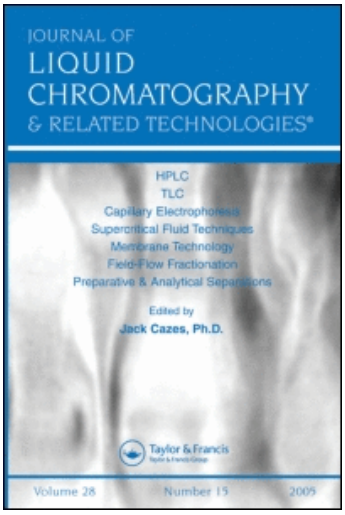

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Use of Solid-Phase Extraction for Improving HPLC Assays of Triterpene Glycosides in Gotu Kola (*Centella asiatica*), Parthenolide in Feverfew (*Tanacetum parthenium*), and Valerenic Acids in Valerian (*Valeriana officinalis*)

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Abstract: The use of solid-phase extraction (SPE) for solving interference problems observed in simple HPLC assays involving nutraceuticals is demonstrated. Anion-exchange SPE is used to remove an interfering compound coeluting with the triterpene glycoside madecassoside in gotu kola (*Centella asiatica*). C_{18} SPE is used to remove an interfering compound coeluting with parthenolide in feverfew (*Tanacetum parthenium*), and as a general cleanup for valerenic acids in valerian (*Valeriana officinalis*). In each case, use of a single ultrasonically assisted extraction was verified by comparing the results with multiple extractions.

Keywords: Solid-phase extraction, HPLC assay, gotu kola, parthenolide, valerenic acids

INTRODUCTION

Although the 1994 Dietary Supplement, Health, and Education Act restricts FDA's oversight for nutraceuticals, well-established companies desiring to provide consistent, high-quality products still seek the development of suitable analytical methods for the assumed therapeutically active substances

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present in each preparation. This desire for "standardization" has resulted in the development of many HPLC methods for the more popular herbal remedies. The Method Validation Program sponsored by the Institute of Nutraceutical Advancement has provided HPLC and GC methods on the Internet for a variety of nutraceuticals.[1] Many more methods for the different nutraceuticals exist in the literature. These herbal medicines, however, present complex matrices that can vary greatly from lot to lot depending on the usual differences in growing conditions, soil content, time and place of harvest, etc. As a result, established methods may suddenly falter when examining a new lot of the same herbal medicine. The point of this paper is to demonstrate that judicious application of solid-phase extraction (SPE) can readily solve some of these problems without resorting to eluent, gradient, or column changes that may not work for the next lot. Three examples are given for initially simple extractions and HPLC analysis: madecassoside and asiaticoside in the chronic venous disease treatment gotu kola (*Centella asiatica*), parthenolide in the antimigrain feverfew (*Tanacetum parthenium*), and valerenic acids in the sleep aid valerian (*Valeriana officinalis*).

The method development strategy was similar in all three cases. First, an appropriate HPLC separation for the commonly assayed active compounds was found in the literature. Then, attempts were made to find the extraction solvent that yielded the greatest amount of extracted analyte in a single ultrasonically assisted extraction. With this solvent, the technique of Anderson and Burney[2] was used to demonstrate that the analyte could be successfully extracted with a single ultrasonically assisted extraction. This consisted of a sample size study followed by a multiple extraction study, as described in Experimental section. Finally, SPE was employed when interference problems arose.

For gotu kola, the HPLC procedure of Inamdar et al.[3] was used except that the HPLC time was shortened by using a shorter column and gradient, since just the determination of the triterpene glycosides, madecassoside, and asiaticoside was sought. Asiatic and madecassic acids were not readily available commercially at the time of development. A simpler extraction procedure was also used. A related procedure[4] was described in a *C. asiatica* review article.[5]

For feverfew, the HPLC system for the determination of parthenolide was based on that found in a Phenomenex Co. (Torrance, CA) application note and also used by Zhou et al.[6] who demonstrated the efficacy of a very simple extraction procedure.

