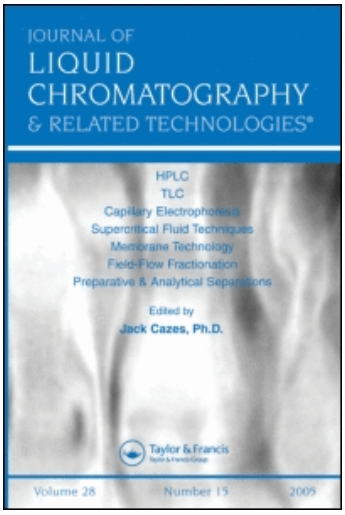

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A RAPID METHOD FOR DETERMINATION OF VITAMINS D_2 AND D_3 IN PHARMACEUTICAL PREPARATIONS BY HPLC

K. Sarioglu[a]; S. S. Celebi[b]; M. Mutlu[a]
[a] Department of Food Engineering, Hacettepe University, Ankara, Turkey [b] Department of Chemical Engineering, Hacettepe University, Ankara, Turkey

Online publication date: 30 April 2001

To cite this Article Sarioglu, K. , Celebi, S. S. and Mutlu, M.(2001) 'A RAPID METHOD FOR DETERMINATION OF VITAMINS D_2 AND D_3 IN PHARMACEUTICAL PREPARATIONS BY HPLC', Journal of Liquid Chromatography & Related Technologies, 24: 7, 973 — 982
To link to this Article: DOI: 10.1081/JLC-100103423
URL: http://dx.doi.org/10.1081/JLC-100103423

J. LIQ. CHROM. & REL. TECHNOL., 24(7), 973–982 (2001)

A RAPID METHOD FOR DETERMINATION OF VITAMINS D_2 AND D_3 IN PHARMACEUTICAL PREPARATIONS BY HPLC

K. Sarioglu,[1] S. S. Celebi,[2] and M. Mutlu[1,]*

[1] Department of Food Engineering,
[2] Department of Chemical Engineering, Hacettepe University, 06532 Ankara, Turkey

ABSTRACT

In this study, a rapid, simple, and economical reversed phase liquid chromatographic method was described for the determination of vitamin D_2 and vitamin D_3 in pharmaceutical preparations. The pharmaceutical formula of vitamin D_2 is prepared by mixing with filling material, and that of vitamin D_3 is prepared by dissolving in herbal oil. Before injection to HPLC the vitamin D_2 sample was extracted with diethyl ether and the extract was cleaned by centrifugation; the vitamin D_3 sample was prepared by dissolving it in an appropriate amount of tetrahydrofuran (THF).

The vitamin content of vitamin D_2 and vitamin D_3 samples was determined by reversed phase liquid chromatography. Ultra Violet-Visible (UV-VIS) detector and C-18 column were used for this purpose. Vitamin D_2 and impurities were completely resolved by the mixture of methanol-acetonitrile as mobile phase with a flow rate of 1.0 mL/min.

* Corresponding author. E-mail: gmehmet@eti.cc.hun.edu.tr

The best resolution of vitamin D_3 from interferences could be achieved by the gradient mixture of methanol and THF as the mobile phase at 1.0 mL/min flow rate.

Mean recoveries of vitamin D_2 ranged from 99.65% to 105.48% with an overall mean of 102.54% for different spiking levels. The limits of detection for vitamin D_2 and vitamin D_3 in their preparation were found to be <0.43 μg/g and <1.02 μg/mL respectively.

INTRODUCTION

Vitamin D_3 and D_2 are very important fat soluble vitamins for human and animal diets. For this reason, they take part in many pharmaceutical preparations, foods, and feed formulation.[1] Due to the biochemical activity of the vitamins, their concentrations in the preparation are always very low. For this reason, a specific and sensitive method was essential for determination of vitamin D_2 and D_3.

Many methods (chromatographic and other techniques) have been developed and are described in the literature.[3–7,9] In the last decade, high performance liquid chromatography (HPLC) was announced as the most suitable technique for determination of trace organic components.[1,3] The main sample was human blood plasma in the majority of these studies.[5,6] Some chromatographic methods were also developed for vitamin D and cholesterol in food and infant formulas.[2]

Most of these methods includes complex stages and long times. Such samples were firstly saponified and then the unsaponified fraction was extracted with an organic solvent.[2,8,10] Finally, the extract was cleaned with solid phase extraction cartridges. These methods were widely used for separation of lipid soluble vitamins from a sample.

