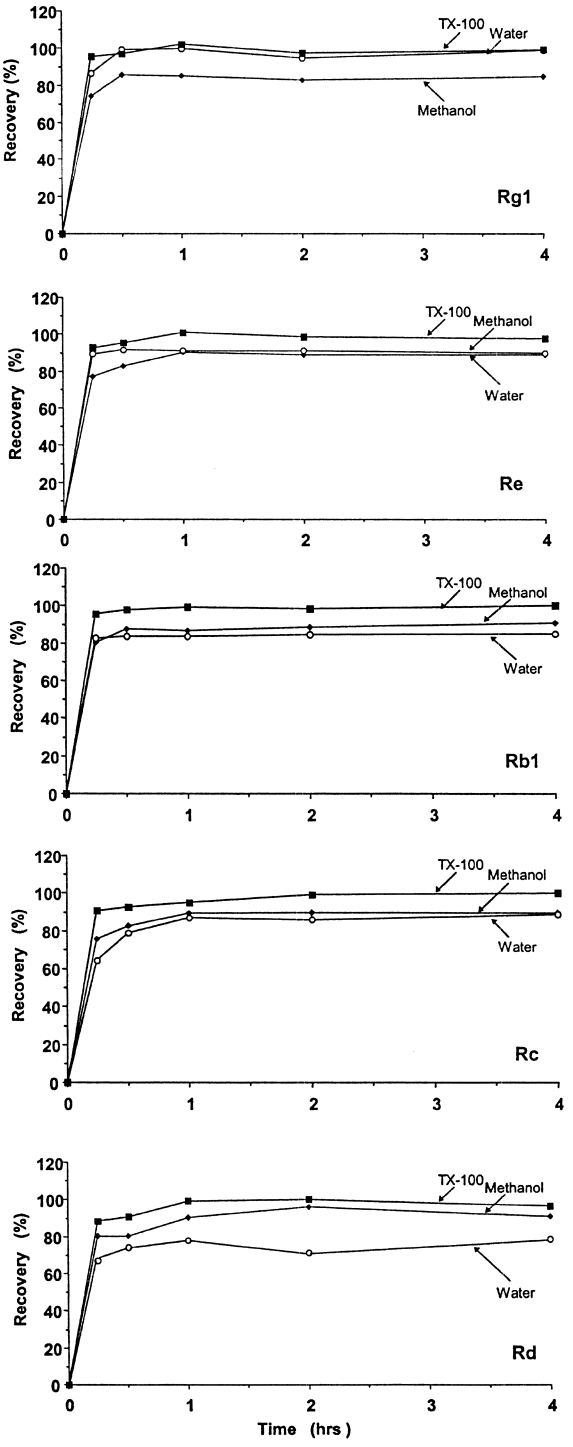

Fig. 2. Comparison of percentage of recovery as a function of time for the five major ginsenosides between three extractants: 10% Triton X-100, methanol and water.

obtained from multiple extractions using methanol as the extractant. Experimental conditions for multiple extractions were as follows: number of extraction =5, volume of methanol per extraction =10 ml, extraction temperature =40°C and time duration per extraction =2 h.

Comparison of the amounts of individual ginsenosides between single and multiple extractions were performed using HPLC peak areas of each of the five ginsenosides. The amounts of ginsenosides obtained from the extract of multiple extractions were considered as reference (i.e., 100% efficiency). Similar experimental procedures were employed for evaluating and validating the recovery of various compounds, including ginsenosides, from crude herb samples [14].

3. Results and discussion

3.1. Optimization of micelle-mediated extraction using experimental design

To the best of our knowledge, only one paper has to date appeared reporting the systematic investigation of the large number of experimental variables (e.g., pH, temperature, surfactant hydrophobicity, ionic strength, analyte concentration, equilibration, centrifugation time, etc.) that affect performances in micelle-mediated extraction [15]. In particular, the use of experimental design methods, which would allow for more efficient/effective optimization of the various experimental variables in micelle-mediated extraction, has never been reported.

Table 1 shows the UD experimental design table employed in the present study to select the optimal micelle-mediated solubilization/purification of ginsenosides from the root of American ginseng (i.e., the first part of the micelle-mediated extraction process). By conducting 24 individual experiments, optimum extraction conditions were determined for five different variables: surfactant type and concentration, volume of the aqueous surfactant solution, extraction time and temperature.