For valerian, an isocratic HPLC system for hydroxyvalerenic, acetoxyvalerenic, and valerenic acids based on that presented in another Phenomenex application note was used. An alternative gradient system for valerenic acids, in addition to valepotriates and baldrinols,[7] is described in a review chapter[8] using a relatively simple extraction procedure.

EXPERIMENTAL

Reagents and Chemicals

Madecassoside and asiaticoside were obtained from Indofine Chemical Co, Somerville, NJ. The former was found to be 97.6% pure, while the latter was 98.2% pure, as determined by area percentage at 210 nm. Gotu kola powder was obtained from Botanical International, Germantown, MD. Parthenolide was obtained from Chromadex, Laguna Hills, CA and was found to be 96.4% pure by area percentage at 215 nm. Feverfew herb powder was obtained from Starwest Botanicals Inc., Rancho Cardova, CA. Valerenic acid was obtained from Chromadex and was found to be 99.4% pure by area percentage at 225 nm. Hydroxyvalerenic, acetoxyvalerenic, and valerenic acids in the root powder were determined versus this standard. Valerian root powder was obtained from Starwest Botanicals Inc. All other reagents were HPLC or ACS quality, and were used without further purification.

Apparatus

Either Varian Bond Elute, Bakerbond or Strata C_{18} SPE columns and Bakerbond or Strata Quaternary Amine anion-exchange SPE columns, all with a 3 cc, 500 mg bed mass were used with a Burdick and Jackson vacuum manifold. All of the columns were conditioned with 2 mL of methanol followed by 2 mL of water prior to use. For some studies, a Bellco Sci Era Hot Shaker water bath set at 50°C was used.

HPLC System

For all three herbs, the HPLC used was a Waters Model 2690 Separations Module with a Model 996 Photodiode Array Detector and a 150 × 4.6 mm, 5 μm Phenomenex Luna C_{18} column. The system was operated with the Millenium32 Chromatography Manager Version 3.00 software. For gotu kola, the 0.3% aqueous phosphoric acid/acetonitrile gradient was as follows: 20–30% acetonitrile in 10 min, changed to 55% acetonitrile in 5 min, returned to 20% acetonitrile in 1 min, and held there for 4 min. The flow rate was 1.4 mL/min with a column temperature of 35°C, an injection volume of 20 μL, and a monitoring wavelength of 210 nm. For feverfew, the eluent was isocratic at 45:55 acetonitrile:water with a 1.5 mL/min flow rate at ambient column temperature, an injection volume of 20 μL, and a monitoring wavelength of 210 nm. For valerian, the eluent was isocratic at 65:35 acetonitrile:aqueous 0.3% phosphoric acid with a 1.5 mL/min flow rate, 25°C column temperature, an injection volume of 20 μL, and a monitoring wavelength of 225 nm.

Preparation of Standard Solutions

One milligram quantities of madecassoside and asiaticoside were accurately weighed into a 5 mL volumetric flask. The solutes were dissolved in 90:10 methanol:water, made up to volume, and serially diluted 2.0:5.0 three times to yield concentrations ranging from 10 to 200 μg/mL. The solutions were found to be stable for at least 10 weeks stored at −20°C. An accurately weighed 1 mg quantity of parthenolide was dissolved in 10.0 mL of methanol and similarly serially diluted to yield concentrations ranging from 5 to 100 μg/mL. The solutions were found to be stable for at least 3 weeks stored at −20°C. Valerenic acid standard solutions were prepared in a similar manner and range as that of parthenolide, except that the solvent was the final extraction solvent (vide infra). The solutions were found to be stable for at least 12 weeks stored at −20°C. Linearity data for all the standards over the aforementioned or a wider range is presented in Table 1.