In this study, we described a rapid and straightforward method for the determination of vitamin D_2 and vitamin D_3 in their commercial pharmaceutical preparation without purification. The described method could also be applied for determination of other vitamin D derivatives, fat soluble vitamins, and sterols, after extraction from their samples.

EXPERIMENTAL

Equipment

A Shimadzu LC-10AED liquid chromatograph was used. It contained a Rheodyne model 7161 injection loop with 20 μL injection volume. Detection was achieved with a Shimadzu SPD-10A UV-VIS detector at 265 nm. A Shimadzu C-R6A Chromatopac model integrator was used with a 1 cm/min chart speed to record the resulting chromatograms.

Column

The column was a Hicrom C_{18} stainless steel, 250 × 4.6 mm (id); it was filled with 5 μm diameter octadecyl reverse phase packing material.

Solvents and Reagents

All solvents were analytical grade and anhydrous. Tetrahydrofuran (THF), and methanol and acetonitrile were HPLC grade. Tetrahydrofuran was supplied from Lab-Scan Analytical Science (Ireland). Methanol and acetonitrile were supplied from J.T. Baker (Holland). Extra pure diethyl ether was used as an extraction solvent. The diethyl ether was obtained from Merck (Germany).

Vitamin D_3 and vitamin D_2 standards were obtained from Sigma (USA) and stored at +4°C until used.

Method

Preparation of Vitamin D_3 and D_2 Standard Solution

Accurately weigh a 0.1 mg Vitamin D_3 (Cholecalciferol) in a 20 mL flask and dissolve in 10 mL THF without heating. All other standard solutions of vitamin D_3 were prepared from this stock solution by diluting with THF in the further stages of study. Vitamin D_2 (Ergocalciferol) standard solution was prepared similar to vitamin D_3 solution. 0.0132 mg vitamin D_2 was accurately weighed in a 20 mL flask and dissolved in 10 mL THF.

Calibration curves were prepared by plotting vitamin concentration (μg/mL) vs. peak area which is shown in Figure 1. For this purpose 2.02, 4.04, 6.06, 8.08, and 10.10 μg/mL vitamin D_3 and 0.65, 1.30, 1.95, and 5.58 μg/mL vitamin D_2 standard solutions were prepared by diluting the stock solutions with THF. These standards were injected into the column, respectively, and peak areas were read from the integrator. Chromatograms of standard solution of vitamin D_2 and D_3 were demonstrated in Figure 2 and Figure 3, respectively.

Determination of Extraction Yield

Particularly, when handling solid samples, extraction yield was important for accuracy of the described method. For this reason, extraction was preceeded by a number of steps. In this study, the vitamin D_2 sample was

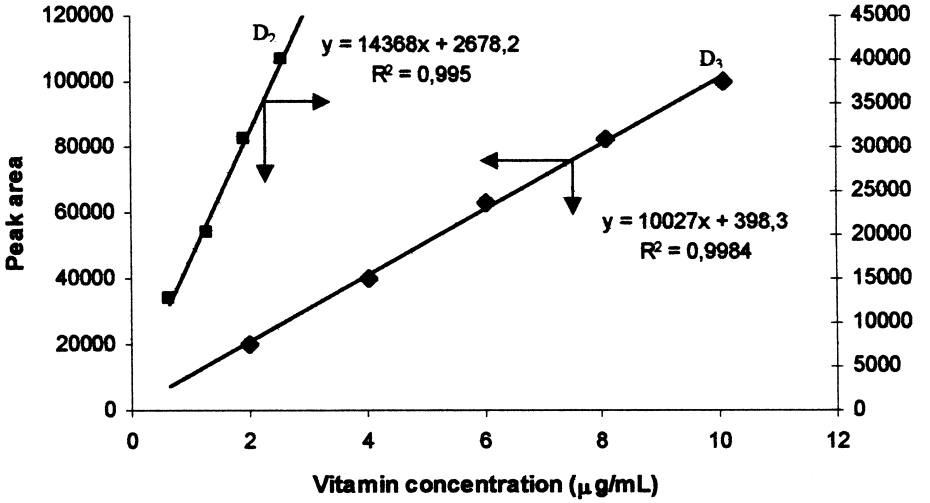

Figure 1. Calibration graphs of vitamin D_2 and D_3.