The average recovery of the five major ginsenosides (Rg1, Re, Rb1, Rc and Rd) for each set of the 24 experiments is shown in Table 1, with values ranging from 75 to 97%. The UD data were subject-

ed to multi-linear regression analysis and two linear equations were obtained for Triton X-114 [Eq. (1)] and Triton X-100 [Eq. (2)], respectively. These two equations relate the average recovery of the five ginsenosides to the experimental variables as follows:

$$R(\%) = 83.4 + 0.2835C + 0.009945V(\log t) \quad (1)$$

$$R(\%) = 83.4 + 0.1874V + 0.004302C \times T + 1.539(\log t) \quad (2)$$

where R = the average percent recovery of the five major ginsenosides; C = surfactant concentration; V = volume of the extractant; T = extraction temperature and t = extraction time. The results obtained in Eqs. (1) and (2) both have statistical significance at the 95% confidence level ($P < 0.05$).

Fig. 1A–D show plots of the effects of various experimental variables on the average recovery of the five ginsenosides using data calculated from Eq. (1) (Triton X-114) and Eq. (2) (Triton X-100), respectively. It is clear that all tested variables have a positive effect on the percent recovery, with increasing recovery values obtained for increasing values of each variable. However, the variables C, V and T show a linear relationship with R (%), while t has a logarithmic relationship. The recovery values obtained using Triton X-100 were in general higher than those of Triton X-114 under the same C, V and t conditions, possibly as a result of the use of extraction temperatures higher than 20°C to facilitate the extraction process.

3.2. Comparison of extraction kinetics and recoveries between different extractants

Fig. 2 show the variation in recovery for the five major ginsenosides from the root of American ginseng as a function of extraction time (over a period of 4 h) for three different extractants: 10% Triton X-100 solution, methanol and water. In these tests, the extraction volume and temperature for the three different solvents were identical and were chosen based on the results calculated from Eq. (2) in which a Triton X-100 concentration of 10%, an extraction volume of 50 ml and extraction temperature of 40°C would provide a recovery value of about 95%, after an extraction time of 4 h in the ultrasonic bath.

It is interesting to note from the data shown in Fig. 2 that, except for Rg1, a general trend can be observed for the other four ginsenosides in which aqueous surfactant solution containing 10% Triton X-100 yields the fastest extraction kinetics as well as highest recovery, followed by methanol and then water. These results are not surprising because although methanol is the solvent of choice when compared to water for the extraction of ginsenosides (due to the very high solubility of gisenosides in methanol) [14], the presence of surfactant micelles of Triton X-100 in water typically provides a solubility-enhancing effect which increases the rate of desorption of relatively non-polar organic compounds from solid substrates [16]. For example, certain surfactants can increase the mass-transfer coefficient for pollutant desorption from soil into water, presumably due to better swelling of the soil organic matter and thus more complete diffusion of the solvent into the solid matrix [17,18]. In the case of Rg1, the higher extraction kinetics and recovery obtained for water when compared to methanol is likely due to the fact that Rg1 is the most hydrophilic ginsenoside compared to Re, Rb1, Rc and Rd.

3.3. Addition of a salting out agent to induce CPE of ginsenosides

Although the most common method to induce CPE is by raising the temperature of the sample solution above the cloud point temperature of the surfactant [2,3], Parish and co-workers [19] have shown that the use of ammonium sulfate to induce phase separation allowed the fractionation of both water-insoluble and water-soluble cellular proteins into the surfactant-rich phase. In our laboratory, we have demonstrated that the use of an appropriate salting out agent allowed the CPE of both hydrophobic and hydrophilic molecules of much lower molecular masses (porphyrins and metallopoprhyrins) using aqueous Triton X-100 solution as the extractant [20,21]. In addition, to providing significant improvement in the recovery of the most hydrophilic porphyrin (uroporphyrin), the addition of salt also made it possible for the CPE process to occur at room temperature for Triton X-100, which

has a relatively high cloud point temperature at about 70°C.