Initial Investigation of Sample Preparation

For feverfew, a solvent study was first performed by comparing assay values of each sample extracted ultrasonically for 30 min with 20.0 mL of methanol:-water solvents, in ratios ranging from 50:50 to 100:0 at ambient temperature. The study was done in three ways: directly without SPE, by passing 2.0 mL of

Table 1. Linearity of gotu kola, feverfew, and valerian standards

Parameter	Madecassoside	Asiaticoside	Parthenolide	Valerenic acid
Range (μg/mL)	10–200	10–200	7–180	1–90
Wavelength (nm)	210	210	210	225
Correlation coefficient	0.999996	0.999993	0.999994	0.999998
Slope, area units (mL/μg)	2,435	2,261	29,759	42,026
Intercept, area units	−421	−494	4,694	2,765
Standard error of the estimate ($S_{y/x}$), area units	681	935	8,831	937
% Intercept[a]	−0.2	−0.2	0.3	0.2
% Variation[b]	0.3	0.5	0.5	0.2

[a](y intercept/$\bar{y}$) × 100, where $\bar{y}$ is the average y[12].
[b]($S_{y/x}/\bar{y}$) × 100.

the sample through a C_{18} SPE column, or by passing 2.0 mL through an anion-exchange SPE column. The latter two effluents were collected in separate 5 mL volumetric flasks. The columns were then washed with 2 mL 30:70 methanol:water, and the total effluent, both load and wash, diluted to 5.0 mL. In addition, a separate data point was obtained by eluting the C_{18} SPE column loaded with the 50:50 methanol:water extraction solvent with 3 mL of methanol and diluting the effluent to 5.0 mL. For valerian, the solvent study was done at ambient temperature with the final SPE procedure described subsequently. For gotu kola, the 90:10 methanol:water solvent employed by Inamdar[3] was used with the appropriate SPE procedure also described subsequently.

Then, for all three herbs, the two-part procedure of Anderson and Burney[2] for validating the efficiency of simple extractions of analytes from herb samples was utilized. It consisted of a sample size study followed by a repeated extraction study. In the first part, the maximum sample weight that could be extracted ultrasonically with 20.0 mL of the selected aqueous methanol solvent without a decrease in assay results was determined. In the second part, single extraction results using the optimum weight were compared with multiple extraction results to estimate percentage extracted (or recovered) in the single extraction. The multiple extractions were commenced by transferring an accurately weighed quantity of sample to a 50 mL centrifuge tube. About 20 mL of extraction solvent was added to the tube and the tube placed in a sonicator for about 30 min. The tube was centrifuged and as much of the supernatant as possible was quantitatively transferred with extraction solvent washings to a 100 mL volumetric flask. The residue was broken-up with a spatula and an additional 20 mL of extraction solvent was added, washing off the spatula. The tube was then sonicated again for 30 min. This procedure was repeated until the sample in the tube had been extracted four times, with all of the extractions collected in the 100 mL volumetric flask, which was then diluted to volume with extraction solvent.

Results obtained by the two extraction procedures were compared using one-way analysis of variance (ANOVA) as calculated with the ORIGIN 6.0 graphic and statistical software package (Microcal, Northampton, MA). Significance of differences between mean values was assessed at the 95% confidence level. The entire single and multiple extraction results for feverfew were compared with conventional Soxhlet extraction results obtained with methanol on 0.5 g samples.

Final Procedure

For gotu kola, a 0.5 g sample was weighed into a 50 mL Erlenmeyer flask, 20.0 mL of 90:10 methanol:water extraction solvent added, and the resulting

solution put in the shaker bath at 50°C for 1 hr. A 2.0 mL aliquot of the cooled, clarified by centrifugation solution was passed through an anion-exchange SPE column, followed by 2 mL of the 90:10 methanol:water extraction solvent. Both the load and the wash were collected in the same 5 mL volumetric flask, which was subsequently diluted to volume with extraction solvent. For one sample, the washed anion-exchange SPE column was eluted with the strong solvent 50:50 methanol:aqueous 0.2 M H_3PO_4[9,10] into a 5 mL volumetric flask, diluted to volume, and examined chromatographically.

For feverfew, a 0.5 g sample was weighed into a 50 mL Erlenmeyer flask, 20.0 mL of 50:50 methanol:water added, and the resulting solution placed in an ultrasonic bath for 30 min. A 2.0 mL aliquot of the cooled, clarified by centrifugation solution was loaded on a C_{18} SPE column. The column was washed with 2 mL 30:70 methanol:water, discarding both the load and the wash solutions. About 3 mL of methanol eluting solvent was added to the column, collecting the effluent in a 5 mL volumetric flask, which was subsequently diluted to volume with methanol.