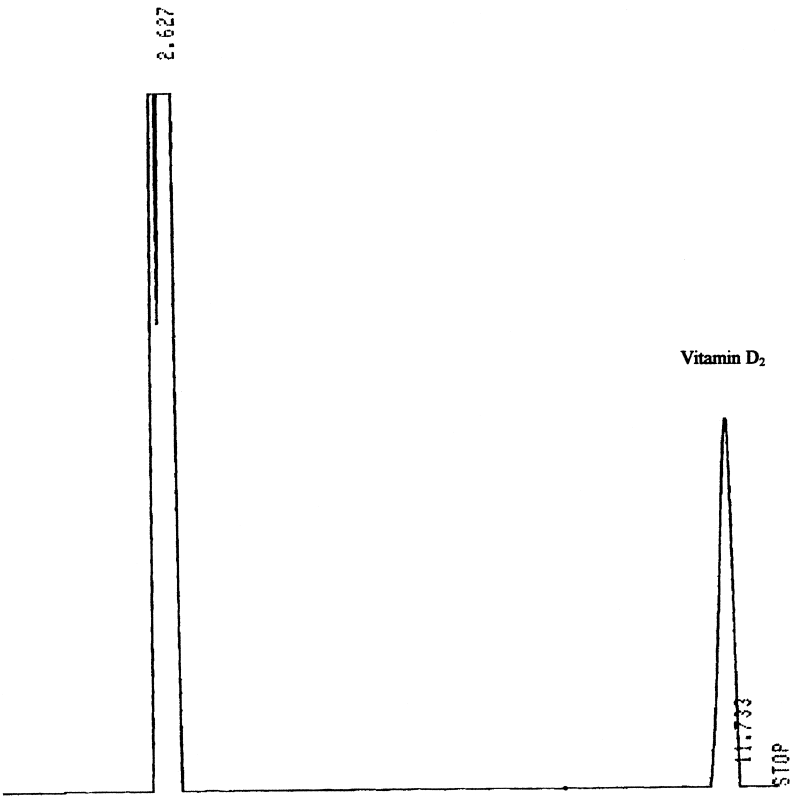

Figure 2. Chromatograms of 2.58 µg/mL vitamin D_2 standard solution.

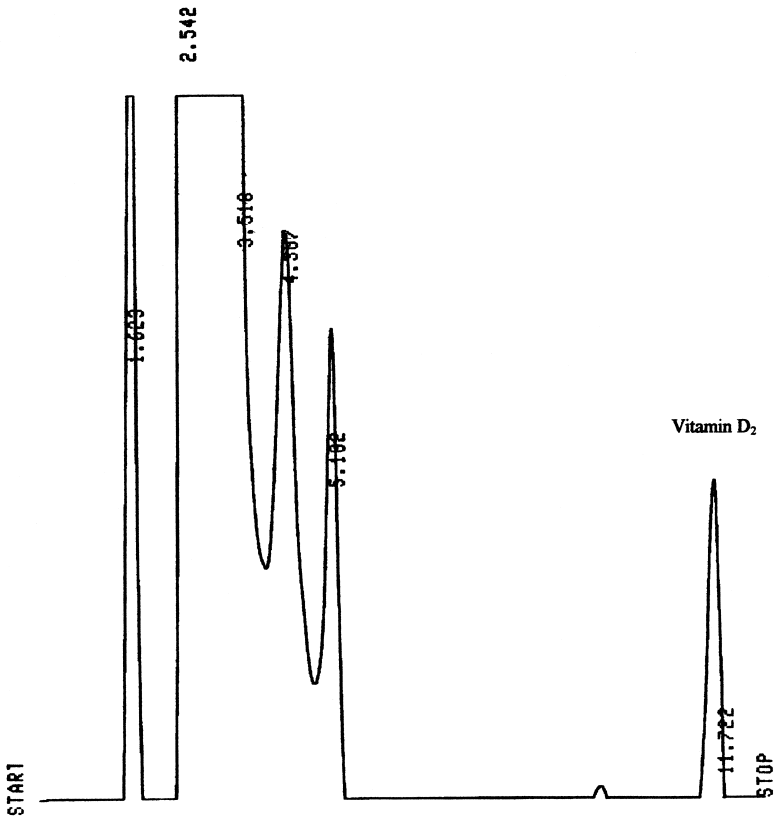

Figure 3. Chromatograms of vitamin D_2 extract.

extracted in four steps. We accurately weighed a 0.6 g homogenized sample in a 20 mL flask and extracted it with 10 mL extra pure diethyl ether in four steps. Each step was analysed by HPLC. 98.10% Vitamin D_2 was recovered in the first and second step. Therefore, a two-step extraction was enough for the sample preparation.