After the first part of the micelle-extraction process (i.e., the use of aqueous Triton X-100 solution for the solubilization and purification of ginsenosides), the effect of salt (sodium sulfate) concentrations on the CPE of ginsenosides from the aqueous surfactant solution into the surfactant rich phase was investigated in the present work. Without the addition of salt (i.e., temperature-induced CPE), it was found that the extraction efficiency (E%) for the more hydrophobic ginsenosides (Rb1, Rc and Rd) fell in the range between 90 and 95% and increased to about 100% when the salt amounts exceeded 1.5 g. On the other hand, the E% for the more hydrophilic ginsenosides (Rg1 and Re) was much lower (70–75%) in the absence of salt; but with the addition of increasing amounts of salt into the sample solution, the E% for these two ginsenosides also approached 100% as shown in Fig. 3A.

Without the addition of salt, the average preconcentration factor (PF) determined for the five ginsenosides was quite low (only about 2), whereas the theoretically calculated PF (assuming $E\% = 100$) for a 10% Triton X-100 solution should be about ten. With a salt amount larger than 1.5 g, the average PF was found to increase to about seven, which is relatively close to the theoretically predicted value [22]. These results can be explained by the effect of salt on the volume of the surfactant-rich phase as shown in Fig. 3B, which shows that the surfactant phase volume decreased significantly as a result of increasing the amount of salt added.

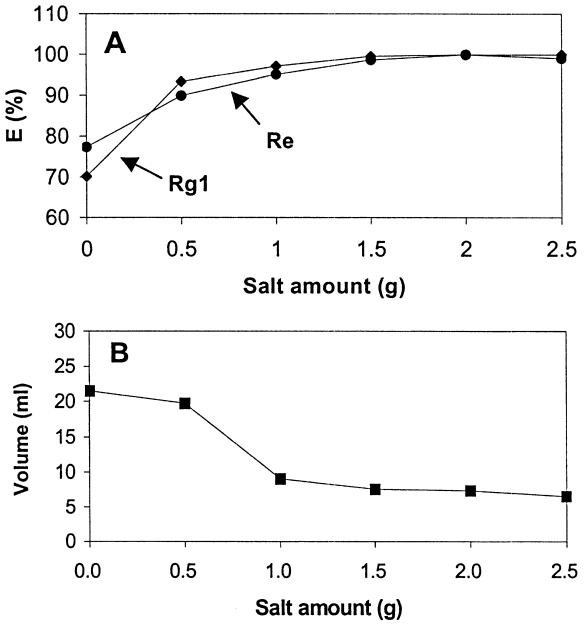

Fig. 3. Effects of the amount of salt (sodium sulfate) on (A) the percentage of ginsenosides (Rg1 and Re) extracted from the bulk aqueous surfactant solution into the surfactant-rich phase (E%) and (B) the volume of the surfactant-rich phase.

It is important to note that with the addition of salt amounts larger than 1.5 g, the volume of the surfactant-rich phase decreased to about 7 ml, which was near the initial volume of Triton X-100 (i.e., about 5 ml) added to 45 ml of aqueous solution (10% Triton X-100, w/v). Therefore, the addition of salt not only promoted the extraction of more hydrophilic ginsenosides into the surfactant-rich phase, but also reduced the overall volume of the surfactant-rich phase, most likely via some type of dehydration mechanism [19–21]. To the best of our knowledge, the effect of salt on the volume of the surfactant-rich phase (phase volume ratio) has never been reported [15].

3.4. HPLC profile of preconcentrated ginsenosides in the surfactant-rich phase

CPE has been employed extensively for the separation and preconcentration of analytes prior to HPLC determinations [4,22,23]. However, relatively high UV absorbances possessed by some commonly available non-ionic surfactants, such as Triton X-100 and X-114, place a limitation on the use of UV absorbance detection when coupling CPE with HPLC, especially for trace analyses.

Fig. 4A shows a HPLC chromatogram of Triton X-100 (blank) present in the surfactant-rich phase. It can be seen that some minor peaks appeared between 20 and 70 min, but the majority of the surfactant components were eluted after 80 min. Thus, an elution window existed between 70 and 80 min.