For valerian, the feverfew procedure was repeated, except that the extraction solvent was 70:30 methanol:water and the SPE elution solvent was 90:10 acetonitrile:water.

RESULTS AND DISCUSSION

Gotu Kola

Examination of the PDA on-the-fly spectrum of madecassoside in the sample chromatogram, obtained after direct extraction of the sample without SPE, yielded a small absorption band with a maximum at about 327 nm that was not present in the standard, as shown in Figure 1. Since the shape of the spectrum band was reminiscent of that observed for some of the caffeoyl tartaric and caffeic acids found in *Echinacea*,[11] the attempt at removal of the interference by anion-exchange SPE was formulated. Figure 2 shows chromatograms without (chromatogram A) and with (chromatogram B) the anion-exchange SPE step. (The former was diluted 2.0–5.0 to yield a concentration commensurate with that obtained from the SPE-treated sample.) The madecassoside spectrum obtained from the SPE-treated sample resembled that of the standard. When the anion-exchange SPE column was eluted with a strong solvent, the resulting solution yielded chromatogram C. This contained the major peaks removed by the SPE step, including the potential interference for madecassoside that absorbs at 327 nm.

Examination of a sample size study indicated an optimum size of 0.25 g at room temperature and 0.5 g at 50°C. Assay values for madecassoside decreased several % at higher sample weights.

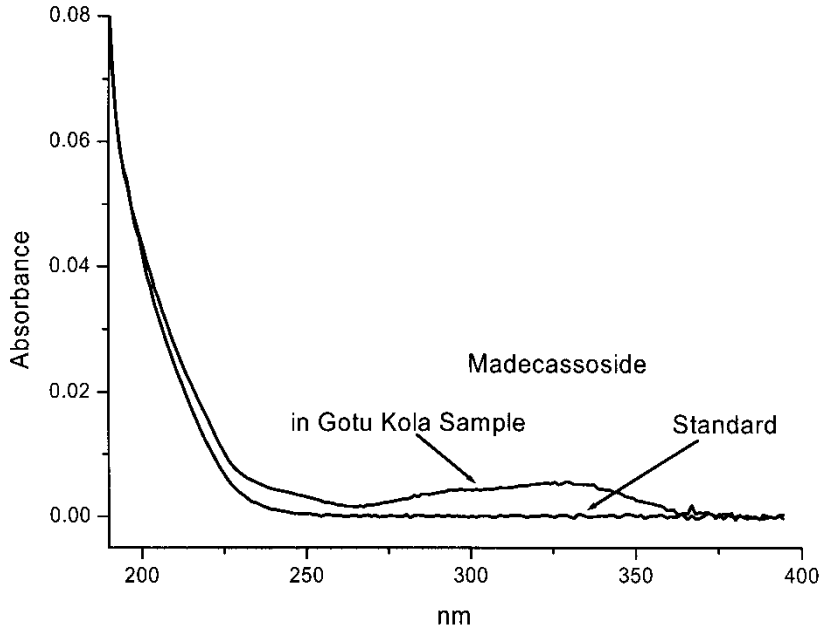

Figure 1. PDA spectra of madecassoside in gotu kola sample and standard.

A comparison of six multiple extractions with six single extractions yielded the results for madecassoside and asiaticoside listed in Table 2. The *F*-test indicated no difference between single and multiple extractions. Assay of the herb sample with and without the SPE procedure indicated the interference would have led to a 10% high bias for madecassoside.