The recovery test was carried out parallel to extraction. For this purpose, a proper amount of vitamin D_2 standard (10.56, 7.92, 5.58, 2.64 μg) was added to the pharmaceutical preparation containing 35.04 μg/g of vitamin D_2. The samples were perpetrated and analysed with HPLC as described below. Recovery percentages were determined to be between 105.48% and 99.65% as shown in Table 1. Because of it being in liquid form, extraction or the recovery test was not necessary for the vitamin D_3 sample. The samples were prepared by directly diluting with THF.

Table 1. Recovery Test of Vitamin D_2 from the Solid Sample

Added Amount of Vitamin D_2 (μg)	Average Amount of Recovered Vitamin D_2 (μg)	Average Percentages of Recovered Vitamin D_2
10.56	11.14 ± 0.96	105.48 ± 9.09
7.92	8.03 ± 0.98	101.40 ± 12.32
5.28	5.47 ± 0.34	103.66 ± 6.49
2.64	2.63 ± 0.41	99.65 ± 15.58

Preparation of Samples

In this study we worked with two kinds of samples. Vitamin D_2 is generally located in the solid system, therefore, the priority of the analysis is to extract the vitamin D_2 with a suitable organic solvent. Several solvents were used for extraction, but the clearest extract was obtained with diethyl ether. Accurately weigh a 0.6 g homogenised sample in a 20 mL flask. Add 10.0 mL pure diethyl ether into the sample and vigorously shake for 10 minutes. Then the solvent-solid mixture was centrifuged at 2500 rpm for 10 minutes.

The decantate was taken, with care, into a 30 mL flask. Extraction was completed in two stages. Total extract volume is 20 mL at the end of extraction steps. Diethyl ether was evaporated with purging nitrogen. The remaining residue was dissolved with 4.0 mL THF.

Preparation of the vitamin D_3 sample is very simple compared to vitamin D_2 sample. For this purpose, accurately measure 1 mL Vitamin D_3 preparation into a 20 mL vial and dilute with 10.0 mL THF. All standard and sample solutions were store at + 4°C before analysis.

Mobile Phases

A mixture of methanol-acetonitrile (40% + 60% v/v) solution was used as a mobile phase for the analyses of vitamin D_2. The rate of mobile phase was set at 1.0 mL/min. The Vitamin D_2 extract was quite clear and did not contain any impurity, which may interfere with the analyte. For this reason, there is no separation problem in vitamin D_2 analyses and the isocratic mobile phase composition of methanol and acetonitrile mixture was enough for separation.

Commercial vitamin D_3 preparation was prepared by dissolving it in herbal oil. Vitamin D_3 was highly soluble in the oil fraction. For this reason, when used as a relatively apolar isocratic mobile phase, separation of a vitamin fraction was

very difficult. The separation of vitamin D_3 could be possible with relatively polar isocratic mobile phases. But, polar mobile phase caused prolongation of elution time of the oil fraction. Therefore, gradient mobile phase composition was used in vitamin D_3 analyses.

The elution was started with 100% of methanol (v/v) for 4 min, followed by the mixture of THF + methanol (13% + 87%, v/v) for 2 min, and finally the mixture of THF + methanol (50% + 50%, v/v) for 5 min at the flow rate of 1 mL/min. The chromatogram of the vitamin D_2 and D_3 pharmaceutical preparation was demonstrated in Figure 4 and Figure 5, respectively.