Fig. 4B shows a chromatogram of preconcentrated ginsenosides present in the surfactant-rich phase using identical CPE conditions as in Fig. 4A. It can be seen that the more non-polar ginsenosides, Rb1, Rc and Rd, appeared at a retention time of about 71,

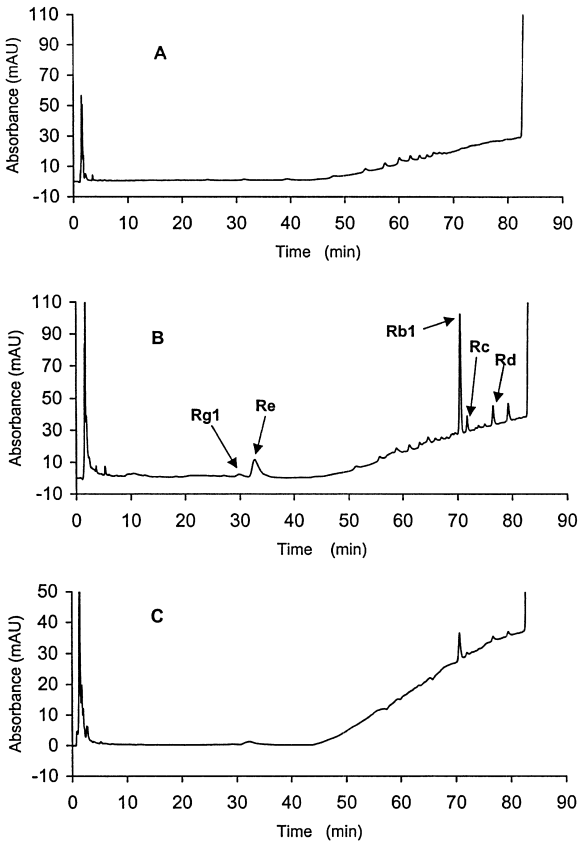

Fig. 4. HPLC chromatograms of the surfactant-rich phase containing (A) Triton X-100 (blank) and (B) Triton X-100 and ginsenosides (preconcentrated sample). In the blank and preconcentrated sample, phase separation was performed by the addition of sodium sulfate into the 10% Triton X-100 solution; (C) chromatogram of the 10% Triton X-100 solution containing the ginsenosides prior to phase separation (non-preconcentrated sample).

72 and 76 min, respectively, with negligible interferences from the blank signals. On the other hand, the more polar ginsenosides, Rg1 and Re, appeared at about 30 and 33 min, respectively. The peak appearing at 80 min arose from an unidentified compound. A minor peak from the blank (Fig. 4A) which appeared at about 33 min happened to overlap the signal from the Re peak; however, this particular interference is minimal when the Re signal is sufficiently large. For comparison, Fig. 4C shows a chromatogram of the aqueous Triton X-100 solution containing the various ginsenosides prior to salt-induced CPE. The increases in peak height/area of the five ginsenosides are shown in Fig. 4B, and when it is compared to Fig. 4C the preconcentration effect of the CPE method is clearly demonstrated. It should be noted that the absorbance scale of Fig. 4B has a sensitivity half that of Fig. 4C, showing an average PF for the five ginsenosides of about seven using a 10% aqueous Triton X-100 solution as the extractant.

4. Conclusions

The present work demonstrated that micelle-mediated extraction/CPE is a potentially powerful tool for the solubilization, purification and/or preconcentration of active ingredients from herbal products. With the aid of an experimental design method such as UD, efficient and rapid extraction of hydrophobic as well as hydrophilic ingredients is possible without needing to use expensive and potentially toxic organic solvents. This capability should be highly valuable in the large-scale purification of herbal products, where the use of aqueous surfactant solutions for extraction is convenient in terms of both cost savings and waste disposal [2,3]. It should be noted that a key step in the purification process would likely to be surfactant removal, which can be carried out by various methods based on exploiting the differences in size, charge and/or hydrophobicity between the surfactant and extracted compounds [24]. For example, a popular method of removing non-ionic surfactants such as Triton X-100 is via hydrophobic adsorption of the surfactants with polystyrene resins [25,26]. The resins are usually added batch-wise to the preparation and removed, together with the bound surfactants, simply by centrifugation or filtration.

It has been recently recognized that polar active ingredients in herbal products (not efficiently extracted by conventional organic solvents) could possess significant pharmaceutical properties [27]. Thus, salt-induced CPE represents an effective tool for the preconcentration of both polar and non-polar compounds prior to determination by chromatography or other techniques for research and/or quality control purposes. To minimize absorbance detection problems inherent in conventional non-ionic surfactants such as Triton X-100, other less commonly used CPE surfactants, including non-ionic [15] as

well as ionic surfactants [28,29] which are not UV active, could be employed. Also, other detection methods, such as electrochemical and fluorescence, have been used for the sensitive and selective detection of various analytes in CPE–HPLC analyses [4,22].

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