Feverfew

Coincidentally, examination of an on-the-fly PDA spectrum of parthenolide in the feverfew sample, extracted without SPE, yielded a spectrum with a band at about 327 nm similar to the interference found for madecassoside in gotu kola herb samples, as shown in Figure 3. The solvent study revealed that not using an SPE procedure would result in a high bias, as shown in Figure 4. Passing the sample solution through a C_{18} SPE column with 100% methanol as the solvent, through an anion-exchange SPE column with any of the solvents studied, or eluting the C_{18} column loaded with 50:50 methanol:water with 100% methanol, all yielded consistent results comparing reasonably well with 6 hr methanol Soxhlet extractions (Table 2). For reasons of economy, the final procedure chosen was elution of the C_{18} SPE column loaded with

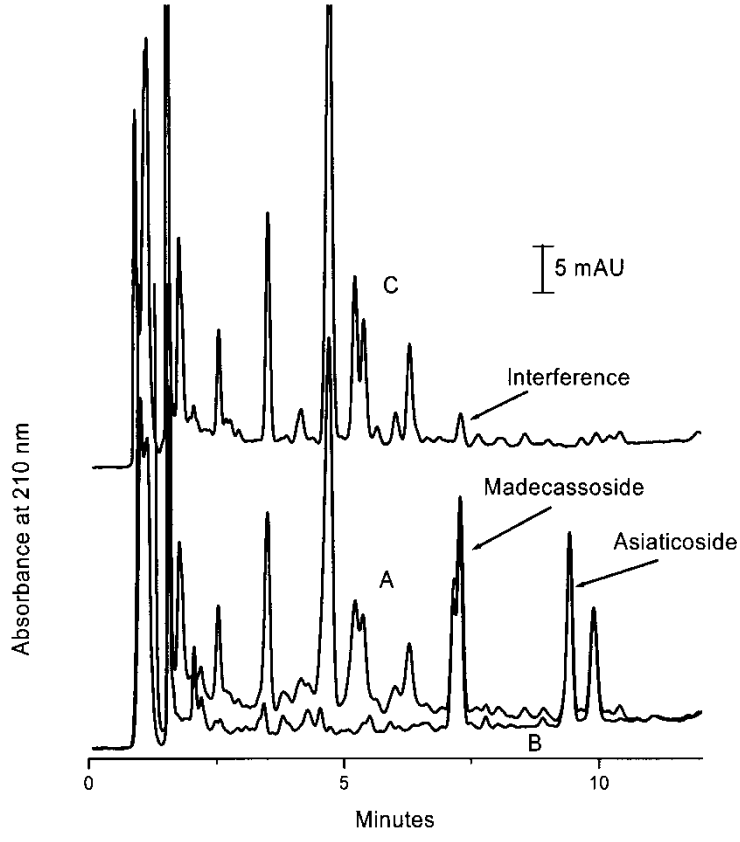

Figure 2. Chromatograms of a gotu kola sample without SPE (A), with anion-exchange SPE (B), and after eluting the anion-exchange SPE column (C). Madecassoside typically yields a doublet peak.

Table 2. Assay values (mg/g), single versus multiple extractions, $n = 6$ for each herb

Extractions	Madecassoside	Asiaticoside	Feverfew[a]
Single	10.1 ± 0.3 (3.0%)	7.2 ± 0.2 (3.0%)	2.83 ± 0.04 (1.4%)
Multiple	10.2 ± 0.4 (3.9%)	7.6 ± 0.3 (3.4%)	2.87 ± 0.03 (0.9%)
	Hydroxyvalerenic acid	Acetoxyvalerenic acid	Valerenic acid
Single	0.058 ± 0.005 (9.2%)	0.94 ± 0.04 (4.3%)	0.54 ± 0.02 (4.1%)
Multiple	0.060 ± 0.006 (9.3%)	0.92 ± 0.04 (4.1%)	0.54 ± 0.03 (4.9%)

[a]For Soxhlet extraction, $n = 4$, the assay is 2.93 ± 0.05 (1.6%).