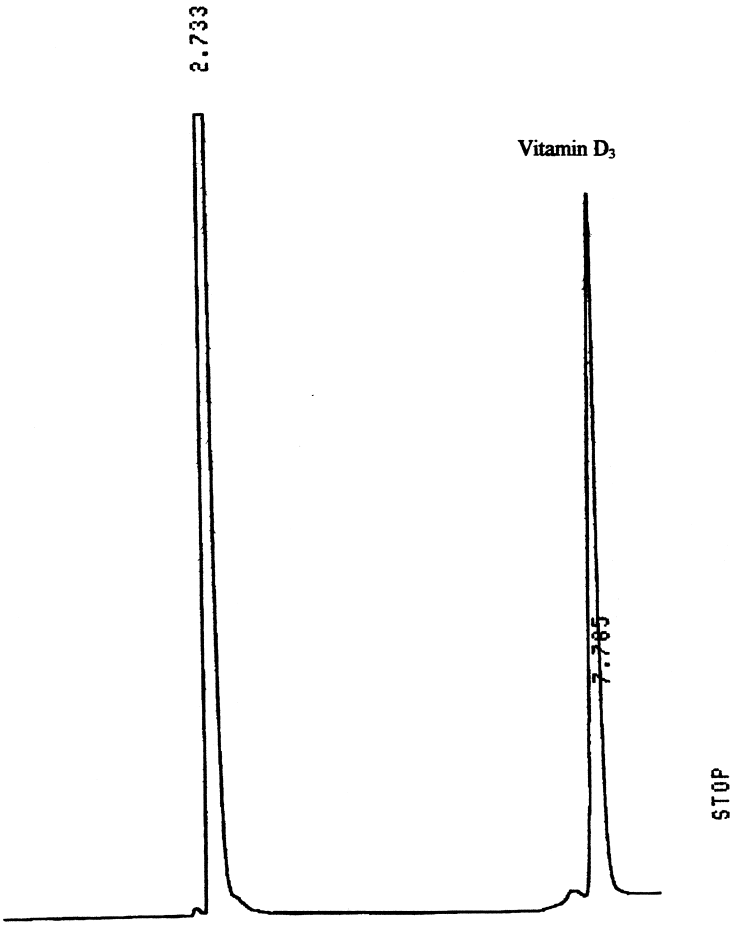

Figure 4. Chromatograms of 9.62 μg/mL vitamin D_3 standard solution.

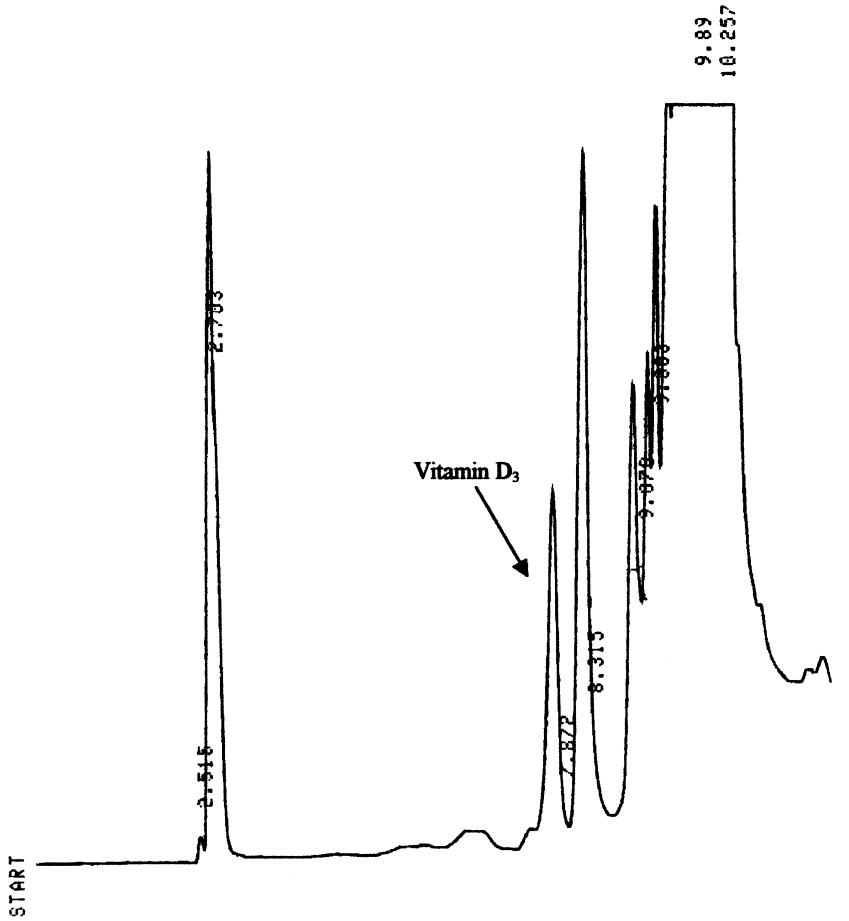

Figure 5. Chromatograms of vitamin D_3 in an oil preparation.

Calculation

After separation of vitamin D_2 and D_3 by reverse phase chromatography the vitamin content is calculated as follows:

Vitamin D_2 (μg/g) = (A × V × 1000)/(S × M)
Vitamin D_3 (μg/mL)= A × S × D/L

Where A= integrated peak area, V= volume of extract (L), S= slope of calibration curve (area/μg/mL), M= mass of sample (g), 1000 = unit conversion factor, D= volume of diluents (mL), L= volume of sample (mL).

RESULTS AND DISCUSSION

In this study, a reverse phase chromatographic method was used for the determination of vitamin D_2 and D_3 in a pharmaceutical preparation. With this method, Vitamin D_3 was easily and accurately determined in complex oil mixtures. Vitamin D_3 is determined as 75.6 μg/mL in the pharmaceutical preparation. Also, the detection limits of the method were tested by adding standard solution to the oil. This method does not include any pre-treatment, extraction, and sample cleaning stages for vitamin D_3. Thus, there isn't any recovery problem with this method. The accuracy of the method was determined as ± 4%. The sensitivity of the method could be increased by decreasing the ratio of dilution, because the amount of the vitamin is relatively increased in the sample. In this way, the concentration of the vitamin is kept in the detection limits.

Also, these results indicate that other fat-soluble vitamins and steroids can easily be detected with the described system from organic materials after extraction. However, fatty acid and triglyceride mixtures could be separated by little modification of the mobile phase composition. Triglycerides were observed at the end of the chromatogram (Figure 5). Changing the solvent composition of the mobile phase can increase the resolution of the peaks at the end of the chromatogram. Modification of mobile phase will increase the resolution of the peaks, which appears at the end of the chromatogram.

In the first stage of elution, an alcohol soluble material, such as butylhydroxyanisole (BHT), was eluted from the column. BHT is used as an antioxidant substance in vitamin D_3 preparations. Vitamin D_3 was eluted from the column in the second stage and an oil fraction, which is highly soluble in THF, was eluted from the column in the last stage. THF concentration was increased continuously in the mobile phase composition until the end of elution. Before the injection of new sample, the column was conditioned with methanol till observing a stable base line. The mobile phase flow rate was set at 1.0 mL/min in all applications.

The mobile phase composition for the eluting vitamin D_2 has been examined in the range of 100% to 20% of methanol ratio in the methanol-acetonitrile mixture. An optimum mobile phase composition was determined on the basis of elution time and resolution of interferences. Finally, the best result was obtained at 40% methanol + 60% acetonitrile. Vitamin D_2 was detected as 35.04 μg/g in the pharmaceutical solid preparation.

Vitamin D_2 and D_3 have high UV absorbance in the specified mobile phase composition. Another fat solvent acetone has high UV absorbance. If this solvent was used as mobile phase with a UV-VIS detector, we not have received a good response for vitamin D_2 and D_3. The accuracy of the detector was also decreased. The response of the SHIDMADZU SPD-10A UV-VIS detector was linear within the range of 0.1–132 μg/mL at 20 μL injection volume.

ACKNOWLEDGMENT

This research has been supported by SEM Laboratory Equipments Inc. (Istanbul/Turkiye).

REFERENCES

1. Fastré, V.R.; Vanhaelen, M. J Chromatogr. **1979,** *179*, 131–142.
2. Reynolds, L.S.; Judd, J.H. Analyst **1984,** *109*, 489–492.
3. Holick, F.M.; Shao, Q.; Liu, W.W.; Chen, C.T. New Eng. J. Med. **1992,** *326–18*, 1178–1181.
4. Wei, S.; Tanaka, H.; Kubo, T.; Ichikawa, M.; Seino, Y. Anal. Biochem. **1994,** *222–2*, 359–365.
5. Song, D.; Kohlhof, K. J. Chromatog. B **1999,** *730*, 141–151.
6. Shimada, K.; Mitamura, K.; Kitama, N. Biomed. Chromatogr. 9 **1995,** *5*, 229–232.
7. *AOAC Official Method 985.27*, *Official Methods of Analysis of AOAC International*, 16th Ed., 4th revision; AOAC International: 481 N. Frederick Ave., Suite 500, Gaithersburg, MD 20877-2417 USA, 1998; 481.
8. *AOAC Official Method 980.26, Official Methods of Analysis of AOAC International*, 16th edition, 4th revision; AOAC International: 481 N. Frederick Ave., Suite 500, Gaithersburg, MD 20877-2417 USA, 1998; 481.
9. *AOAC Official Method 936.14, Official Methods of Analysis of AOAC International*, 16th edition, 4th revision; AOAC International: 481 N. Frederick Ave., Suite 500, Gaithersburg, MD 20877-2417 USA, 1998; 481.
10. *AOAC Official Method 995.05, Official Methods of Analysis of AOAC International*, 16th edition, 4th revision; AOAC International: 481 N. Frederick Ave., Suite 500, Gaithersburg, MD 20877-2417 USA, 1998; 481.

Received October 1, 2000 Manuscript 5404
Accepted October 19, 2000