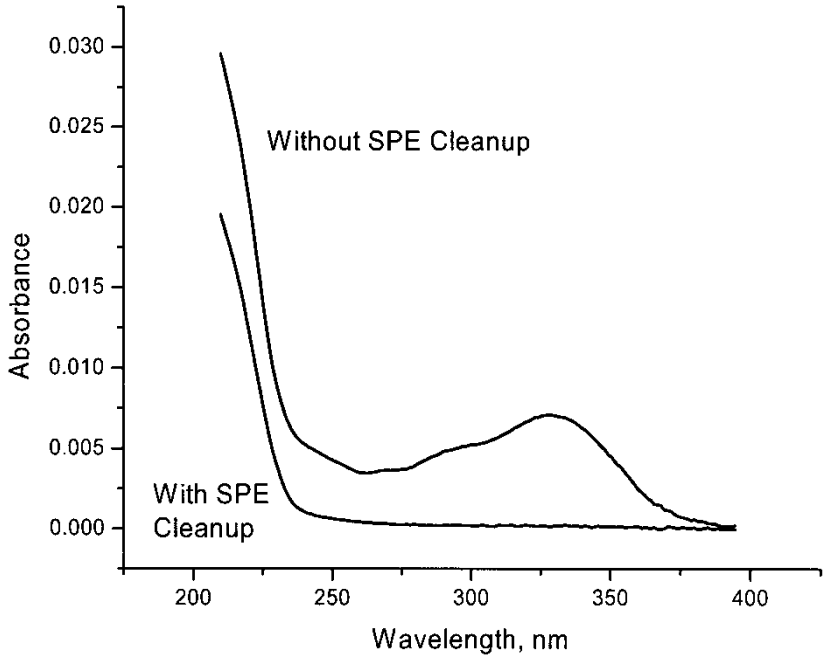

Figure 3. PDA spectra of parthenolide in feverfew sample with and without SPE cleanup.

the 50:50 methanol:water extraction solvent. The final parthenolide peak in the sample yielded a spectrum similar to that of the standard. The chromatogram was substantially cleaned-up (Figure 5).

A sample size study yielded no loss in relative response out to 0.5 g, with a 5% decrease at 1 g. A 0.5 g sample size was chosen.

A comparison of six multiple extractions with six single extractions yielded the results for parthenolide listed in Table 2. The *F*-test indicated no difference between single and multiple extractions.

Valerian

In the case of valerian, preliminary work doing solvent extraction with 70:30 methanol:water without SPE did not indicate any spectral impurities in the valerenic acids. An irreversible decrease in retention time from injection to injection was noted, however, indicating column degradation from dirty sample solutions. Loading of 2 mL of the sample solution (in 70:30 methanol:-water) on an anion-exchange SPE column and eluting with 50:50 methanol:0.2 M aqueous H_3PO_4, similar to a procedure for phenolic acids in *Echinacea*,[9,10] yielded poor recoveries and an incomplete mass balance.

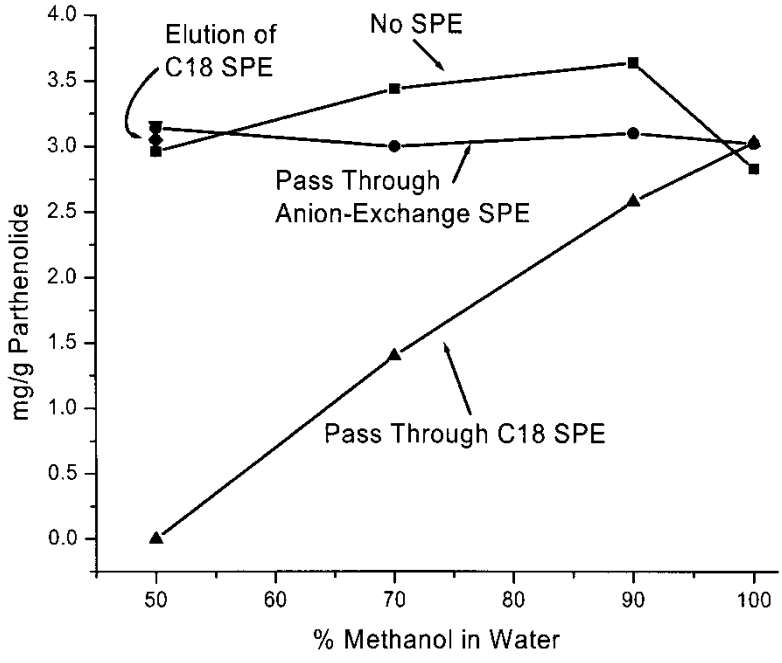

Figure 4. Solvent study for parthenolide extraction from feverfew.

Loading the same sample onto a C_{18} SPE column and eluting with 3 mL of 90:10 acetonitrile:water yielded essentially 100% recovery and a cleaned-up chromatogram, as shown in Figure 6. A solvent study indicated consistent assay results for acetoxy and hydroxyvalerenic acids over the range 50:50–90:10

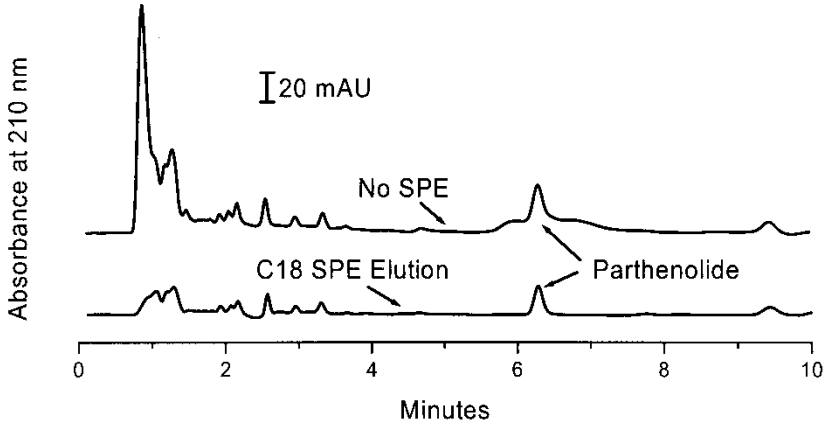

Figure 5. Chromatograms of a feverfew sample with and without SPE.

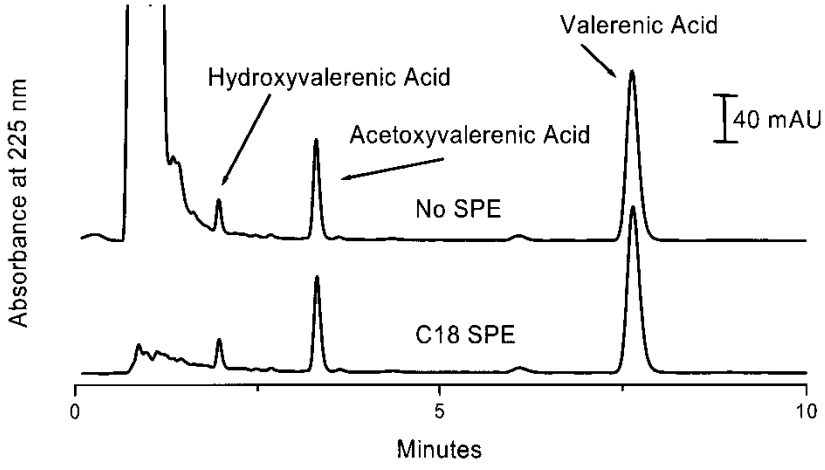

Figure 6. Chromatograms of a valerian sample with and without SPE.

methanol:water as the extraction solvents. For valerenic acid, a 4% decrease in assay results occurred when the solvent strength was reduced from 70:30 to 50:50 methanol:water. A sample size study indicated only a few percentage decrease in assay value as the size was increased beyond 1.0 g. A 0.5 g sample size was chosen.

A comparison of six multiple extractions with six single extractions yielded the results for the three valerenic acids listed in Table 2. The *F*-test indicated no difference between single and multiple extractions.

CONCLUSIONS

The judicious use of SPE can obviate the need for time-consuming eluent, gradient, or column changes when attempting to solve interference problems in nutraceutical assays. When used as a digital technique (either completely retaining or not retaining the analyte), SPE can greatly contribute to the robustness of a method. As in the three cases discussed in this paper, recovery through the columns can be virtually 100%, eliminating many impurities that can interfere with an analyte or accumulate and degrade an analytical column. Indeed, after use, the SPE column is normally highly colored with impurities that would otherwise contaminate the analytical column. In another application, using an *Echinacea* method involving an SPE extraction of 1 g samples, over 970 injections were made (excluding standards) over a period of 14 months on a guard-free analytical C_{18} column with no appreciable increase in pressure, loss of efficiency, or change in retention times.[10]

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